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Effective conservation management of threatened species involves the preservation of both adaptive diversity and evolutionary potential. One of the key steps in forming a cohesive and effective conservation management plan is therefore the analysis of levels of variation across a species distribution, particularly for fragmented species which show discontinuous distributions. Discussion regarding the analysis of fundamental units for conservation has been ongoing. The terms Evolutionarily Significant Unit (ESU) and Management Unit (MU) have been proposed to represent sub-populations which represent distinct units of diversity and therefore have distinct evolutionary potential. While debate around appropriate definition of conservation units continues, most definitions outline the need for integration of both genotypic and phenotypic data. This research aimed to analyse levels of both genotypic and phenotypic variation across isolated populations of the North Island rifleman (*Acanthisitta chloris granti*). The analysis of variation across rifleman populations involved four studies analysing population variation in genetic, morphological and behavioural traits. Results indicate that the North Island distribution of rifleman is characterized by three divergent genetic lineages including the sole insular population, two populations in the south-east of the North Island, and the remaining mainland populations. Several key areas with very low genetic diversity were also identified. Morphological variation was measured in terms of size and plumage colouration and was found to vary on a spatial scale. The population in the Eastern Ranges in particular was characterised by significantly brighter and more colourful male plumage which may be associated with increased levels of sexual selection as a result of competition for mates at high densities. This finding was supported by an analysis of breeding behaviour variation which found that levels of cooperative breeding were found only in this high density population. Structural components of rifleman call vocalisations were also found to vary on a spatial scale, however playback experiments indicated that these variations do not result in reduced conspecific recognition in a territorial context. The results of these analyses were used to provide key recommendations for future conservation management of the rifleman, particularly with regard to the use of translocation. This study provides evidence for the diagnosis of at least three MUs across the North Island, however further analysis incorporating nuclear genetic markers may find evidence of ESUs.
ACKNOWLEDGEMENTS

A huge thank-you goes to my supervisors Dr Stuart Parsons and Dr Mark Hauber for their advice and support through all stages from planning through to writing. Thank-you for helping me navigate the ups and downs of a challenging project and for encouraging me to stick with such a fascinating little study species.

To my advisor Dr Shane Lavery, who agreed to take on the mammoth task of advising a genetics newbie and did so with patience and encouragement at every step, I am incredibly grateful for your assistance on the genetic component of this project.

I am grateful to the reviewers of this dissertation who provided constructive and valuable feedback which enabled me to refine this study.

To Kathryn Lomas, Christina Painting and Sandra Anderson, three of the most intelligent, dynamic and wonderful women I’ve known, thank you for being such incredible colleagues whose opinions and advice I’ve valued hugely, but most of all friends who have kept me company both at work and in the field and always made it fun.

To the students in both the Ecology Lab and the Molecular Ecology Lab at Auckland University, thank you all so much for all of your help with so many stages of this research. The Molecular Ecology Lab in particular were incredibly patient and helpful while I was learning the techniques for my genetic analysis, so thank you to you all for providing me with so much help. An enormous thank you goes to Craig Millar, Selina Patel and Kirsten Thompson who provided me with so much support while I was learning and averted a few crises along the way. I am particularly grateful to Greg Holwell who assisted me with interpretation of one of my hardest chapters and was always on hand with good ideas and supportive comments. I would also like to thank Anne Gasket who kindly allowed me to use her equipment for spectrophotometry and provided advice and encouragement throughout the project.

This research would not have been possible without the support of several funding bodies including the Australasian Society for the Study of Animal Behaviour (ASSAB), the Supporters of Tiritiri Matangi Inc. (SoTM), the New Zealand Ecological Society, Puke Ariki on behalf of the Grant Mason Charitable Trust, the E.B. Firth Charitable Trust and a University of Auckland Doctoral Scholarship.
Numerous people from the Department of Conservation across the North Island provided permitting and logistical support for this project, to whom I am very grateful. Thank you to Lester Bridson, Kellie Mayo and Carolyn Smith who have been so helpful in the ongoing attempts to get this research into Warawara and who provided call samples from the location for analysis. A huge thank you goes to the team at Boundary Stream Mainland Island who looked after me while in the field. I am also grateful to James Griffiths and DoC Wellington who allowed me to visit the Tararuas site and who provided calls from this population for analysis. Thanks also goes to Tamsin Ward-Smith, Andy and Liz Lowe, Warwick and Juliet Hansen and Julian Robertson and the team at Cape Kidnappers who have supported me with advice, access to the site and logistics.

I am hugely grateful to Iwi from all areas of the North Island where I was allowed to work. Thank you for permitting me access to these field sites and for taking the time to discuss my work with me. I’m particularly grateful to Rongo Bentson for discussions on research and conservation of Warawara rifleman.

An enormous thank you goes to Simon and Morag Fordham who have shared many wonderful rifleman experiences with me, have always included me in rifleman activities on Little Barrier Island and Tiritiri Matangi Island and who I’ve had many interesting and enthusiastic discussions with about rifleman conservation and research. I’m also grateful to John Stewart and Kay Milton who helped me with catching rifleman and advice on the project. Thank you to all of the SoTM members who I’ve had the privilege of catching and translocating rifleman with.

I’d like to thank the Stage One teaching team at Auckland University who have been patient and understanding in allowing me to fit teaching commitments around endless fieldwork. I’m particularly grateful to Mandy Harper who is a constant source of support to me and to David Seldon who allowed me to get to the Warawara site. A big thanks also goes to Tessa Holloway and to Sue Skelly who has always been on hand as a shoulder to cry on and a supplier of rescue remedy.

A heart-felt thank you goes to my family who always encourage me and always believe in me and to my friends for putting up with me being a friend in absentia for a long time.

And last but far from least, to my sweetheart Daniel, the biggest thank-you goes to you. Over the course of this research you went from being my boyfriend who gave up life in a country you loved to allow me to pursue this goal, to my fiancé who lost me for months at a time in remote forests without cell phone coverage, to my husband who
tirelessly supported me through the ups and downs that a PhD involves. You’ve watched birds, hugged trees and climbed mountains with me, and never once complained. Thank you for all that you are and all you’ve done for me. Thank goodness I’ve got the rest of my life to make it up to you.
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Introduction
Conservation biology

Conservation biology is a dynamic and developing discipline, however all approaches to conservation focus on a central aim and that is the retention of diversity (Schlapfer and Schmid 1999; Frankham et al. 2002; Frankham 2005). The maintenance of diversity is important on both broad and fine scales. Retention of broad scale biodiversity ensures that key components of ecosystems are kept intact, therefore preserving ecosystem process and function (Simberloff 1998; Hooper et al. 2005; Benayas et al. 2009). Additionally, levels of genotypic and phenotypic diversity within species are vital to their ongoing survival, as increased diversity both lowers extinction risk and increases evolutionary potential (Frankham et al. 1999; Frankham 2005; Bakker et al. 2010).

One of the most common approaches to conservation involves the protection of current levels of diversity and population structure through the establishment of sanctuaries where predator-control and careful management of key resources (e.g. food availability, breeding site availability) ensures that threatened species have an increased likelihood of survival and successful reproduction (Saunders and Norton 2001). While this approach focuses on the maintenance of current population structure and the prevention of further diversity losses, alternative approaches involve pro-active management techniques which seek to reinstitute historical evolutionary processes seen as vital for the evolutionary potential of threatened species (Atkinson 2002).

Translocation is defined by the World Conservation Union (World Conservation Union 1995) as “the release of animals with the intention of establishing, re-establishing or augmenting an existing population”. Translocation therefore attempts to address three predominant aims: 1) the creation of new populations of threatened species in habitats which are deemed likely to contain the appropriate ecological conditions required for successful population establishment, 2) the re-introduction of a threatened species into an area from which the species has recently become regionally extinct, and 3) the supplementation of declining or threatened populations to increase potential survival (Griffith et al. 1989; Armstrong and McLean 1995; Parker 2008). Translocation has been attempted for many species world-wide, and is particularly utilized for species with fragmented distributions that are considered unlikely to re-establish population connectivity naturally. While numerous translocations result in successful establishment of surviving and breeding populations, many have met with mixed success (Griffith et al. 1989; Wolf et al. 1998; Fischer and Lindenmayer 2000). Investigations into the mechanisms responsible for translocation success and failure have typically focussed on the effects of translocation methodology, food availability, habitat variation and predation (Griffith et al. 1989; Armstrong
and Perrott 2000; Blackburn and Duncan 2001). However, both theoretical and empirical studies increasingly support the need for investigation of sub-population structure and variation in guiding appropriate management decisions for threatened and fragmented species (Moritz 2002), particularly with regard to the use of translocation (Frankham et al. 2002). The analysis of levels of variation across threatened species is increasingly considered a vital component to any effective species management plan (Crandall et al. 2000). As conservation management aims to preserve both adaptive variation and evolutionary processes, the preservation of diversity first requires an accurate inventory of levels and patterns of diversity, both between and within populations (Moritz 1994; Moritz 1994b; Frankham 2010). This is particularly so for species with fragmented distributions as population divergence can have significant consequences for the successful mixing of individuals from separated populations (e.g. Olano-Marin et al. 2011). Divergence of fragmented and isolated populations within a species may occur in rapid timeframes, as populations accumulate both adaptive and non-adaptive variation in the absence of the homogenizing effects of gene flow.

The significance of genetic variation for species management

Establishing patterns of genetic connectivity and diversity within species is fundamental to effective conservation management to address the central goals of species management (Moritz 1994; Fraser and Bernatchez 2001). As the fundamental material on which both selective and non-selective forces act, maintenance of genetic variability is vital to a species evolutionary potential, allowing them to adapt to dynamic environmental conditions and therefore avoid extinction (Lande and Shannon 1996). Numerous studies have demonstrated that low levels of genetic variation result in increased extinction risk through the effects of inbreeding (Frankham 1998), the heightened influence of genetic and environmental stochasticity (Reed et al. 2003; Frankham 2005; Bouzat 2010), and a reduction in fitness (Reed and Frankham 2003). Additionally, low genetic diversity results in decreased evolutionary potential as variability allows populations to adapt to changing and challenging environments (Saccheri et al. 1998; Frankham et al. 1999; Bakker et al. 2010). Genetic variation can be reduced as a result of natural and anthropogenic impacts (e.g. Fleischer et al. 2001; Goldberg et al. 2011), selective forces (Kirkpatrick and Nuismer 2004) or genetic drift (Johnson and Seger 2001). The fragmentation and isolation of populations of species with formerly widespread continuous distributions is of particular concern as small, fragmented populations tend to have lower effective population sizes and are therefore affected by genetic drift and genetic stochasticity to a greater degree (Frankham 1996;
Frankham 1997). Small population sizes can lead to fixation of deleterious alleles in rapid timeframes or can result in the stochastic extinction of genetic lineages through drift (Johnson and Seger 2001; Frankham et al. 2002). While populations may appear to have sustainable densities, these processes may result in low effective population sizes, reducing evolutionary potential (Frankham 1996). Small populations with low effective population sizes and low levels of genetic diversity should rarely be considered as appropriate sources for reintroduction attempts (Frankham et al. 1999), demonstrating how analysis of levels of genetic diversity are vital prior to making management decisions.

The assumption of connectivity between isolated populations can also have significant consequences for the future survival and evolution of a threatened species and should therefore be considered prior to any conservation management intervention. The current distribution of a threatened species has often been shown to be an unreliable indicator of historical separation (e.g. Baker et al. 1995; Avise 2000). Additionally, geographically variable phenotypes frequently represent proximate responses to local environmental conditions (Aldrich and James 1991; McKay 2008), and are therefore misrepresentative of actual levels of genetic connectivity between populations. Numerous species which appear to have currently fragmented distributions demonstrate a high level of genetic connectivity when subject to molecular analysis, providing evidence of recent fragmentation (e.g. Ellegren et al. 1999; Avise 2000). The misdiagnosis of conservation units in such species can result in separate management of genetically similar populations, the consequences of which include the unnecessary restriction of natural levels of gene flow which may be vital in the preservation and spread of adaptive diversity. Alternatively, genetic variability is often masked by a lack of phenotypic variation across populations, leading to misidentified conservation units being subject to misguided management practices (e.g. Burbridge et al. 2003; Rhymer et al. 2005). Many species demonstrate cryptic sub-population structure which is not evident using other phenotypic measures (e.g. Daugherty et al. 1990; Burbridge et al. 2003). In these cases, the collective management of populations representing distinct genetic lineages may result in ‘out-breeding depression’ where local adaptations are diluted upon population mixing, resulting in the loss of adaptive history as well as adaptive potential (Huff et al. 2011; Olano-Marin et al. 2011; Dolgener et al. 2012). Both of these cases demonstrate the importance of analysis of levels of gene flow between divided populations. As conservation management aims to maintain both adaptive variants as well as adaptive processes (Crandall et al. 2000), the retention of distinct genetic lineages is balanced by a need to maximize diversity.
The significance of phenotypic variation for species management

Geographically variable ecological conditions are considered a primary driver of allopatric divergence in adaptive phenotypic traits, as populations adapt to varying climates, habitat types, food availability, predation risk and environmental conditions. These varying ecological environmental factors may influence the success of variable feeding morphology, foraging behaviour, predator avoidance mechanisms (e.g. crypsis), signaling traits, and spacing behaviour (Bailey 1978; West-Eberhard 1983; Rice 1987; Grenier and Greenberg 2005; Areta and Repenning 2011; Gur and Gur 2011). As an individual’s morphology, physiology and behaviour is adapted to maximize survival and reproductive success, these ecological variations can cause substantial phenotypic variation across geographically variable environments due to the influence of both natural and sexual selection.

Morphological and behavioural traits that mediate intra-sexual competitive interactions and inter-sexual mate choice decisions are particularly subject to social and sexual selection (Andersson 1994; Catchpole and Slater 2008). The strong effect of sexual selection due to competition for mates is likely to lead to rapid evolution of exaggerated traits and to high levels of variation in traits involved in competition or courtship (Darwin 1871). Population differences in species-specific communication signals which function in competition and courtship are therefore expected to evolve (West-Eberhard 1983). The development of geographic variations in morphological display features such as colouration has particular significance for the management of a fragmented species. Colouration is often the result of a mixture of genetic, physiological, developmental and environmental influences (Hill and McGraw 2006) and is therefore often highly variable both between and within species (Dale 2006; Owens 2006; Benites et al. 2007). The high degree of variability in colour on a fine scale means it is a trait that has evolved to have significant communication value, functioning as a display trait in both intra-sexual competitive interactions (Pryke et al. 2001; Pryke and Andersson 2003; Senar et al. 2005) and inter-sexual mate attraction (Griffith and Pryke 2006; Hill 2006). Colour displays are highly influenced by both natural and sexual selection (Bortolotti 2006; Griffith and Pryke 2006; Senar 2006) and as such, the evolution of geographic variation in plumage colouration is particularly likely. Geographic variation in plumage colour of birds has been found in numerous avian species (Aldrich and James 1991; Hill 1993; Baker 2008; Oatley et al. 2010). As feather colour represents an individual’s genes, physiology and environment, plumage
may be a good indicator of quality in terms of age (Jarvi et al. 1987), status (Senar 2006), territory quality (Wolfenbarger 1999), parental ability (Hill 1991; Siefferman and Hill 2003), nutrition (Evans and Sheldon 2012) or condition (del Cerro et al. 2010) and may also provide information on adaptive ability within certain environments. Assortative mating based on plumage colour has been found in several studies (Hill 1991; MacDougall and Montgomerie 2003; Hill and McGraw 2004; Hill 2006; Freeman-Gallant et al. 2010), demonstrating how geographic variation in colouration may be significant for conservation management of species which use colouration in competitive and courtship displays. Individuals exhibiting non-local colour morphs may therefore experience reduced competitive and/or pairing success.

Habitat-dependent natural selection may be a significant driver of population variation in adult colouration as feather colour may serve as a selective advantage in terms of concealment, advertisement or disguise within particular environments (Bortolotti 2006). As the effectiveness of cryptic colouration is highly habitat-dependent (Endler 1993; Dale and Slagsvold 1996; Gomez and Thery 2004), selective pressure for enhanced camouflage is likely to result in significant geographic variations in colouration. If colouration functions in cryptis, higher predation levels of individuals exhibiting maladapted morphology may significantly impact survival potential (e.g. Cournoyer and Cohen 2011). Geographic variation in patterns of colouration should therefore be carefully considered prior to management of colourful species via translocation.

Like colour signals, vocalisations are an example of a display trait that is subject to both natural and sexual selection (Morton 1975; Catchpole 1982; Catchpole and Slater 2008) and exhibits extensive variability, particularly across spatial scales (Mundinger 1982). Habitat-dependent natural selection may influence vocal signals within species as most vocalisations are adapted for maximized effective transmission. In a forested environment, sound transmission is influenced by numerous scattering surfaces (Wiley and Richards 1982), therefore vocal signals are often adapted to minimise attenuation and thus maximise transmission in long-range signals, or to enhance locatability in short-range signals (Morton 1975; Marten and Marler 1977; Richards and Wiley 1980). The effects of reverberation and scattering on acoustic signals are highly habitat dependent. Geographically variable habitat types therefore exert different selective pressure on signals adapted for communication between conspecifics (Hunter and Krebs 1979; Brown and Handford 2000; Slabbekoorn and Smith 2002b; Ippi et al. 2011). Additionally, non-selective forces such as ‘cultural drift’ may result in divergent
signaling systems across geographic scales, particularly in species which develop vocal repertoires by learning (Jenkins 1978; Lynch et al. 1989). Geographic variations in vocal signals may result from mistakes in the vocal learning process which are assimilated into individual repertoires and then transmitted through populations in rapid timeframes (Nottebohm 1972; Janik and Slater 2000). In vocal learners, the cultural transmission of vocal traits can result in rapid accumulation of variation (e.g. Baker et al. 2003). If populations are isolated with no migration of cultural variants, geographic variations can evolve. These geographic variations in vocal signatures, termed dialects, have significant consequences for the evolutionary potential of separated populations. Vocalisations function for both intra-sexual competitive interactions as well as inter-sexual mate choice decisions (Catchpole and Slater 2008), and convey a vast amount of information to potential receivers including condition (Nowicki et al. 1998; Garamszegi et al. 2003), status (Cuoco and Malacarne 1999), age (Gil et al. 2001) and other measures of ‘quality’ (e.g. Buchanan et al. 1999; Buchanan and Catchpole 2000). Recognition of these cues is vital in mediation of conspecific interactions. In territorial species that advertise or defend territories using vocalisations, the use of inappropriate signals during territorial interactions can result in decreased territorial functionality of variable vocalisations (Balakrishnan and Sorenson 2006; Bolton 2007; Ripmeester et al. 2010). Additionally, as mate choice decisions are often made based on vocal cues (Kroodsma 1976; Catchpole 1980; Searcy 1984; Searcy 1992; MacDougall-Shackleton et al. 2001), individuals singing unfamiliar signals may experience reduced mating success, resulting in significant impacts on reproductive potential.

Finally, geographic variations in mating system and breeding behaviour may represent significant pre-zygotic mating barriers between variable populations. Theoretical analyses on the evolution of mating systems emphasize both life-history characteristics and ecological constraints as contributing substantially to the evolution of particular breeding systems and behaviours (Orians 1969; Emlen and Oring 1977; Arnold and Owens 1998). Geographic variations in mortality, fecundity and density, influenced by population-specific environmental variables, may result in the use of alternative mating systems between populations (Lott 1991). Cooperative breeding systems in particular are associated with certain life history characteristics such as low mortality and sedentariness (Arnold and Owens 1998). Additionally, many cooperative breeders help in response to local ecological constraints, emphasizing a lack of space or vital resources in driving the evolution of cooperation (Emlen 1982a). In this scenario, helpers are making the ‘best of a bad job’ in deriving alternative
benefits from helping others breed due to a restriction in the likelihood of successful independent breeding (Brown 1974; Emlen 1982a; Koenig et al. 1992; Du Plessis et al. 1995; Komdeur et al. 1995; Komdeur 2003b; Kesler et al. 2010). Due to the intrinsic link between resource availability and cooperation under the ecological constraints hypothesis, geographic variation in both resource and territory availability may result in geographically variable breeding systems or levels of cooperation being expressed. Different social and breeding systems are characterized by different levels of reproductive success, reproductive flexibility, resource protection and access, mate guarding, parental care and territoriality (Emlen and Oring 1977; Andersson 1994; Alcock 1998). Therefore, the inter-mixing of individuals from behaviourally divergent populations may be significantly affected by geographic variations in mating system. As the success of translocation relies on survival and reproductive success of founding individuals, an understanding of how breeding systems influence reproductive success is vital in the successful establishment of new or mixed populations via translocation (e.g. Vincent and Sadovy 1998; Norris 2012).

**Conservation biology and the definition of units for conservation**

As the discussion above demonstrates, the analysis of levels of spatial variation for threatened species showing fragmented distributions is vital to appropriate decision-making with regard to conservation management practices, particularly translocation. To attempt to integrate the analysis of spatial variation into the practice of conservation management, researchers have attempted to classify populations of threatened species into fundamental units of diversity, worthy of separate conservation management. Over the history of the conservation biology discipline, discussion regarding the diagnosis of these fundamental units of diversity has been ongoing (Crandall et al. 2000). While traditional conservation biology focused on preservation of taxonomic units (e.g. species and sub-species), a vast number of contemporary studies have concluded that these units may be an unreliable indicator of phylogenetic divergence due to discordance between morphological characters and molecular indices of diversity (e.g. McKay 2008; Donnellan et al. 2009; Roberts et al. 2011). As a consequence, conservation biologists are increasingly seeking a consistent and integrated approach to the definition of key conservation units within species to allow effective conservation management despite taxonomic uncertainty (Crandall et al. 2000). However, the definition of conservation units has been the topic of extensive debate, and a consensus has yet to be reached. Numerous published discussions regarding units for conservation management have focused on use of the terms Evolutionarily Significant Units (ESUs) and
Management Units (MUs) (Ryder 1986; Moritz 1994; Crandall et al. 2000). The analysis and classification of such conservation units addresses both long-term conservation goals related to the evolutionary potential of species and short-term goals associated with the survival of populations. However, while numerous discussions have outlined the need for both genotypic and ecological data in the definition of conservation units (Ryder 1986; Waples 1991; Dizon et al. 1992; Crandall et al. 2000), the majority of analyses classify such units on the basis of genetic data alone, emphasizing the restriction of gene flow in population divergence (Moritz 1994). Despite the potential for population differences in phenotypic, ecological and behavioural traits to represent pre-mating reproductive or dispersal barriers, studies incorporating the analysis of such variations are severely lacking in conservation studies.

**Rifleman (Acanthisitta chloris granti)**

The rifleman is one of only two extant species within the New Zealand wren family Acanthisittidae, Order Passerida. The taxonomic position of the Acanthisittid wrens within the Passerine phylogeny has been historically debated (Forbes 1882; Feduccia 1977; Sibley et al. 1982). However, phylogenetic analyses using molecular markers places the New Zealand wrens as the sole representatives of a third sub-order within the Passeriformes: the Acanthisitti (Ericson et al. 2002). Molecular analyses consistently place the Acanthisittid wrens as the most basal lineage within the passerine phylogeny (Ericson et al. 2002; Barker et al. 2004), with divergence from all other extant passerines dated at approximately 82 mya (Ericson et al. 2002). This divergence date indicates that the lineage originated in Gondwanaland, diverging when the New Zealand land-mass separated from its Gondwanan neighbours. As such, the New Zealand wrens provide some of the most compelling evidence of an ancient Gondwanan element within New Zealand’s biota (Cooper and Millener 1993; Ericson et al. 2002; Worthy et al. 2010).

The Acanthisittidae are entirely endemic to New Zealand and was once comprised of seven species, making it one of the most speciose families in New Zealand (Worthy et al. 2010). Habitat clearance and introduced pests have resulted in the extinction of five of the species, with only the rock wren (Xenicus gilviventris) and the rifleman (Acanthisitta chloris) still extant (Holdaway 1999; Gibbs 2006). Two sub-species of rifleman have been described: the North Island (Acanthisitta chloris granti) and the South Island rifleman (Acanthisitta chloris
chloris). Sub-species classification is predominantly based on colour variation and slight size variations, however examination of multiple specimens indicates that no consistent differentiation separates them (Higgins et al. 2001). No analysis of genetic differentiation between the sub-species has ever been attempted.

Rifleman are New Zealand’s smallest bird and exhibit reverse sexual size dimorphism with males weighing approximately 6 g and females 7 g on average (Higgins et al. 2001). Sexes also vary in colour with obvious dichromatism allowing visual sex identification. Males are described as bright olive green across the head, mantle, scapulars, back and rump, while females are a speckled light and dark brown across the same areas (Hunt and MacLean 1993; Higgins et al. 2001). Both sexes have white or cream under-parts. Juveniles appear similar in colour to females but are characterised by brown mottling (Higgins et al. 2001). Sexual dimorphism in rifleman colouration has been speculated to function in crypsis as dichromatism appears to match the different foraging substrates of males and females during the breeding season (Hunt and MacLean 1993). Females are also found to have longer hind-claws and a more decurved bill compared to males, reflecting the higher proportion of time spent foraging on tree trunks; males who spend more time foraging in the canopy. Both sexes feed on a wide range of small invertebrates, predominantly Coleoptera, Lepidoptera, Orthoptera and Diptera and a large proportion of larvae (Sherley 1985; Lill 1991; Hunt and MacLean 1993).

Rifleman are a monogamous species with stable pairs maintained throughout the year and across seasons (Gray 1969; Sherley 1985). Breeding efforts consist of up to two clutches per season within which 3-5 eggs are typically laid (Gray 1969; Sherley 1985). Rifleman are secondary cavity nesters, building complex spherical nests within existing tree cavities (Gray 1969; Higgins et al. 2001). Females invest heavily in reproduction, producing relatively large eggs at 48 hour intervals (Sherley 1989). Males also invest large amounts of energy in reproduction, contributing equal or higher levels of parental care than females during incubation, nestling and fledgling phases of reproduction, performing the majority of nest-building and nuptial feeding the female (Sherley 1989; Sherley 1994). No evidence of extra-pair paternity or polygamy has been found for populations studied in detail (Sherley 1994), however rifleman are a cooperative species (Gray 1969; Sherley 1990). Young are sexually mature at 9 months and will pair immediately upon independence. However, some individuals remain in natal territories to assist with raising siblings produced in a second clutch (Sherley 1990). Predominantly male helpers are also occasionally seen at unrelated nests and are thought likely to gain access to
mates by assisting with reproduction and pairing with offspring upon independence (Sherley 1990). Male breeders gain the most benefit from the presence of helpers, allowing a reduction in the generally high level of parental care they ordinarily provide (Sherley 1990). Rifleman are a sedentary territorial species, occupying exclusive territories which are predominantly maintained via mutual avoidance (Cameron 1990). Resident territorial adults do not patrol or actively advertise territory boundaries and while territory intrusions are sometimes tolerated (Sherley 1990), they are often responded to by approach and vocal communication and occasional aggression. Vocalisations consist of a small repertoire of simple, high frequency calls (Higgins et al. 2001; Krull et al. 2009) which are used in un-structured combinations for predominantly intra-pair communication. Pairs spend over 90% of time in continuous contact (Sherley 1989), maintained by constant calling by both sexes. No obvious sex differences in calls have been found, however juveniles utter a characteristic call which may provide information on position.

While rifleman are thought likely to have once been found across the majority of New Zealand during periods of extensive forestation (e.g. Gill 1996; Higgins et al. 2001), the distribution of both North and South Island populations is now highly fragmented (Robertson et al. 2007). Populations in the North Island are generally restricted to discontinuous high-altitude mountain ranges where remnants of mature forest remain (Higgins et al. 2001; Robertson et al. 2007). Preferred habitat types include beech (Nothofagus), podocarp, broadleaf and hardwood forests including Totara (Podocarpus totara), Rimu (Dacrydium cupressinum), Tawa (Beilschmiedia tawa), Kamahi (Weinmannia racemosa) and Kauri (Agathis australis) dominated forests (Higgins et al. 2001). Population density varies between regions (Robertson et al. 2007) and while several areas support high density populations of rifleman, noted declines have been observed in multiple regions (Smith and Westbrooke 2004; Elliott et al. 2010) and the sub-species is now recognised as ‘declining’ by the IUCN (IUCN 2012). A cohesive conservation management plan for the North Island sub-species has not been proposed, however the recent discovery of an extremely isolated and low density population in northern New Zealand has demonstrated the need for increased investigation into the potential threats facing the sub-species (Pierce 1994). Ongoing decline has resulted in large areas of regional extinction across the North Island (Robertson et al. 2007), and as a result of this small conservation initiatives have begun to attempt to circumvent further decline of rifleman by reintroducing individuals into areas of local extinction. To date, two North Island sites have been the recipients of rifleman translocated from nearby native source locations. These include a private mainland sanctuary (Cape
Kidnappers and Ocean Beach Wildlife Preserve) located in the Hawkes Bay on the East Coast and a government-owned public island sanctuary (Tiritiri Matangi Island) located in the Hauraki Gulf. Several additional sanctuary sites across the North Island consider reintroduction of rifleman a central part of their future biodiversity plan (e.g. Puketi Forest, Ark in the Park), therefore translocation and reintroduction of rifleman appears to be a highly probable conservation strategy for the future management of the sub-species. Despite these conservation interventions, levels of genotypic and phenotypic diversity between populations of rifleman have never been investigated. Additionally, no attempts have been made to diagnose conservation units for the sub-species to date. As rifleman are a declining and fragmented species, conservation efforts involving translocation are likely to be utilized in the future. It is hoped that the information contained in this thesis will assist in the designation of appropriate units for conservation management and will therefore guide management decisions for the future management of rifleman populations.

**Thesis outline**

The aim of this thesis was to analyse and describe levels of molecular, morphological and behavioural variation between isolated sub-populations of the North Island rifleman and to discuss the results in the context of potential conservation management for the sub-species. This thesis contains results from four studies in which different levels of diversity are analysed to achieve the thesis’s overall aim.

Chapter Two examines levels of within and between-population molecular diversity across the North Island rifleman distribution. Genetic samples across six fragmented populations were analysed using mitochondrial DNA markers to determine patterns of population connectivity and genetic diversity. The findings are discussed in the context of potential natural and anthropogenic forces of distributional change.

Chapter Three describes levels of vocal variation in the simple calls of adult rifleman between six isolated populations across the North Island. Playback experiments are then used to test the functional significance of geographically variable signals in recognition of territorial conspecifics. The results of this analysis are discussed with reference to the genetic results in Chapter Two and I speculate on some potential drivers of
geographic vocal variation which may warrant further research. The implications of the results of this study are briefly discussed.

Chapter Four analyses geographic variation in size and colouration of adult rifleman. General patterns of sexual size dimorphism and sexual dichromatism are analysed and described and geographic variation in the extent of sexual dimorphism is explored and discussed in relation to variable selective forces including natural selection on size variation and sexual selection on colour variation.

Chapter Five explores population variation in breeding biology of rifleman and investigates the effect of ecological constraints on the evolution of cooperative breeding in North Island populations. The study analyses the effects of density and cavity availability on the use of cooperation across four sites representing the source and recipient sites of two recent translocations. The results are discussed in the context of the potential consequences of population variation in breeding behaviour and the evolution of cooperation in rifleman.

Chapter Six provides a summary of the principal findings of this research and discusses the collective results in the context of conservation management of North Island rifleman. The discussion outlines management recommendations for the sub-species, particularly with regard to the use of translocation and discusses historical translocation cases in light of the findings of this research.

References


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CHAPTER TWO: Isolation and genetic divergence between fragmented populations of the North Island rifleman
Introduction

The field of phylogeography focuses on the geographic distribution of genetic lineages of organisms (Avise 2000). Intra-specific phylogeographic analyses are particularly informative as they provide information to both scientists and conservation managers on the distribution of genetic diversity and levels of genetic connectivity across a species distribution (Bermingham and Moritz 1998; Frankham et al. 2002). Investigations into the distribution of intra-specific molecular diversity are common as they provide information on patterns and processes of both microevolution and macroevolution (Avise 2000). Molecular indices of diversity may reveal hidden patterns of diversification between populations that are not necessarily evident using phenotypic measures (e.g. Daugherty et al. 1990; Burbridge et al. 2003) and therefore inform us on patterns of historical gene flow. Locality is often assumed to delimit genetic lineages within species (Avise et al. 1987). Where species exhibit widespread distributions, genetic diversity is often thought to be relatively high. For birds, this is because connectivity across the species’ range is usually assumed, meaning the species is considered one large panmictic population, with a high effective population size (N_e), and thus high within-species genetic diversity. However, small areas of local decline in species with low dispersal ability can result in population fragmentation, which may have significant effects on patterns of diversity across the species distribution (e.g. Bakker et al. 2010). Population fragmentation increases extinction risk as stochastic processes have a stronger influence over small, isolated populations (Avise et al. 1983; Reed et al. 2003; Frankham 2005; Bouzat 2010). Additionally, fragmentation leads to higher risks of inbreeding and the loss of adaptive and evolutionary potential in small and separated populations (Saccheri et al. 1998; Frankham et al. 1999; Bakker et al. 2010). Finally, fragmentation increases population divergence (Phillimore et al. 2008) and therefore has particular consequences for both the evolutionary potential and conservation requirements of species. Species which may appear widespread may therefore in fact be represented by a series of isolated sub-populations with varying demographic and diversity characteristics and are therefore at higher risk of extinction than they first appear (Frankham et al. 2002; Koumoundouros et al. 2009). The analysis of levels of connectivity between populations within a species therefore informs us on both the evolutionary past and evolutionary potential of individual species.

Mitochondrial DNA markers have been shown to be increasingly useful for investigating both species-level phylogenetic patterns and the distribution of within-species phylogroups or cryptic species (Avise et al. 1987;
Avise 2000). While the maternal inheritance of mitochondrial DNA means that patterns of diversity predominantly reflect the distribution of maternal genetic lines (Watanabe et al. 1985), the fast-evolving nature of mitochondrial DNA compared to most nuclear DNA (Brown et al. 1979) means that mitochondrial markers have been useful in identifying patterns of population separation, levels of gene flow and effective population sizes within species (Avise et al. 1987; Frankham et al. 2002).

New Zealand’s geological and cultural history makes it a particularly interesting place for phylogeographic analyses of terrestrial species (Cooper and Millener 1993; Cooper and Cooper 1995; Goldberg et al. 2008). New Zealand’s dynamic geological history includes extensive periods of land submersion, mountain uplift and volcanism as well as repeated glacial cycles which significantly modified land connectivity and distributions of terrestrial species (Fleming 1962b; McGlone et al. 2001; McGlone 2005; Alloway et al. 2007; Goldberg et al. 2008). More recently, anthropogenic impacts have included extensive deforestation and the introduction of invasive mammalian pest species which have had a significant detrimental impact on both the abundance and distribution of native species (Holdaway 1999; Gibbs 2006; Veitch et al. 2011). The analysis of molecular indices of diversity and the distribution of genetic lineages within New Zealand therefore informs us on the relative impacts of both natural and human-induced forces of change.

In this chapter, I investigate levels of genetic connectivity and molecular diversity between populations of the North Island New Zealand rifleman (Acanthisitta chloris granti). The North Island rifleman is of particular interest for phylogeographic studies as it is one of only two extant taxa from the ancient endemic New Zealand wren family, Acanthisittidae. The Acanthisittid wrens are now considered to be the most basal global passerine lineage, and have been placed as the sister sub-order to both oscines and sub-oscines (Ericson et al. 2002; Barker et al. 2004). As such, they are likely to represent one of New Zealand’s few ancient Gondwanan avifaunal species, and the only extant New Zealand passerine suggested to have a vicariant origin (Fleming 1962; Cracraft 1973). While the North Island sub-species of rifleman would once have been found throughout the North Island of New Zealand (Gill 1996; Higgins et al. 2001), they are now characterized by a fragmented distribution, being restricted predominantly to discontinuous high altitude mountain ranges and a single offshore island (Higgins et al. 2001; Robertson et al. 2007). While the rifleman’s contemporary distribution may result from recent anthropogenic impacts such as habitat clearance and regional decline due to invasive pests (e.g.
Beauchamp and Worthy 1988; Millener 1989; Brook 2000), population fragmentation may also have resulted from ancient geological processes which have caused comparable fragmentation in a range of other taxa in New Zealand (e.g. Cooper and Millener 1993; Goldberg et al. 2008; Buckley et al. 2010; Fouquet et al. 2010). The rifleman is currently categorized as being of ‘least concern’ by the IUCN, reflecting the widespread nature of North Island rifleman populations and the high density observed in several of the larger populations (IUCN 2012). However, some populations are recognized as ‘at risk - declining’, with noted declines in several regions of the North Island (Gill 1996; Smith and Westbrooke 2004) and the IUCN has officially recognized the decreasing population trend of rifleman populations in New Zealand (IUCN 2012). Given the low dispersal capability of the species (Higgins et al. 2001), the presence of several isolated and low density populations (e.g. Pierce 1994) has emphasized the need for an investigation into population connectivity and intra-specific phylogeography in this sub-species. Current conservation management for the species is minimal but efforts to date consist of several attempts at regional re-introduction via translocation (e.g. Leech et al. 2007, Withers, S. unpublished data), a practice which is likely to increase in use for future rifleman management strategies, and may have considerable impacts on genetic diversity within the species.

This study aimed to investigate patterns of molecular diversity between geographically separated populations of the North Island rifleman. Given the currently fragmented population structure of North Island rifleman populations, I used molecular indices of inter-population diversity to investigate the following questions:

1) Is there evidence of genetic divergence among isolated mainland and insular sites?
2) If population fragmentation is evident, is it the result of recent anthropogenic impacts, or of historical geological or climatic events?
3) Are rifleman characterized by high genetic diversity or have the impacts of population fragmentation and isolation resulted in low levels of molecular diversity?

Here, I investigate genetic sequence variation using two mitochondrial markers: the Cytochrome Oxidase I region and a portion of the hyper-variable 5’ end of the Control Region, to investigate patterns of haplotype and nucleotide diversity within and among North Island populations and discuss the potential mechanisms of genetic differentiation across the rifleman distribution.
Materials and Methods

Sample collection

Six locations across the North Island of New Zealand were selected for sampling including Little Barrier Island (-36° 19’S, 175° 07’E), Taranaki National Park (-39° 29’S, 174° 06’E), Pureora Forest Park (-38° 57’S, 175° 59’E), Boundary Stream Mainland Island (-39° 06’S, 176° 48’E), Mohi Bush (-39° 51’S, 176° 54’E) and Tararua Forest Park (-40° 86’S, 175° 41’E) (Fig.1). These areas represented Insular, Western, Central, Eastern Ranges, Eastern Coastal and Southern locations respectively. Rifleman were caught within territories using 24mm gauge mist-nets and lure calls. Blood samples were collected using brachial venipuncture and blood was stored in Queen’s lysis buffer (Seutin et al. 1991) and kept at 4°C until DNA extraction.

Fig. 1. North Island sampling sites of rifleman for genetic analysis
**DNA extraction, amplification and sequencing**

Whole genomic DNA was extracted from blood samples using either standard phenol-chloroform extraction procedures, or using a Qiagen DNEasy Blood and Tissue Kit, following the Animal Tissue Modification protocol of the Qiagen Manual. As initial extractions had low DNA yields, the following modifications were made to the protocol: 1) 160µl of SET buffer replaced the recommended 200µl of PBS buffer, 2) 40µl of Proteinase K replaced the recommended 20µl, 3) 30µl of blood in Seutin buffer was used instead of 10µl of whole blood, 4) incubation was carried out overnight rather than for 10 minutes and 5) final elution stages were done with two rounds of 100µl of buffer AE as opposed to 200µl. Polymerase Chain Reactions (PCR) targeted two regions of the mitochondrial genome for amplification. A 751bp region of the Cytochrome Oxidase 1 gene (COI) was targeted using the forward primer AWCF1 (Hebert *et al.* 2004; Patel *et al.* 2010) (5’-CGCYTWAACAYTCYGCCATCTTACC-3’) combined with the reverse primer COIBirdR2 (Hebert *et al.* 2004; Kerr *et al.* 2009)(5’-ACGTGGGAGATAATTCCAAATCCTGG-3’). COI PCRs were carried out in 25µl reactions containing 12.83µl of water, 2.5µl of 10x Reaction Buffer, 1.25 mM of MgCl2, 2.5µl of BSA, 0.2 mM of dNTP’s, 0.17µl of Taq Ti polymerase, 1.25µl of each primer (1.25µM) and 3µl of template DNA (10-40 ng/µl). Thermal cycling conditions for amplification involved an initial partial denaturation phase at 94°C for 2 min and 35 cycles of denaturation at 94°C for 30 s, annealing at 57.5°C for 30 s and extension at 72°C for 30 s, followed by a final extension step at 72°C for 4 min. Primers targeting an approximately 600bp region of the 5’ end of the mt Control Region (mtCR;D-loop) were designed specifically for rifleman using a published mitochondrial genome from the South Island sub-species of rifleman (Accession Number AY325307). The forward primer L16733 (5’-ACTTGGGCACCTCCCCAAAGACCA-3’) was located within the tRNA-Glu region, and the reverse primer H437 was situated within Domain II of the Control Region (5’-GGGTTGCTGATTTCTCGTGAG-3’) (Lavery, unpublished data). PCR reactions contained the same reagent concentrations as for the COI region as detailed above. Thermal cycling conditions were also as for the COI region, but used an annealing temperature of 57°C. PCR products were visualised on a RedSafe-stained 1.6% agarose gel to check for amplification of single fragments of appropriate length before sequencing. Excess primers and nucleotides were removed from PCR products using the SapEx (Shrimp alkaline phosphatase and exonuclease 1) protocol, before carrying out cycle sequencing using Big Dye protocol (Applied Biosystems). Cleanseq (Agentcourt) was used to purify products before sequencing on an ABI3130 automated sequencer, using the reverse primer COIBirdR2 for the COI region and the reverse primer H437 for the Control Region.
**Sequence analysis**

Sequences were viewed and aligned manually using Geneious Pro (version 5.5.6, Biomatters Inc.) All variable sites were confirmed by visual inspection of chromatograms. Resulting alignments were edited and low quality ends were trimmed to create a 377bp alignment for the Control Region and a 652bp alignment for CO1. Separate mitochondrial gene alignments were used for subsequent analyses, plus a concatenated dataset was created by combining Control Region and CO1 sequences. Variable sites were identified using MEGA (version 5.05) to enable manual haplotype designation. Haplotype \( h \) and nucleotide \( \pi \) diversities (Nei 1987) were calculated for individual sample populations using Arlequin v 3.5 (Excoffier and Lischer 2010). To test for neutrality, Tajima’s D and Fu’s F (refs) were calculated for each population and a standard diversity measure of \( \theta_\pi \) was calculated using Arlequin. To calculate an initial estimate of effective population size for each sample population the following formula was used: \( \theta_\pi = 2Ne \mu \), where \( \theta_\pi \) is the diversity index calculated by Arlequin, \( Ne \) is the female effective population size and \( \mu \) is the estimated mutation rate (see below). For each separate alignment, the Akaike Information Criterion (AIC) was used in jModelTest (Guindon and Gascuel 2003; Posada 2008) to select the most appropriate nucleotide substitution model for the data. The best fit model was then used to construct a Neighbour Joining (NJ) tree using Geneious Pro for visual representation of sample relationships. Support for major clades was assessed in a separate analysis using 1000 bootstrap replicates and bootstrap values for predominant branching patterns were displayed on each tree. Branching patterns with bootstrap percentages \( \geq 70\% \) were considered to be strongly supported (Hillis and Bull 1993). To visually represent relationships among haplotypes, a maximum parsimony haplotype network was created for the COI gene, using a median-joining algorithm in Network v4.6.1.0 (Flexus Technology Ltd, 2011). Population divergence was analysed in Arlequin using AMOVA, along with pairwise calculations of \( F_{ST} \) (using haplotype diversities only) and \( \Phi_{ST} \) (including nucleotide divergences), as well as exact tests of frequency differentiation.

**Dating of population divergence**

Dates of population divergence were estimated using the formula \( \mu = d/2t \) where \( \mu \) is the mutation rate, \( d \) is the pair-wise divergence and \( t \) is the time since divergence. Mutation rates are known to vary across different mitochondrial regions (e.g. Ruokonen and Kvist 2002), and while a standard molecular clock rate of 2%/my (0.02) is often used (Brown et al. 1979), analyses have shown that birds often exhibit a slower rate of evolution.
compared to other vertebrates, particularly in protein coding regions (Kessler and Avise 1985). I therefore estimated divergence times for rifleman populations using two alternative estimates of mutation rate for each gene region. Rates of 0.0032 s/s/my (Pacheco et al. 2011) and 0.0075 s/s/my (Pereira and Baker 2006; Eo and DeWoody 2010) were chosen for the COI region based on thorough reviews of avian mitochondrial evolutionary rates. For the Control Region, upper and lower mutation rate estimates of 0.04 s/s/my (Lambert et al. 2002) and 0.20 s/s/my (Hansson et al. 2008; Cibois et al. 2010) were used for divergence dating.

Results

Population nucleotide and haplotype diversity

Sequence data obtained from 90 rifleman representing six populations across the North Island of New Zealand showed evidence of genetic structuring among geographic locations. Two mitochondrial DNA regions were amplified and sequenced, including a 652 bp region of the Cytochrome Oxidase I gene and a 377 bp region of the Control Region. Of the 652 bp COI region, a total of 26 variable nucleotide sites (all involving substitutions) were observed that defined 17 different haplotypes among the six geographic regions (Appendix I) (4% variability). Haplotype and nucleotide diversity varied significantly among populations (Table 1).
Table 1. Measures of population genetic diversity for Cytochrome Oxidase I and Control Region sequences. Significant Tajima’s D (P<0.05) (Tajima 1989) and Fu’s F (P<0.02) (Fu 1997) are highlighted in bold

Considering the COI region first, the Insular population showed no intra-population diversity, with all individuals having identical COI sequences. The Eastern Coastal region had only two haplotypes present, however these were highly differentiated, being characterized by a high nucleotide divergence. The Western population showed the highest haplotype diversity with eight different haplotypes present (0.77 ± 0.07) (Table 1). Most of these were differentiated by only a single base substitution, and therefore intra-population nucleotide diversity was relatively low. The Southern population had high haplotype diversity which was characterized by the highest number of polymorphic sites (13) and showed the highest nucleotide diversity (0.0083 ± 0.005).

The 377 bp 5’ region of the Control Region (mtCR) showed significantly higher variability than COI. This part of the Control Region contained 46 variable sites that were used to characterize 33 haplotypes in total (Appendix II) (12% variability). Five of these variable sites were indels (insertions/deletions). Regional patterns of haplotype diversity were similar to that shown in the COI with Insular and Eastern Coastal populations.
showing lower haplotype variation while Western and Southern populations had the highest diversity (Table 1). Despite having low levels of haplotype diversity, the Eastern Coastal population haplotypes were highly differentiated and the population was characterized by high nucleotide diversity (0.0113 ± 0.007). The haplotypes found in the Southern population were also significantly differentiated with 26 variable sites and high nucleotide diversity. In line with results from the COI, the Insular region had low levels of genetic diversity when considering both haplotype and nucleotide variation (0.0268 ± 0.016).

Tajima’s D (Tajima 1989) and Fu’s F (Fu 1997) were calculated to investigate neutrality within each population. Assuming selective neutrality, positive values may indicate that populations have been through a population bottleneck, whereas negative values may indicate population expansion (e.g. Akey et al. 2004). There was evidence of departure from neutrality in two populations; the Western population had a significantly negative Fu’s F value and the Eastern Coastal population had a significantly negative Tajima’s D value (Table 1). Overall diversity indices (θπ) indicated that the Eastern Coastal and Southern populations had the highest diversity.

Using the formula \( \theta_n = 2Ne\mu \), a measure of female effective population size (Ne(F)) was calculated for all populations using the COI dataset, using a mutation rate of 0.0032 (Pacheco et al. 2011). All populations had relatively high female effective population sizes (Western =178, Eastern Ranges=234, Eastern Coastal=438 and Southern= 844) compared to the Insular and Central populations (Insular = 1, Central=78).

**Phylogenetic analyses**

Appropriate nucleotide substitution models were selected using the AIC criterion for both the COI region and the Control Region using jModel Test. HKY + G was the simplest model selected to explain both datasets, therefore the HKY model (Hasegawa et al. 1985) was used to calculate pair-wise genetic differences in two Neighbour-Joining analyses corresponding to each mitochondrial region (as HKY+G was not available in the software package).
Very clear phylogeographic clustering of sequences was evident from the mtDNA sequences (Figs 2-4). Although both gene fragments provide a very similar pattern overall, there were some differences in the patterns evident from the two fragments sequenced. Therefore, a N-J tree for each fragment is presented separately, and then a combined tree is displayed which uses a concatenated dataset. Three distinct clades are evident in the COI tree (Fig 2): one contains all sequences from the Insular population, the second contains almost all sequences sampled from the Southern and Eastern Coastal populations and the third is made up of all of the sequences from the remaining mainland populations. There is very striking phylogeographic regionalisation of the Insular population, with all individuals sharing a single, diverged COI haplotype. The Southern and Eastern Coastal populations are also clearly delineated, with almost all individuals showing significant divergence from sequences in the remaining mainland populations, and a small but clear divergence from each other. The remaining populations are not phylogeographically divergent, but share only the most common haplotype among populations. All populations show relatively low haplotype diversity, particularly the Insular population, which exhibits no COI diversity at all.

A very similar pattern is displayed in the Control Region sequences, except for two discrepancies (Fig 3). Firstly, as expected, a greater level of both haplotype & nucleotide diversities are exhibited in the Control Region sequences overall. Of note is that the Insular population is now seen to contain some mtDNA diversity which is not observed in the COI data. Secondly, the clade of Control Region sequences from the Insular population appears to be much more closely related to the major clade of mainland populations than was observed in the COI data. On closer inspection of the variable and shared polymorphisms, some of this similarity may be due to a couple of homoplasious substitutions in this hypervariable region or homoplasious length variants in a hyper-variable G-repeat at the terminal end of the sequence. However, regardless of any potential homoplasy, the Control Region sequence clearly shows that the Insular clade of sequences is not as diverged from the major clade as is seen in the COI data. The potential reasons for this and its implications will be discussed further below, but this discrepancy highlights the advantage of analysing sequences from more than one mtDNA region.

As both mtDNA fragments appear to be evolving under the same model (HKY + G), both COI and Control Region sequences were combined (for those individuals with both mitochondrial regions sequenced to high
quality), to provide an overall tree. The phylogenetic patterns from the combined sequences (Fig 4), confirm the
general patterns seen in the COI data: that the North Island rifleman distribution is characterized by three
 genetically divergent and geographically restricted clades, including all Insular populations (hereafter referred to
 as the ‘Insular clade’), the majority of the Eastern Coastal and Southern populations (hereafter referred to as the
 ‘South-Eastern clade’, and all remaining sequences from the mainland population (hereafter referred to as the
 ‘Mainland clade’).
Fig. 2. Neighbour Joining Tree of COI sequences from six populations of North Island rifleman. Distances were calculated using the HKY model. Bootstrap support for neighbour joining analyses are shown for nodes that appeared in >70% of bootstrap replicates.
Fig. 3. Neighbour Joining Tree of Control Region sequences from six populations of North Island rifleman. Distances were calculated using the HKY model of substitution. Bootstrap support for neighbour joining analyses are shown for nodes that appeared in >70% of bootstrap replicates.
Fig. 4. Neighbour Joining Tree created using a concatenated dataset of COI and Control Region sequences from six populations of North Island rifleman. Distances were calculated using the HKY model of substitution. Bootstrap support for neighbour joining analyses are shown for nodes that appeared in >70% of bootstrap replicates.
**Geographic patterns of haplotype sharing**

Geographic patterns of haplotype sharing were minimal for both the COI and Control Region regions. Of the 17 haplotypes represented by COI sequences, only a single haplotype (Haplotype 3) was shared between more than one population (Fig. 5). To visually characterize haplotype relationships, a statistical parsimony network analysis was constructed using sequences from the COI region using Network (Flexus Technology, 2011) (Fig. 5, inset). The most common haplotype was haplotype 3. As the haplotype at the centre of the Mainland clade, haplotype 3 is the most likely to represent the ancestral haplotype (Crandall and Templeton 1993). Most mainland haplotypes diverged from this ancestral haplotype by only a single substitution (Fig. 5, inset).

Haplotype 1 was highly differentiated from the ancestral and associated haplotypes. Haplotypes 14, 15 and 16 were also significantly divergent from all other haplotypes. When coloured according to geographic region, some geographic patterns of haplotype relationships are evident (Fig. 6). The ancestral haplotype 3 is both the most common and the most widely distributed haplotype, being found in Western, Central, Eastern Ranges and Southern populations. The remaining haplotypes associated with Western, Central and Eastern Ranges regions are all closely related to this ancestral haplotype demonstrating a higher level of past connectivity among most mainland populations. The exceptions to this include the highly differentiated clade of haplotypes 14, 15 and 16, found in the Eastern Coastal and the Southern populations (the South-Eastern clade). While these geographic regions appear to have haplotypes that are highly divergent from other mainland haplotypes, both populations also possess a single haplotype from the major ancestral clade. Haplotype 1 was also highly diverged from all other haplotypes and characterized all individuals from the Insular population.
Fig. 5. Geographic distribution of COI haplotypes across the North Island with sample size represented by the size of each pie. Inset shows the COI haplotype network with identical colours representing haplotypes (shared haplotypes indicated by *)
Fig. 6. Maximum Parsimony Network for the COI region. Sizes of circles represent the number of individuals with each haplotype. Line lengths reflect the number of base substitutions.

Patterns of haplotype sharing for the Control Region showed high diversity with only one haplotype shared between two populations (Haplotype 17 shared between Central and Eastern Range populations) (Fig. 7). A statistical parsimony network analysis constructed using the Control Region alignment was highly reticulated. This resulting network was complex and did not reveal haplotype relationships any more clearly than that seen in the tree in Figure 3 and therefore is not displayed here.
Fig. 7. Geographic distribution of Control Region haplotypes across the North Island with sample size represented by the size of each pie (shared haplotypes indicated by *)
Population genetic divergence

Genetic divergence among populations was evident from statistical AMOVA tests performed in Arlequin. For the COI region, haplotype diversity across the North Island of New Zealand showed significant population differentiation ($F_{ST} = 0.49$, $P<0.0001$). Almost all pair-wise comparisons produced significant $F_{ST}$ values. Following a strict Bonferroni correction ($\alpha=0.003$), all remained significant except for the pair-wise contrasts of the Western vs Central populations ($F_{ST}=0.08$, $P=0.0628$) and Central vs Eastern Range populations ($F_{ST}=0.27$, $P=0.0032$) (Table 2). Genetic differentiation was also highly significant among sample locations when considering nucleotide diversity ($\Phi_{ST}=0.80$, $P<0.0001$). Pair-wise comparisons showed that genetic divergence across the North Island was significant for all population contrasts except for comparisons of the Western and Central, Central and Eastern Ranges, and Southern and Eastern Coastal contrasts (Table 2).

Table 2. $F_{ST}$ values (below diagonal) and $\Phi_{ST}$ values (above diagonal) for the COI. Significant results ($P<0.05$) are in bold. Significant results following strict Bonferroni correction ($P<0.003$) are indicated with *

<table>
<thead>
<tr>
<th>Population</th>
<th>Insular</th>
<th>Western</th>
<th>Central</th>
<th>Eastern Ranges</th>
<th>Eastern Coastal</th>
<th>Southern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insular</td>
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<td></td>
<td></td>
<td>0.94*</td>
<td>0.99*</td>
<td>0.92*</td>
</tr>
<tr>
<td>Western</td>
<td>0.59*</td>
<td></td>
<td>0.12</td>
<td>0.26*</td>
<td>0.86*</td>
<td>0.71*</td>
</tr>
<tr>
<td>Central</td>
<td>0.84*</td>
<td>0.08</td>
<td></td>
<td>0.27</td>
<td>0.86*</td>
<td>0.64*</td>
</tr>
<tr>
<td>Eastern Ranges</td>
<td>0.69*</td>
<td>0.17*</td>
<td>0.27</td>
<td></td>
<td>0.84*</td>
<td>0.67*</td>
</tr>
<tr>
<td>Eastern Coastal</td>
<td>0.93*</td>
<td>0.42*</td>
<td>0.62*</td>
<td>0.48*</td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>Southern</td>
<td>0.78*</td>
<td>0.27*</td>
<td>0.41*</td>
<td>0.33*</td>
<td>0.56*</td>
<td></td>
</tr>
</tbody>
</table>

AMOVA analysis for the Control Region showed a lower but still significant $F_{ST}$ value ($F_{ST}=0.23$, $P<0.0001$) for haplotype diversity compared to the COI region, due to the high level of within-population variation demonstrated by the Control Region (77% of the variation). All pair-wise comparisons were significant apart from Central and Eastern Ranges divergence as well as contrasts of the Southern population with all other mainland populations except the Eastern Coastal area (Table 3). AMOVA analysis also showed significant population divergence when considering nucleotide diversity with a significant $\Phi_{ST}$ of 0.71 ($P<0.0001$). When considering nucleotide diversity, population differentiation was significant for all pair-wise comparisons except when contrasting the Central and Eastern Ranges populations (Table 3).
Table 3. \( F_{ST} \) values (below diagonal) and \( \Phi_{ST} \) values (above diagonal) for the Control Region. Significant results (P<0.05) are in bold. Significant results following strict Bonferroni correction (P<0.003) are indicated with *

<table>
<thead>
<tr>
<th>Population</th>
<th>Insular</th>
<th>Western</th>
<th>Central</th>
<th>Eastern Ranges</th>
<th>Eastern Coastal</th>
<th>Southern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insular</td>
<td></td>
<td>0.42*</td>
<td>0.62*</td>
<td>0.59*</td>
<td>0.91*</td>
<td>0.81*</td>
</tr>
<tr>
<td>Western</td>
<td>0.21*</td>
<td></td>
<td>0.17*</td>
<td>0.17*</td>
<td>0.85*</td>
<td>0.73*</td>
</tr>
<tr>
<td>Central</td>
<td>0.25*</td>
<td>0.11*</td>
<td></td>
<td>0.09</td>
<td>0.84*</td>
<td>0.66*</td>
</tr>
<tr>
<td>Eastern Ranges</td>
<td>0.31*</td>
<td>0.18*</td>
<td>0.13</td>
<td></td>
<td>0.85*</td>
<td>0.71*</td>
</tr>
<tr>
<td>Eastern Coastal</td>
<td>0.38*</td>
<td>0.24*</td>
<td>0.30</td>
<td>0.36*</td>
<td></td>
<td>0.35*</td>
</tr>
<tr>
<td>Southern</td>
<td>0.23*</td>
<td>0.08</td>
<td>0.11</td>
<td>0.19</td>
<td>0.27*</td>
<td></td>
</tr>
</tbody>
</table>

The concatenated dataset was used to derive overall population divergence statistics. The overall \( F_{ST} \) value showed significantly different haplotype variation between populations (\( F_{ST} = 0.20, P<0.0001 \)). Pair-wise comparisons were all significant except comparisons between Central and Western populations and between the Southern population and all mainland sites (Table 4). The overall AMOVA \( \Phi_{ST} \) result for nucleotide divergence showed highly significant population isolation (\( \Phi_{ST} = 0.74, P<0.0001 \)). Pair-wise comparisons were all significant at the \( \alpha=0.05 \) level. After applying Bonferroni correction (\( \alpha=0.003 \)), three populations were no longer significant including Western vs Central, Central vs Eastern Ranges and Eastern Coastal vs Southern comparisons. The Insular population showed the highest levels of divergence for all pair-wise comparisons with mainland populations. The Eastern Coastal population also showed highly significant levels of divergence from all populations except the Southern population (Table 4).

Table 4. \( F_{ST} \) values (below diagonal) and \( \Phi_{ST} \) values (above diagonal) for the concatenated dataset. Significant results (P<0.05) are in bold. Significant results following strict Bonferroni correction (P<0.003) are indicated with *

<table>
<thead>
<tr>
<th>Population</th>
<th>Insular</th>
<th>Western</th>
<th>Central</th>
<th>Eastern Ranges</th>
<th>Eastern Coastal</th>
<th>Southern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insular</td>
<td></td>
<td>0.83*</td>
<td>0.90*</td>
<td>0.84*</td>
<td>0.90*</td>
<td>0.80*</td>
</tr>
<tr>
<td>Western</td>
<td>0.20*</td>
<td></td>
<td>0.16</td>
<td>0.24*</td>
<td>0.84*</td>
<td>0.73*</td>
</tr>
<tr>
<td>Central</td>
<td>0.24*</td>
<td>0.09</td>
<td></td>
<td>0.17</td>
<td>0.82*</td>
<td>0.65*</td>
</tr>
<tr>
<td>Eastern Ranges</td>
<td>0.30*</td>
<td>0.15*</td>
<td>0.18*</td>
<td></td>
<td>0.81*</td>
<td>0.68*</td>
</tr>
<tr>
<td>Eastern Coastal</td>
<td>0.33*</td>
<td>0.18*</td>
<td>0.21*</td>
<td>0.28*</td>
<td></td>
<td>0.24</td>
</tr>
<tr>
<td>Southern</td>
<td>0.23*</td>
<td>0.07</td>
<td>0.09</td>
<td>0.17</td>
<td>0.20</td>
<td></td>
</tr>
</tbody>
</table>
**Pair-wise divergence and dating estimates**

Pair-wise comparisons between populations were calculated using the Tamura distance method and a gamma level of 0.015 in Arlequin as the closest approximation to the HKY + G model as selected by jModel test for the COI dataset. Comparisons based on the Control Region divergence data were calculated using the Tamura distance method and a gamma level of 0.306. For the COI data, the Insular population showed consistently high levels of divergence from all mainland populations (0.89-1.46%). Distances between mainland sites were generally low (0.02-0.07%). The exceptions to this pattern were the high levels of divergence between the Eastern Coastal and Southern populations when compared to the remaining mainland populations. Divergence of the Southern population to all Western, Central and Eastern Ranges populations varied from 0.88% - 0.94%. The Eastern Coastal population had the highest levels of divergence from all other populations (1.21%-1.50%) except the Southern population (0.13%). Interestingly, the most significant divergence between all populations was between the Eastern Ranges and the Eastern Coastal populations (1.50%) (Table 5).

Table 5. Corrected (net) average pairwise COI nucleotide divergences between populations (below diagonal) and corresponding percentage pairwise differences (diagonal and above) following pair-wise contrasts between populations for the COI region

<table>
<thead>
<tr>
<th>Population</th>
<th>Insular</th>
<th>Western</th>
<th>Central</th>
<th>Eastern Ranges</th>
<th>Eastern Coastal</th>
<th>Southern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insular</td>
<td>0.00%</td>
<td>1.41%</td>
<td>1.41%</td>
<td>1.46%</td>
<td>1.21%</td>
<td>0.89%</td>
</tr>
<tr>
<td>Western</td>
<td>9.21</td>
<td>0.17%</td>
<td>0.02%</td>
<td>0.07%</td>
<td>1.45%</td>
<td>0.88%</td>
</tr>
<tr>
<td>Central</td>
<td>9.22</td>
<td>0.16%</td>
<td>0.08%</td>
<td>0.07%</td>
<td>1.46%</td>
<td>0.89%</td>
</tr>
<tr>
<td>Eastern Ranges</td>
<td>9.53</td>
<td>0.45%</td>
<td>0.46%</td>
<td>0.23%</td>
<td>1.50%</td>
<td>0.94%</td>
</tr>
<tr>
<td>Eastern Coastal</td>
<td>7.86</td>
<td>9.44%</td>
<td>9.52%</td>
<td>9.83%</td>
<td>0.42%</td>
<td>0.13%</td>
</tr>
<tr>
<td>Southern</td>
<td>5.77</td>
<td>5.74%</td>
<td>5.81%</td>
<td>6.11%</td>
<td>0.88%</td>
<td>0.83%</td>
</tr>
</tbody>
</table>

Analysis of the Control Region dataset produced similar patterns to the COI except for the two discrepancies mentioned in previous analyses: the higher levels of variability characterizing each population and the alternative positioning of the Insular population closer to the mainland sites (Table 6). The Insular population showed medium levels of divergence from mainland sites (0.27%-0.43%) apart from the Eastern Coastal and Southern populations (3.17%-4.28%). Eastern Coastal and Southern populations were highly derived from all other populations (Southern 2.91-3.17%, Eastern Coastal 3.86-4.28%) and showed a medium level of divergence from one another (0.83%).
Table 6. Corrected average pairwise difference (below diagonal) and corresponding percentage pairwise differences (diagonal and above) following pair-wise contrasts between populations for the Control Region

<table>
<thead>
<tr>
<th>Population</th>
<th>Insular</th>
<th>Western</th>
<th>Central</th>
<th>Eastern Ranges</th>
<th>Eastern Coastal</th>
<th>Southern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insular</td>
<td>0.14%</td>
<td>0.27%</td>
<td>0.42%</td>
<td>0.43%</td>
<td>4.28%</td>
<td>3.17%</td>
</tr>
<tr>
<td>Western</td>
<td>1.01</td>
<td>0.59%</td>
<td>0.12%</td>
<td>0.12%</td>
<td>3.86%</td>
<td>2.75%</td>
</tr>
<tr>
<td>Central</td>
<td>1.59</td>
<td>0.44%</td>
<td>0.60%</td>
<td>0.05%</td>
<td>3.96%</td>
<td>2.85%</td>
</tr>
<tr>
<td>Eastern Ranges</td>
<td>1.64</td>
<td>0.44%</td>
<td>0.20%</td>
<td>0.55%</td>
<td>4.03%</td>
<td>2.91%</td>
</tr>
<tr>
<td>Eastern Coastal</td>
<td>16.15</td>
<td>14.57</td>
<td>14.94</td>
<td>15.19</td>
<td>0.93%</td>
<td>0.83%</td>
</tr>
<tr>
<td>Southern</td>
<td>11.96</td>
<td>10.38</td>
<td>10.76</td>
<td>10.96</td>
<td>3.13</td>
<td>2.54%</td>
</tr>
</tbody>
</table>

To date the divergence between isolated populations of rifleman, two alternative mutation rates were used for each mitochondrial region. Estimated population divergence dates varied widely depending on the gene region and the mutation rate used (Table 7). Upper estimates using the most reliable mutation rate for the COI (0.0032 s/s/my) indicated that separation of the Insular population occurred approximately 2 mya, as did divergence of both the Eastern Coastal and Southern populations. Populations across the Central mainland including Western, Central and Eastern Ranges had significantly more recent divergence estimates ranging from 30,000-100,000 years ago. Divergence dates using the Control Region data were significantly more recent, ranging from 1kya-500kya.
Table 7. Estimates of population divergence dates based on two alternative substitution rates for the COI and Control Region mitochondrial regions

<table>
<thead>
<tr>
<th></th>
<th>Cytochrome Oxidase I</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µ</td>
<td>0.0032 s/s/my</td>
<td>0.0075 s/s/my</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insular</td>
<td>West</td>
<td>Central</td>
<td>Eastern R</td>
<td>Eastern C</td>
</tr>
<tr>
<td>Insular</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western</td>
<td>2.208</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>2.209</td>
<td>0.038</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eastern R</td>
<td>2.283</td>
<td>0.108</td>
<td>0.110</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Eastern C</td>
<td>1.883</td>
<td>2.263</td>
<td>2.281</td>
<td>2.356</td>
<td>0.000</td>
</tr>
<tr>
<td>Southern</td>
<td>1.383</td>
<td>1.376</td>
<td>1.392</td>
<td>1.465</td>
<td>0.211</td>
</tr>
<tr>
<td></td>
<td>Control Region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µ</td>
<td>0.04 s/s/my</td>
<td>0.2 s/s/my</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insular</td>
<td>West</td>
<td>Central</td>
<td>Eastern R</td>
<td>Eastern C</td>
</tr>
<tr>
<td>Insular</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western</td>
<td>0.033</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>0.053</td>
<td>0.015</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eastern R</td>
<td>0.054</td>
<td>0.015</td>
<td>0.007</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Eastern C</td>
<td>0.536</td>
<td>0.483</td>
<td>0.495</td>
<td>0.504</td>
<td>0.000</td>
</tr>
<tr>
<td>Southern</td>
<td>0.396</td>
<td>0.344</td>
<td>0.357</td>
<td>0.363</td>
<td>0.104</td>
</tr>
</tbody>
</table>

Discussion

General patterns of diversity in North Island rifleman

The results of this analysis indicate that geographically isolated North Island rifleman populations are generally characterised by low intra-population levels of both haplotype and nucleotide diversity. This pattern of low intra-population genetic diversity is not uncommon in New Zealand taxa, which have experienced a series of events likely to drastically reduce population sizes. New Zealand has been subjected to significant periods of marine transgression throughout history, particularly during the Oligocene, during which only a small proportion of New Zealand is likely to have been emergent (Fleming 1962b; Cooper and Cooper 1995). Investigations into the impacts of this event on levels of diversity in surviving species indicate that the
Oligocene submersion resulted in a significant genetic bottleneck for several taxa, including the New Zealand wrens (Cooper and Cooper 1995). Following the Oligocene submersion, the New Zealand landscape has been affected by periods of inundation, mountain uplift, volcanism and glaciation, all of which are considered forces of change that have resulted in the division of populations and the depletion of genetic variation in terrestrial species (Baker et al. 1995; Trewick 2001; Lloyd 2003; Buckley et al. 2009; Buckley et al. 2010; Goldberg et al. 2011). The paucity of genetic variation demonstrated for North Island rifleman populations is therefore likely to be reflective of the series of restrictive influences on New Zealand land taxa through history.

Both haplotype and nucleotide diversity indices of variation for both the COI and Control Region of the mitochondrial genome showed significant genetic differentiation between populations, indicating a lack of contemporary gene flow between current populations. All populations of rifleman across the North Island contained a unique set of haplotypes and only a single haplotype was shared across multiple populations for both the COI and Control Region data. Phylogenetic analyses identified three distinct clades across the North Island, consisting of an Insular clade, a South-Eastern clade and a Mainland clade.

*Divergence between Insular and Mainland populations*

Network, Phylogenetic and AMOVA analyses consistently showed significant divergence of the Insular population from mainland sites. Phylogenetic Neighbour Joining analyses positioned the Insular site differently depending on which mitochondrial region was used. Control Region sequences suggest a closer relationship between the Insular and mainland sites than suggested by the COI sequences. This discrepancy highlights the importance of using multiple gene regions in intra-specific phylogeographic analyses, as emphasized by other authors (e.g. Mila et al. 2007). The higher diversity characterized by the Control Region sequences compared to COI sequences is consistent with both theoretical and empirical predictions; as a non-coding region, the Control Region is theoretically subject to decreased stabilizing selection and typically shows higher variability than coding regions in other taxa (Ruokonen and Kvist 2002; Mila et al. 2007). However, the results obtained from the Control Region data may also reflect homoplasy. The placement of the Insular site within the mainland clade appears to be explained predominantly by a higher sequence similarity with four individual from the Western population. While Control Region sequences had higher diversity compared to COI sequences, two of the three variable sites with shared nucleotides between Insular and Western populations were indels (insertion/deletion) within long G-repeat regions of the Control Region. High-repeat regions may exhibit hyper-
variability due to slippage in the DNA replication process (Viguera et al. 2001). Therefore, these shared indels may be an artifact of saturation at hyper-variable sites, rather than reflecting a more recent shared ancestry or gene flow. Homoplasy has long been recognized as a potential complexity in phylogenetic analyses as it can often misguide interpretation of patterns of connectivity among phylogenetic groups (Galtier et al. 2006).

While the COI region of the mitochondrial genome is known as an effective means of ‘barcoding’ species (Kerr et al. 2007), high levels of intraspecific variation for COI are uncommon in birds (Hebert et al. 2004). This conserved nature of the COI mitochondrial gene makes intraspecific phylogeographic studies relatively difficult unless deep divergence is evident. The results of the analysis performed here indicate that rifleman appear to have enough intra-specific variation in the COI region to indicate deep divergence times between populations.

Data from both the COI and the concatenated dataset indicate that the Insular population is significantly diverged from all mainland populations. AMOVA FST and ΦST values were highly significant for all pair-wise comparisons and pair-wise divergence between the Insular and mainland sites varied between 0.89% and 1.46%, approaching the upper limits of within-species divergence in other birds (Hebert et al. 2004). This consistent separation of insular and mainland sites indicates historically restricted gene flow. Island sites are often characterized by divergence from mainland sites due to the presence of dispersal barriers which may decrease levels of gene flow between island and mainland locations (e.g. Alcaide et al. 2009). The level of differentiation between the Insular and mainland sites is comparable to that seen in highly diverged populations separated by significant geographic barriers (e.g. Friesen et al. 2007; Hare et al. 2008) and in some cases approach levels of differentiation found between separate species (e.g. kiwi, Baker et al. 1995; Burbridge et al. 2003). Divergence dating indicated that Insular rifleman have been isolated from the mainland for a significant period of time. Little Barrier Island is an emergent volcano, with an origin dated at approximately 3 million years ago (mya) with subsequent eruptions approximately 1.6 mya (Lindsay et al. 1999). Geological and botanical evidence indicates that the island has geochemical and floristic affinities with the Coromandel ranges on the mainland (Hamilton and Atkinson 1961; Lindsay et al. 1999). Further evidence from molecular studies on Oligosoma skinks and distributional data on other terrestrial species indicate that Little Barrier Island and the mainland Coromandel region are likely to have formed a land-bridge at least once during the Pleistocene, during which glacial periods would have caused significant lowering of sea-levels (Turbott 1961; Chapple et al. 2008; Hare et al. 2008). There is substantial controversy over the use of molecular clocks in dating divergence times between populations or species (Hedges and Kumar 2004) and therefore the dating estimates conveyed here
should be interpreted with caution. However the estimates from this study are calculated using a mutation rate specifically for the COI gene region in passerines and derived from an extensive mitochondrial genome review across numerous avian orders (Pacheco et al. 2011). Additionally, the dating estimate of approximately 2 million years obtained in this study for divergence between the Insular site and all mainland sites is consistent with the geological evidence for the establishment of the island from volcanic origins. While this estimate predates the supposed second volcanic period of Little Barrier Island (Lindsay et al. 1999), the age of lineage separation is generally more recent than haplotype coalescence indicates, as haplotypes diverge from a common ancestor prior to the population divergence (Edwards 1997). Rifleman may have colonized the island from established populations on the mainland via a land-bridge with the Coromandel Peninsula following the re-establishment of forest cover after volcanic dormancy. Coromandel populations of rifleman are now regionally extinct (Robertson et al. 2007), therefore patterns of gene flow between Insular and mainland Coromandel sites could not be investigated. However the clear phylogenetic separation of the Insular population in this analysis suggests that Little Barrier Island was subsequently isolated from the mainland by rising sea levels, and has remained so ever since. The presence of monophyletic groups distinguished by large phylogenetic gaps is likely to be the result of long-term zoogeographic barriers to dispersal (Avise et al. 1987). As rifleman are poor fliers (Higgins et al. 2001) the isolation of Little Barrier Island via a significant marine barrier appears to have been sufficient to result in the deeply diverged separation of the population shown by the mitochondrial data.

It is possible that the level of divergence found for the Insular population has been inflated by extreme genetic drift in a population that has suffered a strong bottleneck, thus artificially inflating the estimates of divergence time. Genetic drift is likely to result in higher levels of fixation of slightly deleterious mutations in small populations (Johnson and Seger 2001). Changes may therefore accumulate and be fixed in small isolated populations faster than in populations with higher effective population sizes. While the effects of genetic drift on levels of divergence between comparative populations may have inflated the dating estimates presented here, it is clear that the Insular population is significantly genetically divergent from all other mainland populations and therefore appears to have highly restricted gene flow historically.

In addition to the significant divergence between the Insular and mainland sites, the Insular population was characterized by very low genetic diversity, particularly for the COI data. While no within-population genetic diversity was evident from COI sequences, the addition of the Control Region sequences showed that more than
one haplotype is present within the population. Therefore, while genetic diversity within the Insular population is still very low, there is evidence of multiple maternal lines retained within the population. Both haplotype and nucleotide diversity were extremely low in the Insular population. The antiquity of the lineage combined with the low diversity evident in both COI and Control Region indicates that the population has been subject to a genetic bottleneck, which appears to have persisted until recent times. Isolated island sites are often found to have lower levels of diversity compared to continuous mainland populations as small, isolated populations are more susceptible to lineage extinction via genetic drift and selective sweeps (Avise et al. 1984; Frankham 1997; Clegg et al. 2002; Hansson and Richardson 2005). This may result in faster acquisition of reciprocal monophyly (Templeton 1980; Neigel and Avise 1986). While the estimates of effective population size in this study should be interpreted with caution, the results indicate that the Insular rifleman population has an extremely low effective population size. The bottleneck may have begun some time ago, however the island has a recent history of anthropogenic impacts which have caused genetic bottlenecks in other species (e.g. tuatara, MacAvoy et al. 2007). Invasive species such as rats (*Rattus exulans*), dogs (*Canis lupus familiaris*), goats (*Capra hircus*) and cats (*Felis catus*) were introduced to Little Barrier Island prior to its designation as a sanctuary in 1895 (Watson 1961), and reached significant population sizes before the last invasive species were eradicated in 2004. Invasive species have had an extremely detrimental impact on most native species in New Zealand (Veitch et al. 2011) and it is likely that the combined effects of invasive pest species and the loss of the mature canopy trees used by rifleman for nesting (Gray 1969) had a significant impact on the population size of Little Barrier Island rifleman in recent time. The fact that the Insular population sampled here is extremely genetically depauperate may therefore be a result of recent anthropogenic impacts, although the small habitat size is likely to have also had an ongoing effect.

**Genetic connectivity through the mainland**

Isolation of the Southern and Eastern Coastal populations

The analysis of genetic connectivity of North Island rifleman populations across the mainland unexpectedly identified a deep and consistent divergence of the Southern and Eastern Coastal populations from all other mainland sites. Haplotype network, phylogenetic and AMOVA results were highly consistent in the significant separation of these populations from all other mainland sites, indicating an historical lack of connectivity between the South-Eastern part of the North Island and the remainder of the mainland. $\Phi_{ST}$ values were highly
significant and supported a deep divergence between the South-Eastern populations and the remainder of the mainland populations. The significant majority of individuals from the Southern and Eastern Coastal populations had haplotypes which differed from all other mainland populations by 17 fixed substitutions for the Control Region and 11 fixed substitutions for the COI and showed deep divergences in phylogenetic analyses. Highly significant $\Phi_{ST}$ values combined with dating estimates indicate that these populations have been isolated from all other mainland populations for 1-2 million years, indicating an historical lack of gene flow since the late Pliocene/early Pleistocene. During the late Pliocene the lower third of the North Island was inundated between the Wanganui-Hawkes Bay basins (Fleming 1962b; Trewick and Bland 2011). Rifleman from the Southern population were characterized by the highest levels of molecular diversity across the North Island range indicating that the population may not have suffered a significant contraction during this time which supports the suggestion that the Wellington area (represented by the Southern population) was emergent throughout this time (Fleming 1962b; Trewick and Bland 2011). The high level of genetic divergence evident between the Southern population and all other mainland sites (except the Eastern Coastal region) is likely to have resulted from the inundation of the lower North Island which would have completely restricted gene flow between the Southern population and those situated north of the inundation. It is possible that gene flow may have occurred between the southern North Island populations and populations resident in the northern South Island. Both geological and biological evidence from a range of taxa including kiwi (*Apterix australis*) (Baker *et al.* 1995), *Oligosoma* skinks (Greaves *et al.* 2007; O’Neill *et al.* 2008) and moa (Dinornithiformes) (Bunce *et al.* 2009) indicate that a land-bridge connected the top of the South Island to the bottom of the North Island during the late Pliocene and possibly into the Pleistocene (Trewick and Bland 2011). Further sequence analysis including Northern South Island populations will help to clarify this hypothesis.

The sequence analysis performed here indicates that the Eastern Coastal population may have been colonized from the larger Southern population. It is estimated that links between the South of the North Island and the South-East coast occurred approximately 1 mya (Bunce *et al.* 2009; Trewick and Bland 2011), supporting the possible colonization from the Southern population to the Eastern Coastal population during the mid-Pleistocene. Alternatively, populations of rifleman may have remained in the Eastern Coastal region during the submersion by surviving on emergent islands (Trewick and Bland 2011), re-establishing gene flow with the Southern population as sea levels dropped and continuous forest was re-established. The presence of endemic species of tree weta (*Hemideina trewicki*) restricted to this region support the emergence and isolation of this
part of the North Island during the inundation of the lower North Island (Trewick and Bland 2011). The levels of divergence between Eastern Coastal and other mainland populations are the highest across all pair-wise population comparisons, providing support for the long-term isolation of this population.

Contemporary patterns of genetic differentiation between the Southern and Eastern Coastal populations indicate a lack of current gene flow, with divergence estimated in the late Pleistocene. While the two populations are closely connected in both haplotype network and phylogenetic analyses, they share no contemporary haplotypes and form two distinct sub-clades within the various phylogenetic trees. Following potential colonization of the Eastern Coastal region from the south, the populations appear to have been isolated during the late Pleistocene, perhaps as a result of restriction to glacial refugia (e.g. Buckley et al. 2009). Despite apparent divergence in the late Pleistocene, my analysis indicates that both the Southern and Eastern Coastal populations contain a small number of migrants which appear to have originated from other mainland populations. Both populations have 1-2 individuals with a haplotype that is closely related to the mainland ancestral (most common) haplotype and are significantly divergent from all other individuals within their resident populations (Fig. 4). The haplotypes of these individuals differ from the ancestral haplotype by a single substitution and are therefore likely to be evidence of recent migration between populations in the south-east and the remainder of the mainland populations. This result is interesting as it indicates a series of diverse historical influences on patterns of connectivity across the mainland. While the late Pliocene inundation may have caused significant separation between the South-East populations and the remainder of the mainland, inter-glacial periods following the reduction in sea levels within the Pleistocene may have caused forest corridors to be re-established, allowing genetic connectivity. Overall, the patterns of genetic diversity suggest a dramatic change in the paths of connectivity between the mainland populations over time.

While the Southern population is characterized by relatively high levels of diversity compared to all other North Island populations, with the exception of the single individual with a mainland haplotype, all individuals within the Eastern Coastal population share an identical COI haplotype and only two haplotypes are present for the Control Region data. This result indicates that the population has undergone a genetic bottleneck at some stage, possibly as a result of the founder event following colonization or due to recent anthropogenic impacts. While the effective population size of this population is among one of the highest estimated (based on the formula $\theta = 2Ne\mu$), the single individual with a significantly divergent haplotype artificially inflates estimates of diversity.
and effective population size in this population using this method. Population sub-structure and migration rates have been shown to affect estimates of Ne (Waples 2010), indicating that these estimates should be treated with caution.

Continuity through the central mainland

The results of this analysis indicate relatively recent genetic connectivity between the remaining mainland populations across the North Island. No significant phylogenetic divergence was detected among the Western, Central and Eastern Range populations, with shallow branch lengths characteristic of the entire ‘mainland’ clade. While populations share only a single haplotype for both the COI and Control Region sequences, all haplotypes were closely related by variations of only 1-2 nucleotide substitutions from the ancestral haplotype. The shallow divergence evident in all analyses resulted in more contemporary divergence date estimates (30,000-100,000 years ago) indicating that these populations have had ongoing gene flow until the mid-late Pleistocene. The shallow differentiation of sequences and star-shaped haplotype network evident from analysis of the mainland clade are both evidence of population expansion (Rogers and Harpending 1992; Dixon 2011), possibly as a result of expansion from Pleistocene glacial refugia. The central North Island during the Pleistocene was subject to the impacts of both glacial maximums and high levels of volcanism (Trewick and Bland 2011). Glacial cycles caused mature forest to be restricted to Northland during the height of glacial periods through the Pleistocene, with small, isolated patches of forest refugia elsewhere (McGlone et al. 2001; Buckley et al. 2009), while volcanic episodes caused substantial range disruption through the Central Plateau (Trewick and Bland 2011). The effects of both glacial cycles and volcanism on the current distribution of New Zealand taxa have caused several species to exhibit molecular characteristics of population restriction and expansion through the central mainland of the North Island (Lloyd 2003; Murphy et al. 2006; Chapple et al. 2008; Hare et al. 2008; Fouquet et al. 2010; Goldberg et al. 2011). As a mature forest species that are reliant on mature native trees for nesting and foraging requirements (Higgins et al. 2001), rifleman would have been restricted to forest refugia during Pleistocene glacial and volcanic periods. Given the higher levels of gene flow between mainland rifleman populations, and the evidence of range expansion, two alternative explanations for the phylogeographic patterns seen are possible: 1) that rifleman were restricted to a single large refugium during Pleistocene range restriction, subsequently expanding from this refugium during inter-glacials (e.g. Buckley et al. 2009; Goldberg et al. 2011) or 2) that rifleman were restricted to multiple isolated regional refugia, re-establishing connectivity as mature forest expanded during warming phases and following volcanic cessation.
Given the relatively low diversity across this central range and the shallow coalescence evident from these results, it seems likely that rifleman were located within a single refugium and have subsequently expanded from this common refuge following forest re-establishment. There is evidence that in current times, a very low level of gene flow has occurred between these populations, demonstrated by the significantly diverged haplotype frequencies between the Western and Eastern populations which are likely to have been intermittently connected through the Central population.

Patterns of diversity across the mainland populations indicated low overall diversity, however the Western population had a relatively high diversity of closely related haplotypes compared to Central and Eastern Range sites. Of additional interest is the presence of non-synonymous COI mutations only in the Western population. Six individuals in the Western population contained non-synonymous mutations, four of which shared the same mutation. While these four individuals may share the same mitochondrial DNA due to relatedness, they were sampled from several distant regions within the population, indicating that they are unlikely to be close relatives. Additionally, Fu’s F was significantly negative for the Western population which may indicate either population expansion or selection. The Western site is situated within a restricted forest on Mount Egmont, which was subjected to a series of volcanic eruptions through the late Pleistocene, until approximately 7000 years ago (Trewick and Bland 2011). The star-shaped pattern of haplotype relationships in the Western population is indicative of population expansion, and is likely to be the result of recent colonisation of the mountain following volcanic dormancy. However, the presence of non-synonymous mutations isolated to the Western population may mean that the Western population has been subject to unique selective forces. Further analyses will be needed to clarify this. While an increased number of nonsynonymous mutations may reflect higher levels of genetic drift in a small population causing fixation of slightly deleterious mutations (Johnson and Seger 2001), the Western population has some of the highest levels of diversity and highest population sizes of the North Island rifleman range; therefore this drift explanation is less likely to explain the non-synonymous variation observed within the Western population.
Timing of fragmentation

The intraspecific phylogeographic analyses reported here appear to overwhelmingly support the influence of historical geological and climatological forces in shaping patterns of population divergence among North Island rifleman populations. The separation of three main clades within the North Island rifleman distribution indicates three deeply divergent lineages with historically reduced gene flow. While dating estimates should be interpreted with caution, the extensive divergence evident from network, phylogenetic and AMOVA analyses indicate that late Pliocene and early Pleistocene events have caused substantial disruption to the distribution of rifleman populations. However, habitat fragmentation and the introduction of invasive pest species have reduced the range of many New Zealand taxa (e.g. Beauchamp and Worthy 1988; Millener 1989; Brook 2000) and are likely to have substantially contributed to further declines of rifleman populations across the North Island. Current levels of gene flow are clearly lower than historic levels of gene flow, indicating possible effects of both habitat fragmentation and predator impacts. The low levels of diversity evident from both the Insular and Eastern Coastal populations provide two examples of the possible effects of these impacts and the ongoing decline of populations acts to promote the fragmentation and isolation of current populations.

The evidence supports several historic periods of importance in the creation of the contemporary genetic landscape of North Island rifleman. Fluctuating sea levels during the late Pliocene/early Pleistocene appear to have contributed to the creation and isolation of the Insular population from all other mainland populations and the separation of the South-Eastern part of the mainland North Island. During the Pleistocene, glacial fluctuations and periods of extensive volcanism are likely to have caused rifleman populations to contract to common refugia, forcing the species to undergo a genetic bottleneck through most of its range. Inter-glacials may have been characterized by the expansion of mature forest cover, allowing the colonization of new populations (e.g. the Eastern Coastal population) and the re-establishment of connectivity between previously isolated populations before late Pleistocene glaciations may have re-located barriers to gene flow, removing connectivity between Southern & Eastern Coastal populations, but re-establishing limited connections between these and the remaining mainland populations. Finally, the contemporary impacts of invasive species and habitat clearance have served to emphasize any existing isolation between rifleman populations and are likely to have caused lower contemporary gene flow among all populations and recent significant genetic bottlenecks in some populations (e.g. the Insular population).
Conclusions and future analyses

Analysis of molecular indices of diversity across separated rifleman populations uncovered significant divergence between three geographic regions of the North Island. These include the Insular site, the South-Eastern populations (Eastern Coastal and Southern populations) and the remaining Mainland populations (Western, Central and Eastern Ranges populations) (Fig 8).

Figure 8. Summary of population connectivity from genetic analyses. Three clades were identified across the North Island distribution including the Insular population, Mainland populations and the South-Eastern populations (from North to South).

Population divergence appears to relate predominantly to natural geological and climatic events, including marine inundation, glaciation and volcanism throughout the late Pliocene and Pleistocene. Recent anthropogenic impacts serve to exacerbate the effects of fragmentation and may have contributed to genetic
bottlenecks in several key areas. This analysis has provided important information on the evolutionary history of rifleman in New Zealand and the relative impacts of various mechanisms of change for the New Zealand landscape, however further work should address the following:

1) Analysis of nuclear molecular markers should be used in addition to the mitochondrial analysis performed in this study

2) The availability of fossil or museum specimens from the recently-extinct Coromandel population should be investigated. These samples could address the hypothesis that the Insular population is derived from an historical Coromandel population

3) Additional sampling of northern South Island samples would allow a test of the hypothesis that South-Eastern populations shared gene flow with the South Island during the Pleistocene bridging of the Cook Strait

4) Sampling should be extended. Previous preliminary morphological work has suggested that the sole remnant Northland population may represent a separate sub-species (Pierce 1994). Additional sampling of this population should investigate the divergence between this population and other mainland and insular locations. In addition, intermediate sites between currently sampled locations should be targeted to investigate levels of gene flow over smaller geographic distances. Along with increased sample sizes of the central mainland populations, and use of nuclear data, this would clarify the extent of current gene flow among mainland populations.

5) The dating estimates provided here are approximations based on published mutation rates for the gene regions used in this study. Coalescence modelling would permit a more comprehensive analysis of specific mutation rates, effective population sizes, demographic changes over time, levels of gene flow and divergence dating which would more specifically address some of the hypotheses outlined in this study (e.g. Mailund et al. 2011).
References


APPENDIX I. Variable nucleotide sites characterizing 17 mitochondrial DNA haplotypes for the Cytochrome Oxidase I gene

| Nucleotide Position | 12 | 24 | 36 | 78 | 104 | 186 | 210 | 248 | 252 | 267 | 279 | 309 | 321 | 338 | 353 | 354 | 519 | 537 | 551 | 566 | 567 | 579 | 582 | 611 | 618 |
|---------------------|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| HAP1                | C  | A  | A  | G  | G  | A   | G   | G   | T   | C   | T   | C   | T   | A   | T   | A   | G   | T   | A   | T   | A   | T   | A   | T   | C   | T   |
APPENDIX II. Variable nucleotide sites characterizing 33 haplotypes for a 377 bp section of the Control Region

| Nucleotide Position | HAP1 | HAP2 | HAP3 | HAP4 | HAP5 | HAP6 | HAP7 | HAP8 | HAP9 | HAP10 | HAP11 | HAP12 | HAP13 | HAP14 | HAP15 | HAP16 | HAP17 | HAP18 | HAP19 | HAP20 | HAP21 | HAP22 | HAP23 | HAP24 | HAP25 | HAP26 | HAP27 | HAP28 | HAP29 | HAP30 | HAP31 | HAP32 | HAP33 |
|---------------------|------|------|------|------|------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 1 1 1 1 1 1 1 1 1 | G    | G    | T    | A    | A    | A    | A    | G    | T    | G     | A     | G     | A     | G     | C     | T     | A     | C     | T     | A     | T     | C     | G     | C     | A     | G     | C     | T     | G     | A     | T     | C     | T     | -     | -     | A     | T     |
| 2 3 5 5 5 6 8 9 0 0 | 1 1 1 2 2 3 5 6 7 8 | 9 0 1 2 4 5 5 8 8 | 1 1 1 3 3 4 4 4 5 5 | 5 6 0 1 2 3 5 7 6 7 |
| 2 2 2 2 2 2 2 2 2 2 | 3 3 3 3 3 3 3 3 3 3 | 3 3 3 3 3 3 3 3 3 3 | 3 3 3 3 3 3 3 3 3 3 | 3 3 3 3 3 3 3 3 3 3 |

Control Region (377bp)
HAP2


HAP2


HAP2


HAP3


HAP3


HAP3

. . . . . . . . . . . . . . . G . T . . . C . G . . . . . . . . . . . . . . . G - - -
CHAPTER THREE: Geographic variation in structural characteristics of North Island rifleman vocalisations
**Introduction**

The description and analysis of variation in behavioural traits such as vocalisation is an important first step in any investigation into the drivers of evolutionary change in the communication systems of birds. While avian vocalisations can vary on numerous levels both within and between species (Kroodsma and Miller 1982; Catchpole and Slater 2008), the analysis of geographic variation in vocal signals within a species is particularly informative for any discussion on the evolution of species-specific communication traits (Becker 1982; Mundinger 1982; Irwin and Price 1999). Additionally, the description of geographic variation in communication traits is vital in informing conservation management plans for threatened species, as regional variations may impact the ability of individuals from variable environments to successfully breed (Rowe 2007; Guerra et al. 2008; Laiolo et al. 2008).

In general, avian vocal development may be influenced to different extents by innate genetic or physiological mechanisms as well as social experience, learning and memory (Kroodsma 1982; Kroodsma 1996; Catchpole and Slater 2008). As such, vocal traits show evidence of an evolutionary history affected by both selective and non-selective forces of change and are particularly likely to vary between populations with increasing geographic distances (Mundinger 1982; Beecher and Brenowitz 2005). Geographic variations in avian vocalisations have been investigated in an extensive variety of species, and the existence of spatial variants, often termed ‘dialects’ is a common and widespread phenomenon (Mundinger 1982).

In the oscine passerines or ‘songbirds’, the description and analysis of geographically variable songs has been widely documented. Song learning has been found to be a ubiquitous trait across the oscine passerines (Kroodsma 1982; Catchpole and Slater 2008). Learned songs have the potential to rapidly diverge across spatial scales due to isolation by distance combined with the propensity for rapid vocal change both within and across generations in these species (Lynch et al. 1989; Baker 1994; Baker et al. 2001; Irwin et al. 2001). Vocal variants evolve rapidly in such species due to learning errors during song ontogeny combined with social learning which may spread and stabilise population-specific vocalisations (Jenkins and Baker 1984; Lynch et al. 1989). The function of song in male competition and female mate choice (Catchpole 1982; Catchpole and Slater 2008) contributes to the speed at which geographic variations spread and stabilise, as sexual selection for
shared vocal features causes individuals to adapt and specialise to common repertoires (MacDougall-Shackleton et al. 2001; Nowicki et al. 2001; Ellers and Slabbekoorn 2003; Haavie et al. 2004; Nelson and Soha 2004), reinforcing dialect boundaries. For this reason, geographic variation in learned songs has been extensively investigated in this group and has important consequences for population divergence and speciation (Payne et al. 2000; Irwin et al. 2001; Kirkpatrick and Ravigne 2002; Slabbekoorn and Smith 2002a; Ellers and Slabbekoorn 2003; Lachlan and Servedio 2004).

Studies on vocal variation in sub-oscines or non-passerines are less common, however they provide important comparative systems with which to test theories concerning the evolution of vocal learning and the development of variation within species (e.g. Kroodsma 1996; Lovell and Lein 2004; Robertson et al. 2009). While there are some notable exceptions (Psittaciformes, Apodiformes and Charadriiformes) (Kroodsma and Baylis 1982), most sub-oscine passerines and non-passerines are thought to exhibit innate vocal development, with few species using vocal learning as part of vocal ontogeny (Kroodsma and Baylis 1982). The development of spatial vocal variants in these species is usually explained as an effect of isolation and genetic drift (e.g. Bretagnolle and Genevois 1997; Isler et al. 2005; Nyari 2007). Alternatively, environmental variation creating variable selection pressure on signal design may result in geographic variations in innate or physiologically determined vocal traits (Richards and Wiley 1980; Wiley and Richards 1982; Brown and Handford 2000; Lohr et al. 2003). Numerous studies on species with learned vocal traits have demonstrated how geographic vocal variations may evolve due to spatial variations in environmental features including habitat structure, predator presence or level of acoustic competition (Hunter and Krebs 1979; Doutrelant et al. 1999; Doutrelant et al. 2000; Doutrelant and Lambrechts 2001; Leader et al. 2002; Slabbekoorn and Smith 2002a; Ripmeester et al. 2010). In the same way, non-learned vocal traits may also be subject to similar forces of environmentally-driven natural selection (e.g. Ippi et al. 2011), although it is likely that population divergence will require longer evolutionary timeframes for non-learned traits. Calls in sub-oscine or non-passerine species are often used to mediate territorial interactions or mate-choice decisions (Falls and McNicholl 1979; Clapperton and Jenkins 1987; Speirs and Davis 1991; Bard et al. 2002), indicating that the implications of geographic variation in call structure and design in these groups may be underestimated.
In this chapter, I investigate geographic variation in the calls of the North Island rifleman (*Acathisitta chloris granti*). Rifleman are a particularly interesting species on which to focus for studies of vocal variation.

Rifleman are one of only two extant species within the Acanthisittidae family which has been identified as the most basal global passerine lineage and sister sub-order to both sub-oscines and oscines (Ericson *et al.* 2002). Vocal ontogeny has never been studied in the rifleman, and their basal phylogenetic position to the sub-oscine and oscine divergence means that the mechanism of vocal development for the species is completely unknown. However, as the divergence of this family predates the oscine-sub-oscine divergence, it is likely that rifleman exhibit innate vocal development as seen in most other avian lineages and this will be assumed in this study.

The sub-species distribution is currently widespread but highly fragmented, with populations generally isolated to discontinuous mountain ranges across the North Island (Pierce 1994; Smith and Westbrooke 2004; Robertson *et al.* 2007). Rifleman flight morphology is characterised by reduced wing size and a reduced tail, therefore they have limited flight capacity and are assumed to be relatively poor dispersers (Higgins *et al.* 2001). Gene flow between isolated populations is therefore highly unlikely given the current sub-population structure. Rifleman are considered a rare vicariant component of the New Zealand avifaunal assemblage (Cooper and Millener 1993; Ericson *et al.* 2002; Worthy *et al.* 2010), however it is unknown whether the currently fragmented distribution of rifleman is a product of ancient geological processes which have caused comparable fragmentation in other species’ distributions (Cooper and Millener 1993), or if it is due to more recent anthropological impacts such as human-mediated predator invasions and habitat clearance. The evidence presented in this thesis (see Chapter Two) represents the first analysis of molecular population variation on the sub-species and suggests that populations have been isolated for an extensive period of time and that the current distribution is largely influenced by ancient geological processes. Rifleman are a socially monogamous and cooperatively breeding species (Gray 1969; Sherley 1985) who occupy territories which are generally maintained via mutual avoidance (Cameron 1990) but at times have overlapping boundaries (Cameron 1990, pers. obs.). Both male and female rifleman use a small repertoire of simple, high frequency calls (Krull *et al.* 2009) to maintain contact within pairs and family groups and to mediate inter-group territorial interactions (Sherley 1985; Higgins *et al.* 2001). While the repertoire of rifleman has been described as a series of ‘contact calls’ (Sherley 1985; Higgins *et al.* 2001), until now a complete description of the call repertoire and any analysis of call function or variation has never been attempted for the species. Rifleman do not appear to patrol or advertise territory boundaries using calls (Sherley 1985), but both sexes respond to playback of conspecific
calls within territory boundaries and therefore vocalisations appear to be used in negotiation and defence of territory boundaries.

The aim of this study is to investigate geographic variation in the fine-scale structural and temporal components of adult rifleman vocalisations. The findings of this study are potentially important on several levels. As the basal lineage in the passerine phylogeny, a description of geographic vocal variation across the sub-species distribution may inform on general theories regarding vocal learning and development. Additionally, as individual variations in vocal behaviour influence territory establishment and mate attraction in many species, population variations may create a barrier to gene flow between variable populations (Leader et al. 2002; Nelson and Soha 2004; Balakrishnan and Sorenson 2006), acting as a pre-mating reproductive barrier (Baker 1982; MacDougall-Shackleton and MacDougall-Shackleton 2001). As such, vocal variability on broad scales is implicated in population divergence and perhaps in some cases, speciation (Payne et al. 2000; Irwin et al. 2001; Kirkpatrick and Ravigne 2002; Slabbekoorn and Smith 2002a; Ellers and Slabbekoorn 2003; Lachlan and Servedio 2004). The evolution of geographically variable vocal signatures in species which exhibit spatially discontinuous distributions are particularly likely to have important consequences for the evolutionary potential of the species as a whole, as population division is a powerful driving force of speciation (Phillimore et al. 2008). The characterisation of geographic variation in vocal signals is therefore critical to investigations into the evolution of population divergence in most bird species which use acoustic systems for communication. This is particularly so for species which show naturally or anthropogenically fragmented distributions, as vocal variation across spatial scales may have conservation implications (Rowe 2007; Guerra et al. 2008; Laiolo et al. 2008).

Using spectral analysis of digitised rifleman calls from six geographically separated regions this study seeks to address the following questions:

1) Is there evidence of geographic variation in fine-scale structural or temporal components of rifleman calls?

2) Could geographic variation in the calls of adult rifleman result in decreased recognition in territorial defensive signalling?
I predicted that the protracted period of isolation between current rifleman populations found in Chapter Two, combined with low dispersal capability in adults would result in the evolution of geographical variation in vocal signals in this species. To investigate the biological significance of call variation, I then performed playback experiments using resident territorial male rifleman. If calls are functional in mediating both inter-pair and extra-pair interactions within the territory, geographically variable signals may result in reduced recognition between conspecifics in a territorial context.

**Methods**

*Research sites*

Vocal variation across the local rifleman sub-species’ distribution was sampled in six geographically separated populations across the North Island in New Zealand. Locations available for these analyses included Warawara Forest (-35° 35’S, 173° 25’E), Little Barrier Island (-36° 19’S, 175° 07’E), Taranaki National Park (-39° 29’S, 174° 06’E), Pureora Forest Park (-38° 57’S, 175° 59’E), Boundary Stream Mainland Island (-39° 06’S, 176° 48’E) and Tararua Forest Park (-40° 86’S, 175° 41’E) (Fig.1). These sites represent Northern, Insular, Western, Central, Eastern and Southern regions of the North Island. While all sites are forested high-altitude regions, they each differ in both their level of isolation from other sample locations and in habitat features such as forest density and level of browsing and predator impacts. Rifleman also occur at different densities in each site. All study regions are managed by the New Zealand Department of Conservation (DoC). All activities and data collection carried out as part of this research were permitted by both the New Zealand Department of Conservation (Banding permit 2010/025; Regional bird handling permits WE-25869-FAU, NO-26310-FAU, AK-27236-FAU, WK-28729-RES, WA-27986-FAU) and The University of Auckland Ethics Committee (R762).
Vocal variation

Recordings

Rifleman were located by monitoring established walking tracks and predator-control trap-lines used to maintain mustelid and rodent control regimes and, therefore, provide high levels of area-coverage of the habitat. Rifleman were detected by ear or by sight and followed to obtain recordings which were continued until target individuals were lost. Position fixes for each recording site were noted using a Garmin GPSMAP 60CSx GPS device (Garmin Ltd.). Using observations of approximate territory sizes at each site, this allowed me to make conservative judgements regarding individual identification to ensure recordings represented genuine individual replicates, despite the lack of individual identity information via banding. Recordings were made using a 722 Digital Audio Recorder (Sound Devices, LLC) or a Sony MZ-R909 Minidisk Walkman (Sony Inc.), recorded at a sample rate of 44.1 kHz with 16 bit precision. A Sennheiser microphone (model K6 ME 66) (Wennebostel, Wedemark, Germany) with a frequency response of 40-20,000 Hz ± 2.5 dB was also used. Calls recorded from Tararua Forest Park were obtained using five minute recording periods at randomly positioned sample
locations along established loop tracks within the forest park. Sample locations were situated at a minimum of 250 m apart to ensure independence. Recordings were made using an Olympus LS-10 PCM field recorder (Olympus Corporation) in mp3 format with a sample rate of 44.1 khz with 16-bit precision and an in-built microphone with a frequency response of 70-20,000 Hz. As rifleman are small birds (5 – 8 grams (Higgins et al. 2001)) and adult males and females spend up to 90% of their time in close contact with each other (Sherley 1985), distinguishing calls of males and females within a continuous recording was not possible. Calls are therefore categorized based on an independent recording and are considered to represent a single biological unit for analysis, even if a pair were present during the recording. Rifleman produce a limited call repertoire of three contact call elements, an aggressive chat, a solicitation chatter, a nest visitation call, a feeding call, an alert call and an alarm call (Krull et al. 2009; Withers, S. unpublished data). I selected the three contact call types (the ‘Chuck’, ‘Zip’ and ‘Pip’, Fig. 2) for this investigation as they are used in all conspecific interactions.

![Fig. 2. Spectrogram image (frequency versus time) of the ‘Chuck’, ‘Zip’ and ‘Pip’ calls demonstrating temporal and spectral characteristics of contact calls](image)

Analysis

Digital recordings were transferred and visualized using Raven Pro Version 1.3Interactive Sound Analysis Software (Cornell Lab of Ornithology, Bioacoustics Research Program). Only recordings with minimal background noise and a high signal-to-noise ratio were selected for analysis. As noise did not overlap with rifleman call signals, filtering was not necessary. Both waveform and spectrogram views were used to visualize recorded calls and measurements were made using Raven’s Measurement tools. Spectrograms were created...
using a Hann Window with a Fast Fourier Transform (FFT) of 1024 points and 50% overlap, giving a frequency resolution of 43.1 Hz. Time measurements were taken from the Waveform view using the Spectrogram view as a guide. To analyse whether inter-individual variation was greater than intra-individual variation, an ANOVA was performed using all individuals with more than five calls recorded. A significant result in assessing individually consistent call parameters from this analysis (see Results) then justified using a single example of each call type to represent each individual/recording site. Three contact calls (the Chuck, Pip and Zip) were measured for Minimum Frequency (Hz), Maximum Frequency (Hz), Bandwidth (Hz) (Maximum Frequency - Minimum Frequency), Quarter 1 Frequency (Hz), Quarter 3 Frequency (Hz), Peak Frequency (Hz) and Centre Frequency (Hz) and Duration (s) (Table 1).

All statistical analyses were performed in JMP Version 8 (SAS Institute Inc.). Data normality and equality of variances for each call measurement within each population were tested using the Shapiro-Wilks test and the Levene’s Test, respectively. Appropriate transformations were made for any non-normal data. Welch’s Test was used as a substitute for ANOVA for any data showing unequal variance. As many measurement variables were correlated, separate Principal Component Analyses (PCA) for each call type were performed on original non-transformed data to reduce dimensionality and co-linearity. To determine whether calls could be categorized according to their source population, Principal Components were then used in a Linear Discriminant Function Analysis (DFA) for each call type. Due to the small sample size, the Northern (Warawara) population was removed from all analyses on the Zip call.

Table 1. Call parameters measured in analysis of rifleman calls

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Unit</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum Frequency</td>
<td>Hertz (Hz)</td>
<td>Lowest Frequency</td>
</tr>
<tr>
<td>Maximum Frequency</td>
<td>Hertz (Hz)</td>
<td>Highest Frequency</td>
</tr>
<tr>
<td>Quarter 1 Frequency</td>
<td>Hertz (Hz)</td>
<td>Mean frequency for the first quarter of the call</td>
</tr>
<tr>
<td>Quarter 3 Frequency</td>
<td>Hertz (Hz)</td>
<td>Mean frequency for the third quarter of the call</td>
</tr>
<tr>
<td>Peak Frequency</td>
<td>Hertz (Hz)</td>
<td>Frequency with maximum amplitude</td>
</tr>
<tr>
<td>Centre Frequency</td>
<td>Hertz (Hz)</td>
<td>Frequency at the half-time point of the call</td>
</tr>
<tr>
<td>Bandwidth</td>
<td>Hertz (Hz)</td>
<td>Maximum frequency minus Minimum Frequency</td>
</tr>
<tr>
<td>Duration</td>
<td>Seconds (s)</td>
<td>End Time minus Start Time</td>
</tr>
</tbody>
</table>
Playback Experiments

Study sites

Boundary Stream Mainland Island, in the Hawkes Bay region of North Island, New Zealand (Fig. 3) was selected as the location for playback experiments. This site has some of the highest densities of rifleman in the North Island (Robertson et al. 2007), making it ideal for vocal playback experiments. Calls selected for playback treatments were taken from Little Barrier Island (Hauturu) in the Hauraki Gulf (Insular), Pureora Forest Park in the Central Plateau (Central) and Boundary Stream Mainland Island in the Eastern ranges (Eastern Ranges). These sites produced a gradient of distances from the target location, representing Foreign Far, Foreign Near and Local treatments (Fig. 3).

Recordings for playback

Recordings for playback were taken from the three North Island sites described above, representing three treatment types. Details of recording methods are described above. Only uncompressed sound files were used for playback stimuli. GPS locations for all recordings were noted to ensure that playback locations were
selected to represent individuals naive to treatment stimuli. Recordings for playback were made in the first half of the breeding season in 2008, 2009 and 2010.

**Stimulus preparation**

Recordings with minimal background noise and a high signal-to-noise ratio, without being overloaded were chosen for creation of playback stimuli. Two call types were selected for use in playback stimuli, the Pip and the Zip. These call types were chosen as they represent the calls used most often during rifleman conspecific interaction. The Chuck call was not used due to difficulties in normalizing such highly broadband calls while still maintaining an adequate signal-to-noise ratio (Fig. 2). To select calls for playback, recordings were viewed in Spectrogram form using Raven Pro. Spectrograms were created using a Hann Window with an FFT value of 512 points. Overlap was set to 50% giving a frequency resolution of 86.1 Hz. A single call of each type (Zip or Pip) was selected from each recording where available. To avoid pseudoreplication, a pool of ten different calls of each type was selected for each population, ensuring that every call within all call types was from a different individual (based on GPS locations and known territory sizes, see above). Calls were then filtered to remove all noise below 6 kHz, to avoid playing target individuals location-specific ‘noise signatures’ (Baker and Logue 2003). Calls were normalized using R Analysis Software (Version 2.12.0, The R Foundation for Statistical Computing) to ensure that all stimuli were at an equal amplitude. A 20 s playback file was then generated by selecting a single Pip call and a single Zip call and placing them within a blank file, repeating each call four times in total. Each playback stimulus was therefore identical in length and overall structure, with only the calls themselves varying. Due to the constant movement of focal rifleman, natural sound intensity or amplitude is difficult to estimate, and has never been investigated for this species. However, my observations have shown that rifleman often produce the same calls with varying amplitude. Amplitude of the stimuli used in playback was therefore calibrated by ear at a distance of eight meters to ensure that playback was within the natural range of rifleman calls.

**Treatments**

Two controls and three treatments were used for playback experiments. Control 1 consisted of speaker noise (i.e. static) and was used to ensure that any differences in responses were not the product of different reactions...
to the speaker itself. Control 2 was an endemic heterospecific bird species’ call, the whitehead (*Mohoua albicilla*), that was normalized to the same amplitude as all rifleman calls. Whitehead was selected as the species is sympatric with rifleman in most sites where rifleman occur, including at Boundary Stream Mainland Island (Robertson et al. 2007). However, they are rarely involved in direct aggressive interactions with rifleman and were therefore unlikely to cause a stress response. The three treatments represented calls from populations with varying degrees of separation from the target population, and consisted of a Local (Boundary Stream), Foreign Near (Pureora Forest: distance 121 km) or Foreign Far (Little Barrier Island: distance 362 km) sourced recordings.

**Playback procedure**

Playback experiments were carried out in the first half of the breeding season (November 2011) when birds are either nest building or incubating (Gray 1969; Sherley 1985). Calls were broadcast using an iPod Nano (Apple Inc.) or Zen Neeon MP3 player (Creative Technology Ltd.), connected to a Divoom iTour-20 speaker (Divoom Technology Co. Ltd.) which was placed on the ground. The speaker was placed within the territory boundaries to simulate territory intrusion. Territory positions and boundaries were known from recording and monitoring research carried out in previous seasons. Observations were carried out from a vantage point situated 8 meters from the speaker. As rifleman rarely fly into open, exposed areas, the speaker was always placed beneath a tree with adequate cover, ensuring that targeted individuals could approach the speaker whilst still being within cover. While both male and female rifleman are responsive to playback, only male rifleman were targeted for playback experiments as they are typically more reliably responsive compared to females (pers. obs). Playback was not initiated if the target individual was within five meters of the speaker. Thirty territorial males were targeted for playback in total. Each male received both controls and a single treatment type (Local, Foreign Near or Foreign Far), resulting in 10 target males per treatment type. All 10 males within each treatment type were played different playback files to ensure no pseudoreplication (Kroodsma et al. 2001). For all males presented with the Local treatment, I ensured that the territory location of the target male was not within the same neighbourhood (defined by regional walking tracks) as the recording location. I could therefore be confident that no male had previous experience with the playback file used. The same treatment type was not played in adjacent territories, ensuring that neighbouring birds could not hear playback experiments in other territories and habituate to the treatment type. Playbacks were performed between the hours of 0800 and 1800
and were only carried out on clear days with minimal wind. Each target individual was played Control 1, Control 2 and a single Treatment type in randomized order (random sampling without replacement) to control for the effects of habituation. Each playback sequence lasted nine minutes in total. The 20 s playback file was looped continuously for a two minute playback period, followed by a two minute observation period. Five minutes were then allowed between each playback sequence to ensure target individuals returned to normal behaviour (non-vigilant foraging or preening). Each individual was therefore exposed to a cumulative trial time of 27 minutes.

Responses

Rifleman approach towards the playback was deemed a response if the target individual came within 5 m of the speaker and exhibited ‘searching’ behaviour (see below for a definition of searching). The presence or absence of rifleman within both a five m radius and a two meter radius of the speaker during either the playback period or the observation period was recorded. The closest approach distance within the total four minute observation time (two minute playback plus two minute post-playback observation) and the time taken to reach this closest distance was then noted. The presence or absence of searching behaviour was recorded, defined as the directed movement of individuals without foraging or preening, often looking around with the body flattened). The time at which the individual departed the playback area was also recorded. Departure was defined as being the moment when the target individual left the five meter radius and stopped searching behaviour, returning to normal foraging or preening.

Playback Statistical Analysis

Because focal male rifleman did not respond to playback controls with an approach to the speaker or with searching behaviour, comparable data on closest approach distance and time to approach were not available to allow an analysis between responses to controls and treatments. The complete absence of an approach response was therefore deemed ‘no response’ for control playbacks and further analyses were conducted solely on the three treatments including Local, Foreign Near and Foreign Far. Treatment effects on the Closest Distance response were analysed using a Kruskal-Wallis non-parametric test, while treatment effects on the Time to Closest Distance response were analysed using an ANOVA.
**Results**

**Geographic Vocal Variation**

North Island rifleman populations showed geographic differentiation in several call characteristics for all three call types (Table 2). Measurements of call parameters were generally correlated (correlation statistic > 0.4) (Table 3). As a MANOVA could not be performed on the correlated variables, geographic differences in call measurements were compared using a series of one-way ANOVA’s with Tukey Kramer comparisons. In the case of unequal variances, Welch’s Test was performed. Variation between individuals was found to be significantly higher than variation within individuals for all measurements for all call types (Welch’s Test, \( F > 5.9456, P < 0.0001 \)). This justified including a single representative call from each individual, and including individuals which had just one call replicate recorded.

Table 2. Results of analysis of variance tests of eight acoustic variables between six populations. Significant results are shown in bold.

<table>
<thead>
<tr>
<th>Acoustic Variable</th>
<th>Chuck F_102</th>
<th>Chuck P</th>
<th>Pip F_97</th>
<th>Pip P</th>
<th>Zip F_76</th>
<th>Zip P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum Frequency (Hz)</td>
<td>10.87</td>
<td>&lt;0.0001</td>
<td>2.43</td>
<td>0.0412</td>
<td>3.50</td>
<td>0.0115</td>
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<tr>
<td>Maximum Frequency (Hz)</td>
<td>3.45</td>
<td>0.0066</td>
<td>1.40</td>
<td>0.2564</td>
<td>3.83</td>
<td>0.0071</td>
</tr>
<tr>
<td>Bandwidth (Hz)</td>
<td>1.605</td>
<td>0.1669</td>
<td>2.26</td>
<td>0.0811</td>
<td>&lt;0.0001</td>
<td>6.92</td>
</tr>
<tr>
<td>Duration (s)</td>
<td>1.38</td>
<td>0.2375</td>
<td>7.35</td>
<td>&lt;0.0001</td>
<td>3.94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Q1 Frequency (Hz)</td>
<td>11.015</td>
<td>&lt;0.0001</td>
<td>1.19</td>
<td>0.3411</td>
<td>6.40</td>
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<td>Q3 Frequency (Hz)</td>
<td>8.44</td>
<td>&lt;0.0001</td>
<td>1.03</td>
<td>0.4235</td>
<td>3.13</td>
<td>0.0099</td>
</tr>
<tr>
<td>Peak Frequency (Hz)</td>
<td>7.25</td>
<td>&lt;0.0001</td>
<td>1.06</td>
<td>0.4051</td>
<td>4.98</td>
<td>0.0014</td>
</tr>
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<td>Centre Frequency (Hz)</td>
<td>10.83</td>
<td>&lt;0.0001</td>
<td>0.97</td>
<td>0.4532</td>
<td>4.83</td>
<td>0.0017</td>
</tr>
</tbody>
</table>
Table 3. Correlation matrices for Chuck, Pip and Zip calls (top to bottom) showing correlation coefficients between call parameter measurements

<table>
<thead>
<tr>
<th>Chuck</th>
<th>Min Freq</th>
<th>Max Freq</th>
<th>Bandwidth</th>
<th>Duration</th>
<th>Q1 Freq</th>
<th>Q3 Freq</th>
<th>Peak Freq</th>
<th>Centre Freq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min Freq</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max Freq</td>
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<td>Duration</td>
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<td>Q1 Freq</td>
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<td>0.06</td>
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<td>Q3 Freq</td>
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<td>0.10</td>
<td>0.69</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>Peak Freq</td>
<td>0.37</td>
<td>0.29</td>
<td>0.04</td>
<td>0.26</td>
<td>0.78</td>
<td>0.64</td>
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</tr>
<tr>
<td>Centre Freq</td>
<td>0.56</td>
<td>0.50</td>
<td>0.12</td>
<td>0.18</td>
<td>0.93</td>
<td>0.82</td>
<td>0.82</td>
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</table>

<table>
<thead>
<tr>
<th>Pip</th>
<th>Min Freq</th>
<th>Max Freq</th>
<th>Bandwidth</th>
<th>Duration</th>
<th>Q1 Freq</th>
<th>Q3 Freq</th>
<th>Peak Freq</th>
<th>Centre Freq</th>
</tr>
</thead>
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<tr>
<td>Min Freq</td>
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<tr>
<td>Max Freq</td>
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<tr>
<td>Bandwidth</td>
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<tr>
<td>Duration</td>
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<td>-0.31</td>
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<tr>
<td>Q1 Freq</td>
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<td>0.85</td>
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<tr>
<td>Q3 Freq</td>
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<td>0.98</td>
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<td>Peak Freq</td>
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<td>0.89</td>
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</tr>
<tr>
<td>Centre Freq</td>
<td>0.54</td>
<td>0.94</td>
<td>0.18</td>
<td>-0.22</td>
<td>0.95</td>
<td>0.98</td>
<td>0.95</td>
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<table>
<thead>
<tr>
<th>Zip</th>
<th>Min Freq</th>
<th>Max Freq</th>
<th>Bandwidth</th>
<th>Duration</th>
<th>Q1 Freq</th>
<th>Q3 Freq</th>
<th>Peak Freq</th>
<th>Centre Freq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min Freq</td>
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<td></td>
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<td>Max Freq</td>
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<tr>
<td>Q1 Freq</td>
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<td>0.43</td>
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<tr>
<td>Q3 Freq</td>
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<td>0.06</td>
<td>0.01</td>
<td>0.86</td>
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</tr>
<tr>
<td>Peak Freq</td>
<td>0.50</td>
<td>0.42</td>
<td>0.03</td>
<td>0.21</td>
<td>0.91</td>
<td>0.85</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Centre Freq</td>
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<td>0.45</td>
<td>-0.02</td>
<td>0.13</td>
<td>0.97</td>
<td>0.93</td>
<td>0.94</td>
<td>1</td>
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</table>

Six call measurements showed significant population differences for the Chuck call (Table 2). The Eastern population had significantly lower values for Maximum Frequency when compared to the Northern population (Table 4, Fig. 4). The Eastern and Central populations had lower Minimum, Quarter 1, Peak and Centre Frequencies than the Southern population and lower Quarter 3 frequencies compared to Northern, Insular and Southern populations. The Central population also had lower Quarter 1 and Centre frequencies compared to the Northern population. For the Pip call, only two measurements were found to be significantly variable between populations (Table 2). The Eastern population had a significantly lower minimum frequency and shorter calls compared to the Southern (Table 5, Fig. 5). The Western site also had longer calls compared to the Eastern site. For the Zip call, all measurements were significantly different between populations. Eastern and Insular sites
had significantly lower frequencies for Minimum, Quarter 1, Peak and Centre Frequencies compared to the
Southern site (Table 6, Fig. 6). The Southern population had a significantly narrower bandwidth than Western,
Central and Insular regions. The Western population had a significantly higher maximum frequency compared
to the Eastern population.
Table 4. Summary statistics for Chuck call from six geographically separated populations. Values are presented as means ± S.E.M

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Minimum Frequency (Hz)</th>
<th>Maximum Frequency (Hz)</th>
<th>Bandwidth (Hz)</th>
<th>Duration (s)</th>
<th>Quarter 1 Frequency (Hz)</th>
<th>Quarter 3 Frequency (Hz)</th>
<th>Peak Frequency (Hz)</th>
<th>Centre Frequency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern (Warawara)</td>
<td>6</td>
<td>6915 ± 315</td>
<td>14632 ± 480</td>
<td>7717 ± 510</td>
<td>0.016 ± 0.001</td>
<td>8836 ± 204</td>
<td>10781 ± 243</td>
<td>9324 ± 403</td>
<td>9640 ± 207</td>
</tr>
<tr>
<td>Insular (Little Barrier Island)</td>
<td>21</td>
<td>6556 ± 155</td>
<td>13658 ± 283</td>
<td>7103 ± 250</td>
<td>0.017 ± 0.001</td>
<td>8622 ± 200</td>
<td>10720 ± 208</td>
<td>9558 ± 356</td>
<td>9564 ± 207</td>
</tr>
<tr>
<td>Western (Taranaki)</td>
<td>7</td>
<td>6920 ± 218</td>
<td>14257 ± 350</td>
<td>7337 ± 385</td>
<td>0.015 ± 0.002</td>
<td>8515 ± 247</td>
<td>10275 ± 86</td>
<td>8810 ± 422</td>
<td>9358 ± 153</td>
</tr>
<tr>
<td>Central (Pureora)</td>
<td>14</td>
<td>6179 ± 140</td>
<td>13149 ± 377</td>
<td>6970 ± 346</td>
<td>0.014 ± 0.001</td>
<td>7881 ± 214</td>
<td>9745 ± 210</td>
<td>8207 ± 232</td>
<td>8650 ± 175</td>
</tr>
<tr>
<td>Eastern (Boundary Stream)</td>
<td>36</td>
<td>6021 ± 85</td>
<td>13187 ± 127</td>
<td>7166 ± 139</td>
<td>0.017 ± 0.001</td>
<td>8143 ± 100</td>
<td>9905 ± 87</td>
<td>8624 ± 124</td>
<td>8924 ± 88</td>
</tr>
<tr>
<td>Southern (Tararuas)</td>
<td>19</td>
<td>7220 ± 183</td>
<td>13789 ± 205</td>
<td>6569 ± 199</td>
<td>0.016 ± 0.001</td>
<td>9404 ± 122</td>
<td>10735 ± 119</td>
<td>9948 ± 146</td>
<td>10023 ± 131</td>
</tr>
</tbody>
</table>

Table 5. Summary statistics for Pip call from six geographically separated populations. Values are presented as means ± S.E.M

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Minimum Frequency (Hz)</th>
<th>Maximum Frequency (Hz)</th>
<th>Bandwidth (Hz)</th>
<th>Duration (s)</th>
<th>Quarter 1 Frequency (Hz)</th>
<th>Quarter 3 Frequency (Hz)</th>
<th>Peak Frequency (Hz)</th>
<th>Centre Frequency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern (Warawara)</td>
<td>7</td>
<td>7420 ± 338</td>
<td>10522 ± 123</td>
<td>3102 ± 438</td>
<td>0.034 ± 0.002</td>
<td>9782 ± 139</td>
<td>10108 ± 117</td>
<td>10034 ± 110</td>
<td>9973 ± 123</td>
</tr>
<tr>
<td>Insular (Little Barrier Island)</td>
<td>13</td>
<td>7552 ± 135</td>
<td>10619 ± 74</td>
<td>3067 ± 123</td>
<td>0.031 ± 0.001</td>
<td>9768 ± 81</td>
<td>10179 ± 76</td>
<td>10056 ± 91</td>
<td>10015 ± 81</td>
</tr>
<tr>
<td>Western (Taranaki)</td>
<td>6</td>
<td>7240 ± 235</td>
<td>10242 ± 229</td>
<td>3002 ± 217</td>
<td>0.040 ± 0.003</td>
<td>9554 ± 195</td>
<td>9870 ± 241</td>
<td>9848 ± 249</td>
<td>9769 ± 227</td>
</tr>
<tr>
<td>Central (Pureora)</td>
<td>15</td>
<td>7436 ± 218</td>
<td>10588 ± 132</td>
<td>3152 ± 178</td>
<td>0.033 ± 0.001</td>
<td>9710 ± 99</td>
<td>10175 ± 121</td>
<td>10006 ± 109</td>
<td>9983 ± 108</td>
</tr>
<tr>
<td>Eastern (Boundary Stream)</td>
<td>32</td>
<td>7331 ± 117</td>
<td>10702 ± 125</td>
<td>3371 ± 100</td>
<td>0.029 ± 0.001</td>
<td>9699 ± 106</td>
<td>10255 ± 116</td>
<td>10121 ± 124</td>
<td>10040 ± 109</td>
</tr>
<tr>
<td>Southern (Tararuas)</td>
<td>24</td>
<td>7944 ± 154</td>
<td>10805 ± 90</td>
<td>2860 ± 109</td>
<td>0.036 ± 0.001</td>
<td>9938 ± 77</td>
<td>10352 ± 90</td>
<td>10280 ± 97</td>
<td>10200 ± 88</td>
</tr>
</tbody>
</table>

Table 6. Summary statistics for Zip call from six geographically separated populations. Values are presented as means ± S.E.M

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Minimum Frequency (Hz)</th>
<th>Maximum Frequency (Hz)</th>
<th>Bandwidth (Hz)</th>
<th>Duration (s)</th>
<th>Quarter 1 Frequency (Hz)</th>
<th>Quarter 3 Frequency (Hz)</th>
<th>Peak Frequency (Hz)</th>
<th>Centre Frequency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insular (Little Barrier Island)</td>
<td>9</td>
<td>7356 ± 272</td>
<td>14258 ± 331</td>
<td>6902 ± 310</td>
<td>0.035 ± 0.003</td>
<td>9470 ± 246</td>
<td>10073 ± 230</td>
<td>9717 ± 222</td>
<td>9771 ± 223</td>
</tr>
<tr>
<td>Western (Taranaki)</td>
<td>10</td>
<td>7619 ± 270</td>
<td>14655 ± 389</td>
<td>7037 ± 317</td>
<td>0.039 ± 0.004</td>
<td>9888 ± 243</td>
<td>10633 ± 241</td>
<td>10211 ± 266</td>
<td>10181 ± 235</td>
</tr>
<tr>
<td>Central (Pureora)</td>
<td>6</td>
<td>7813 ± 402</td>
<td>14788 ± 331</td>
<td>6976 ± 294</td>
<td>0.032 ± 0.004</td>
<td>9683 ± 365</td>
<td>10365 ± 308</td>
<td>9934 ± 450</td>
<td>9991 ± 354</td>
</tr>
<tr>
<td>Eastern (Boundary Stream)</td>
<td>33</td>
<td>7540 ± 98</td>
<td>13809 ± 108</td>
<td>6270 ± 129</td>
<td>0.028 ± 0.001</td>
<td>9322 ± 83</td>
<td>10294 ± 88</td>
<td>9720 ± 95</td>
<td>9766 ± 80</td>
</tr>
<tr>
<td>Southern (Tararuas)</td>
<td>20</td>
<td>8114 ± 145</td>
<td>13863 ± 164</td>
<td>5749 ± 163</td>
<td>0.035 ± 0.002</td>
<td>10011 ± 165</td>
<td>10584 ± 152</td>
<td>10302 ± 167</td>
<td>10276 ± 157</td>
</tr>
</tbody>
</table>
Fig. 4. Chuck call measurements showing significant differences between populations. Bars show means ± s.e.m. Results of Tukey-Kramer post-hoc comparisons are shown (ABC)
Fig. 5. Pip call measurements showing significant differences between populations. Bars show means ± s.e.m. Results of Tukey-Kramer post-hoc comparisons are shown (ABC)

Fig. 6. Zip call measurements showing significant differences between populations. Bars show means ± s.e.m. Results of Tukey-Kramer post-hoc comparisons are shown (ABC)
Fig. 6 continued. Zip call measurements showing significant differences between populations. Bars show means ± s.e.m. Results of Tukey-Kramer post-hoc comparisons are shown (ABC)

To simplify comparisons between populations and orthogonalize variables, three separate principal components analyses were performed for each call type. For the Chuck call, 84% of the variation in the data was explained by the first three principal components. The first principal component (PC1) was positively associated with Quarter 1, Centre and Quarter 3 Frequencies and the Peak Frequency. PC2 was associated with both Maximum Frequency and Bandwidth and PC3 was predominantly explained by an increase in Duration (Table 7). Population variation is evident when PC1 is plotted against PC2 (Fig 7). The Eastern and Central populations have lower PC1 values (Q1, Q3, Peak and Centre Frequencies) compared to other populations. The Southern population has higher PC1 scores, but lower PC2 scores compared to all other populations, demonstrating a lower Maximum Frequency and Bandwidth.
For the Pip call, the first three principal components explained 96% of the variation. PC1 was associated with all frequency parameters except Minimum Frequency and Bandwidth. PC2 was explained predominantly by an increase in Bandwidth and a decrease in Minimum Frequency and PC3 was associated almost entirely with Duration (Table 7). When PC1 and PC2 are plotted, the Western and Southern populations appear to have the lowest and highest PC1 scores (Maximum, Q1, Q3, Peak and Centre Frequencies), respectively (Fig 8). The Southern population repeats the pattern found in the Chuck call, with higher PC1 (Frequency) and lower PC2 (Bandwidth) scores compared to all other populations.

For the Zip call, 91% of the variation was explained by the first three principal components. Factor loadings on the principal components showed that PC1 was explained by Quarter 1, Centre, Quarter 3 and Peak Frequencies, PC2 by Bandwidth and PC3 by Duration (Table 7). Plotting PC1 against PC2 results in Eastern and Insular populations having lower PC1 (Q1, Q3, Peak and Centre Frequencies) scores compared to other populations (Fig. 9). The Southern population is again characterised by higher PC1 (Frequency) and lower PC2 (Bandwidth and Maximum Frequency) scores than other populations.

Table 7. Factor loadings/eigenvalues of call variables with the first three principal components (PC1, PC2 and PC3) of each call type. Predominant associations are highlighted in bold

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>CHUCK PC1</th>
<th>CHUCK PC2</th>
<th>CHUCK PC3</th>
<th>PIP PC1</th>
<th>PIP PC2</th>
<th>PIP PC3</th>
<th>ZIP PC1</th>
<th>ZIP PC2</th>
<th>ZIP PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum Frequency</td>
<td>0.33</td>
<td>-0.26</td>
<td>-0.38</td>
<td>0.27</td>
<td>-0.61</td>
<td>0.07</td>
<td>0.32</td>
<td>-0.36</td>
<td>0.31</td>
</tr>
<tr>
<td>(Hz)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Frequency</td>
<td>0.32</td>
<td>0.53</td>
<td>-0.11</td>
<td>0.43</td>
<td>0.07</td>
<td>-0.06</td>
<td>0.27</td>
<td>0.45</td>
<td>0.51</td>
</tr>
<tr>
<td>(Hz)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bandwidth (Hz)</td>
<td>0.10</td>
<td>0.76</td>
<td>0.17</td>
<td>0.05</td>
<td>0.77</td>
<td>-0.13</td>
<td>0.00</td>
<td>0.71</td>
<td>0.23</td>
</tr>
<tr>
<td>Duration (s)</td>
<td>0.11</td>
<td>-0.15</td>
<td>0.88</td>
<td>-0.13</td>
<td>0.13</td>
<td>0.96</td>
<td>0.15</td>
<td>0.40</td>
<td>-0.72</td>
</tr>
<tr>
<td>Q1 Frequency</td>
<td>0.46</td>
<td>-0.13</td>
<td>-0.01</td>
<td>0.41</td>
<td>0.04</td>
<td>0.19</td>
<td>0.46</td>
<td>-0.05</td>
<td>-0.15</td>
</tr>
<tr>
<td>Q3 Frequency</td>
<td>0.42</td>
<td>0.03</td>
<td>-0.08</td>
<td>0.44</td>
<td>0.06</td>
<td>-0.02</td>
<td>0.44</td>
<td>-0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>Peak Frequency</td>
<td>0.40</td>
<td>-0.16</td>
<td>0.20</td>
<td>0.42</td>
<td>0.11</td>
<td>0.09</td>
<td>0.44</td>
<td>0.00</td>
<td>-0.19</td>
</tr>
<tr>
<td>Centre Frequency</td>
<td>0.47</td>
<td>-0.08</td>
<td>0.00</td>
<td>0.44</td>
<td>0.06</td>
<td>0.07</td>
<td>0.46</td>
<td>-0.06</td>
<td>-0.16</td>
</tr>
</tbody>
</table>
Fig. 7. Geographic variation in the Chuck call. Plot of population mean scores (error bars represent ± S.E.M.) from the first (PC1) and second (PC2) principal components. Collectively, PC1 and PC2 explain 72% of the variation in Chuck call measurement data.
Fig. 8. Geographic variation in the Pip call. Plot of population mean scores (error bars represent ± S.E.M.) from the first (PC1) and second (PC2) principal components. Collectively, PC1 and PC2 explain 85% of the variation in Pip call measurement data.
To test whether call variables could be used to discriminate between populations, the first three Principal Components (describing a minimum of 80% of the variation) for each call type were placed into three separate Discriminant Function Analyses corresponding to call types. Discriminant Functions produced from Principal Components for all three call types were not good predictors of source population. For the Chuck call, only 51% of calls were classified correctly using the first three Principal Components. Western, Eastern, Southern and Central populations had the highest classification rates (71%, 67%, 63% and 50% respectively) while Northern and Insular sites had low successful classification rates (33% and 14%, respectively). The first canonical discriminant function was negatively correlated with PC1 (Frequency) and positively correlated with PC3 (Duration) (Table 8). The second canonical function was associated predominantly with PC3 (Duration) while the third was correlated with PC2 (Bandwidth) and PC3 (Duration). The overall discriminant function analysis for the Pip call had a successful classification rate of 60%. Northern and Western populations had high
classification rates (86% and 83%) while Eastern, Insular, Central and Southern sites had lower successful classification rates (59%, 69%, 40% and 54%, respectively). The first canonical discriminant function correlated strongly with PC3 (Duration). The second function correlated most strongly with PC1 (Frequency), while the third was strongly associated with PC2 (Bandwidth). For the Zip call, the overall classification success rate of the Discriminant Function was 47%. While all populations had low successful classification rates, Southern, Eastern and Western populations had the highest success rates (56%, 52% and 50%, respectively), while Insular and Central populations had lower success rates (22% and 33% respectively). PC2 (Bandwidth) and PC3 (decreasing Duration) had stronger associations with the first canonical discriminant function. The second function was explained predominantly by a positive association with PC2 (Bandwidth), while the third canonical function was strongly associated with PC3 (decreasing Duration).

Table 8. Correlation coefficients of principal components with each canonical discriminant function for all three call types

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>CHUCK</th>
<th>PIP</th>
<th>ZIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Canon1</td>
<td>Canon2</td>
<td>Canon3</td>
</tr>
<tr>
<td>PC1</td>
<td>-0.57</td>
<td>0.07</td>
<td>0.25</td>
</tr>
<tr>
<td>PC2</td>
<td>0.34</td>
<td>-0.51</td>
<td>0.57</td>
</tr>
<tr>
<td>PC3</td>
<td>0.56</td>
<td>0.78</td>
<td>0.49</td>
</tr>
</tbody>
</table>

**Playback Experiments**

Male rifleman were not responsive (defined by speaker approach and searching behaviour) to either the speaker noise control or the whitehead control. Therefore, all subsequent analyses were done using the three treatment types only. All individuals responded to playback with searching behaviour and by approaching within 5 m of the speaker for all three treatment types. Eighty percent of individuals for all treatment types approached within 2 m of the speaker. Therefore, no effect of treatment was detected for these three response variables. While there appeared to be a trend toward closer approach distances with increasing distance from the source recording population, no statistical differences were found (Kruskal-Wallis Test, $X^2 = 1.76, P = 0.4143$) (Fig 10A). Similarly, focal individuals spent longer in response to calls from more distance source recording populations, however this result was not statistically significant (ANOVA, $F = 1.73, P = 0.1961$) (Fig. 10B).
Fig. 10. The closest approach distance (A) and total response time (B) in response to playback for Foreign Far (Little Barrier Island), Foreign Near (Pureora) and Local (Boundary Stream) treatments, showing mean values per treatment type ± s.e.m.

A test of response to Local versus all Foreign treatments combined again did not result in a significant difference for either Closest Distance response (Kruskal-Wallis Test, $X^2 = 0.96, P = 0.3275$) or Time to Closest Distance response (ANOVA, $F=2.29, P=0.1413$).

**Discussion**

*Geographic variation in rifleman calls*

Two of the three adult calls (the Chuck and Zip calls) analysed in this study were significantly variable between populations for a number of call measurements, particularly in frequency parameters. The Pip call was less variable between populations, but exhibited significant differentiation in Minimum Frequency and Duration. Analyses of population variation in individual vocal measurements indicated that individuals from the Eastern population used relatively low frequencies for all three call types compared to other populations. Southern populations showed the opposite trend, using higher frequencies for most frequency-related parameters for all call types. This use of higher frequencies (particularly for Minimum Frequency) resulted in a consistently reduced bandwidth in Southern populations for all call types, particularly the Zip call. Measures of duration
showed similar trends across both the Pip and Zip call types with a generally decreasing duration moving across the central North Island from Western to Eastern populations, and Southern populations having a medium-level duration in comparison to other populations.

Evidence of geographic variation in non-learned acoustic signals is most commonly explained by evolutionary drivers including genetic drift or population variation in environmental natural selection pressures (Isler et al. 2005; Campbell et al. 2010). A correspondence between molecular and vocal trait divergence across populations has been interpreted as evidence supporting the influence of population isolation followed by drift in creation of geographic vocal variation. The results of this study provide some support for a concordance between genetic and behavioural data. Results of Principal Components Analyses indicated that the Southern population was consistently separated from other populations on the basis of the two primary principal components PC1 and PC2 (representing higher frequency parameters and lower bandwidth respectively) for all three call types. This marked separation of the Southern population from other North Island populations is consistent with the genetic evidence presented in Chapter Two. The molecular analysis presented in this thesis indicates that the Southern population has been separated from other mainland populations for over two million years, the cause of which is likely to be the complete marine inundation of the lower third of the North Island. However, while the results of the vocal analysis presented in the current chapter appear to indicate that genetic divergence is accompanied by vocal divergence in the Southern population, other population comparisons do not show the same concordance between datasets. For example, the Insular population has also been found to exhibit marked molecular divergence from all mainland populations, however does not show a corresponding vocal divergence. Additionally, despite the significant variation demonstrated across fine-scale spectral parameters of rifleman calls, Discriminant Function Analyses failed to result in successful population classification, indicating that population variations are not consistent enough to demonstrate marked and reliable population vocal divergence.

Given the extensive period of time for which populations of North Island rifleman have been isolated (see Chapter Two), it seems remarkable that only fine-scale and inconsistent geographic variations in vocal calls are found in this study. It is possible that high variation across individuals within populations is obscuring any evidence of consistent population variations, as has been found in previous studies (Odom and Mennill 2012).
Error measurements for Principal Components Analyses for all three call types showed substantial variation across individuals in this study. It is possible that intra-population individual variations in call parameters may lead to weakened population-specific vocal traits and contributes to the lack of consistent and marked geographic variation in North Island rifleman. Further research on individual variation of adult calls is warranted to address this hypothesis. Individual variation analysis of the Zip call is particularly warranted as it is the most common call uttered by rifleman while foraging with conspecifics (see reference to the ‘ssip’ call in Higgins et al. (2001)). Additionally, a variation of the Zip call (higher amplitude and broader bandwidth) is used immediately prior to nest entry during shared incubation. This call is uttered upon arrival at the nest entrance and appears to be the stimulus for incubation turnover as males and females take turns at incubation. As nests are closed cavities which do not allow visual identification of nest visitors (Higgins et al. 2001) it may contain both position and identification information.

Alternatively, innate calls of rifleman could be subject to stabilising selection for consistent recognition of species-specific vocalisations, while genetic drift may explain the fine-scale variations across populations. Character shifts of innate vocal signals within populations should be uncommon due to stabilizing selection for sounds which convey species identity, particularly if they are functional for conspecific recognition (Miller 1982). As the penalties for calling with the incorrect signal could be significant for territorial defence and pair-bonding (Becker 1982), population variations in these types of signals are less likely to evolve via drift. Species-specific vocal signatures should be found in all vocalisations of a species and should vary little within populations (Emlen 1972; Becker 1982). Rifleman have a small and simple call repertoire made of short, high frequency elements. The high frequency used by rifleman for all vocalisations and the lack of significant frequency overlap between rifleman calls and other avifauna within each population mean that frequency range may be a highly species-specific vocal characteristic for rifleman, as has been demonstrated in other species (Becker 1982). If this consistently high frequency range provides sufficient information to conspecifics regarding species identification, individual rifleman may show individual or population-level changes within this frequency range. This type of plasticity has been found in white-throated sparrows where individual identity features vary within a general species-specific frequency range (Brooks and Falls 1975a). If slight variations in the frequency and temporal parameters of rifleman calls do not convey a selective disadvantage on individuals in terms of conspecific recognition, populations in isolation may accumulate phenotypic variations.
in vocalisations via drift. Many species have been demonstrated to have a relatively high level of plasticity in various vocal features (Becker 1982). While the results outlined in this study indicate that populations are divergent in the fine-scale acoustic features of contact calls, multivariate analyses could not successfully classify calls into populations based on acoustic measures and playback experiments indicated that these variations are not functionally significant to territorial male rifleman, supporting the fact that fine-scale variations may be the result of drift.

Given the lack of concordance between the genetic and vocal datasets across most populations in this study, it is possible that population variations may be explained via natural selection. Discordance between genetic and behavioural data in non-learned traits has been cited as evidence in favour of selective mechanisms of population divergence (Nicholls et al. 2006; Odom and Mennill 2012). For vocalisations that are not culturally inherited, these selection pressures are likely to be environmental, particularly in relation to adaptation for enhanced signal transmission in different habitats. Forest habitats represent a complex acoustic environment due to a high density of scattering surfaces, which increase reverberation of sound waves (Wiley and Richards 1982) and both degrade and attenuate vocal signals (Richards and Wiley 1980; Wiley and Richards 1982). Selective pressure for enhanced signal transfer to conspecifics should therefore lead to vocal signals that are designed to maximise or optimise transmission within a particular habitat (the acoustic adaptation hypothesis) (Morton 1975; Marten and Marler 1977) or within a particular avian assemblage (the character shift hypothesis) (Miller 1982; Doutrelant and Lambrechts 2001), leading to fine-scale variations in populations occupying different physical or social environments. Support for the such signal-design hypotheses has been found in several species (Morton 1975; Wiley and Richards 1982; Doutrelant et al. 1999; Doutrelant and Lambrechts 2001; Slabbekoorn and Smith 2002b), including sub-oscine species with innate vocal ontogeny (Ippi et al. 2011). In this study, no data on population variation in habitat structure or levels of acoustic competition was collected therefore an analysis of the effects of habitat selection or acoustic competition on signal design across populations is not possible here and I can provide only speculative comments. Further investigation into the possibility of signal optimisation (as opposed to transmission) is recommended in attempting future explanations for fine-scale geographic variation in rifleman calls. The function of rifleman vocalisations has never been investigated in depth but has been speculated to be primarily for intra-pair contact communication (Higgins et al. 2001). Rifleman do not patrol or actively defend territory boundaries using vocalisations
Instead, territories appear to be maintained predominantly through mutual avoidance (Cameron 1990) and vocal interactions following territory intrusion. Additionally, rifleman are a long-term monogamous species, pairing for life unless one of the pair dies (Higgins et al. 2001). Calls therefore do not appear to function in long-range territory advertisement or in long-range seasonal mate attraction, although they are likely to be used in short-range territory and pair-bond maintenance interactions. Rifleman pairs perform most daily activities together, spending up to 90% of their time in close contact with one another (Sherley 1985). These associations are accompanied by constant vocalisations made up predominantly of the three call types investigated in this study. As small birds, that are cryptically coloured (Hunt and MacLean 1993; Higgins et al. 2001), visual cues may not be useful for foraging rifleman pairs to maintain contact within a forested environment. Vocal signals are therefore a highly effective way of keeping track of one another within a complex habitat. As New Zealand’s smallest passerine (Higgins et al. 2001) rifleman are likely to experience a significant constraint on the range of frequencies they are able to produce (Ryan and Brenowitz 1985; Seddon 2005), perhaps explaining the extremely high frequency range used by rifleman, with calls ranging up to 15 kHz (Krull et al. 2009; this study). High frequency signals are maladapted for increased transmission due to the increase in attenuation of higher frequencies in forested environments (Morton 1975). Additionally, hearing thresholds of passerines have been shown to rise steeply at higher frequencies (Dooling 1982), therefore frequencies higher than 4 kHz are not likely to permit long-range communication (Dooling 1982; Wiley and Richards 1982). This restriction to the use of high frequencies produced at low amplitude means that rifleman calls are likely to be ineffective in long-range broadcast signalling. While small body size may be a constraint on the use of optimal frequencies, higher frequencies in small species may be an advantage in short-range interactions as they carry more directionality (Konishi 1973; Richards and Wiley 1980) and frequency modulations can provide position information to receivers due to frequency-dependent attenuation (Slabbekoorn et al. 2002). The small body size combined with the territorial spacing behaviour of the species suggests that rifleman calls are restricted to short-range communications, and are therefore adapted for position communication. Some characteristics of the rifleman call repertoire support this hypothesis. Both the Pip and the Zip calls are characterised by relatively rapid frequency modulation, which has been shown to be an effective means of conveying both position information (via frequency dependent attenuation) and maximizing transmission (Wiley and Richards 1982). While the Chuck call is also highly frequency modulated, the rapid broadband nature of the call makes it relatively atonal. Wide-spectrum signals with sharp amplitude changes (including sudden onsets and terminations) have been shown to maximize locatability (Wiley and Richards...
This call is the only call frequently observed being produced during flight and therefore may be predominantly functional for communication of position between foraging pairs. Playback experiments demonstrated that vocal advertisement of territory intrusion elicits a defensive response from resident territorial male rifleman over controls including silence or heterospecific vocalisations. Resident territory holders are likely to use vocalisations to monitor the position of neighbours and respond to intrusion with defensive searching behaviours, physical approach and vocalisation. These findings again support the suggestion that rifleman calls are functional in short-range communication of identity and position between conspecifics as opposed to being adapted for long-range signal transmission. Further research into the significance of habitat-selection hypotheses for geographic variation in rifleman calls should therefore focus on fine-scale variations in habitat structure across micro-environments.

Significance of geographic variation in rifleman calls

The playback experiments performed in this study demonstrated that territorial male rifleman respond to all vocal territorial intrusions with aggressive behaviour. The consistent response of male rifleman to vocal territory intrusion supports the suggestion that rifleman calls are functional in territory maintenance and position communication. Numerous studies have demonstrated that geographic variations in vocalisations can result in decreased functionality in territory and mate attraction displays. Males singing or calling variable vocal signals may experience higher levels of aggression from neighbouring males or a reduction in the efficacy of their territorial displays (Leader et al. 2002; Lovell and Lein 2004; Nelson and Soha 2004). Additionally, mate choice in response to male vocalisations may result in assortative mating, whereby females prefer to mate with males singing or calling using familiar vocal traits (MacDougall-Shackleton et al. 2001; Vehrencamp et al. 2003; Rowe 2007). The reduction in effective functionality of territorial or mate attraction vocalisations due to geographic variation has significant consequences for the evolution of population differentiation. The effects of sexual selection on dispersal of both males and females have been demonstrated to be a powerful driver of population divergence (Baker and Mewaldt 1978; Baker 1994; Irwin et al. 2001; Naguib et al. 2001; Kirkpatrick and Ravigne 2002; Slabbekoorn and Smith 2002a), particularly when populations are geographically isolated and have little gene flow between them.
While rifleman calls appear to function predominantly for short-range interactions, and do not function in long-range territory advertisement or in seasonal mate attraction, it is likely that they are involved in territory perimeter and pair-bond maintenance. Resident males responded to all playback stimuli with an aggressive defensive response. These playback results indicate that fine-scale statistical differences in the acoustic parameters of calls between populations are not biologically salient enough to result in modulating discrimination, recognition, and response and are currently unlikely to represent a barrier to population mixing. However, continued isolation of fragmented populations may promote ongoing population divergence in vocal signals. If signals continue to differentiate between populations, dispersing or translocated males using different calls may experience different levels of aggression from neighbours which may impact their ability to adequately maintain territory boundaries and pair-bonds (e.g. Parker et al. 2012).

References


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CHAPTER FOUR: Geographic variation in sexual size dimorphism and dichromatism in adult North Island rifleman
Introduction

Phenotypic morphological traits such as size and colouration are the result of a mixture of genetic, physiological, developmental and environmental influences in birds (Hill and McGraw 2006) and are therefore often highly variable both between individuals within a specific population and between populations within species (Dale 2006; Owens 2006; Benites et al. 2007). This variation evolves due to the fact that both size and colour are variously influenced by both genes and the environment (Hill and McGraw 2006) and as such can be affected by forces of both natural and sexual selection (Bortolotti 2006; Griffith and Pryke 2006; Senar 2006).

Size and colour variation both between sexes and across populations may evolve due to the influence of natural selection. Sexual dimorphism in size and colouration has been shown to evolve in response to natural selection pressures where sexes differ in key aspects of their behaviour. For example, numerous avian species exhibit sexual dimorphism in size or feeding morphology where the sexes have specialised in their resource exploitation habits (Slatkin 1984; Shine 1989; Kruger 2005; Chenoweth et al. 2008; Gonzalez-Gomez and Estades 2009). The use of different habitats or consumption of different diets can lead to the sexes exhibiting marked variation in their physiology, allowing each sex to adapt to a different foraging niche and therefore maximise their energetic uptake. This use of different habitats may also lead to variations in colour morphology across the sexes in some species. Where colouration is used as a means of crypsis, the use of different components of the habitat may result in sexual dichromatism as each sex adapts to become more camouflaged within their respective environment (Martin and Badyaev 1996; Gotmark et al. 1997; Igic et al. 2010). Finally, differential reproductive roles and demands often result in sexual dimorphism in birds. For example, the need for females to maximise their reproductive capacity has been shown to result in female-biased sexual size dimorphism in some species as larger females are assumed to have higher reproductive potential (Head 1995). While more common in invertebrates (Head 1995; Kraushaar and Blanckenhorn 2002; Stephens and Wiens 2008), evidence in favour of the Fecundity Selection Hypothesis in birds has been found where female size or mass correlates with egg size or clutch mass in several avian species (Christians 2002; Lislevand et al. 2009).

Geographic variation in size and colour within the sexes may also evolve due to population-specific variation in the extent and direction of natural selection (Aldrich and James 1991; Hill 1993; Baker 2008; Oatley et al. 2010). Traits evolved to enable individuals to effectively forage and/or avoid predation will be highly habitat-
dependent, leading to geographically variable size and colour traits where populations occupy distinct habitats (e.g. Mumme et al. 2006). Size in particular has been shown to be highly influenced by population-specific environmental features. Bergmann’s rule, the general observation that within species, individuals tend to be larger at cooler temperatures, has been shown to hold for birds in cross-species reviews (Ashton 2002), demonstrating how population variations in size can evolve due to differences in environment.

Sexual dimorphism and geographic variations in size and colouration may also evolve due to the influence of sexual selection. The theory that sexual dimorphism has evolved due to sexual selection has been detailed extensively by Darwin (1871). The most robust support for this hypothesis has come from comparative studies indicating that levels of sexual dimorphism are related to mating systems: polygynous mating systems assumed to experience high levels of sexual selection have higher levels of sexual dimorphism compared to monogamous systems (Moller 1985; Fairbairn 1990; Moller and Birkhead 1994; Cuervo and Moller 1999; Szekely et al. 2000; Dunn et al. 2001). On a finer scale, within-species studies have also linked levels of dimorphism to specific forms of sexual selection (Andersson 1993; Kraushaar and Blanckenhorn 2002; Bonduriansky 2007). Size and colour are both used as signalling traits in many avian species (Andersson 1994), providing information on quality to receivers and therefore dictating outcomes of both intra-sexual competitive interactions as well as inter-sexual mate choice decisions. Assortative mating based on these traits has been shown to influence pairing and reproductive success (e.g. Hill 1991; Hill and McGraw 2004; Hill 2006; Christensen and Kleindorfer 2007), demonstrating how sexual selection may drive the evolution of sexual dimorphism in size and colouration. Sexual selection may also result in geographic variations in both size and colour due to population-specific variations in the extent and direction of selective forces (Badyaev et al. 2000; Figuerola and Green 2000). For example, geographically variable levels of mate competition may result in more intense sexual selection in some populations when compared to others (Badyaev et al. 2000).

The description and analysis of levels of variation in size and colouration, and levels of sexual dimorphism in these traits, have both theoretical and practical impacts, particularly for managed species. If an individual’s size or colouration is adapted for a particular environment, the movement of individuals between different habitat types via translocation may have serious consequences for the potential survival and reproduction of establishing populations. Additionally, as assortative mating preferences are often based on size and colour there may be significant implications for the future evolutionary potential of populations differentiated by
variable size and colour morphology. Mate choice and competitive decisions based on size and colour may result in pre-mating isolation between individuals exhibiting geographically variable phenotypic traits (Price 1998). The successful mixing of individuals between divergent populations, whether by natural dispersal processes or by artificial conservation management practices (e.g. translocation), may be significantly affected by these variations, emphasizing the need for detailed analysis of intra-specific patterns of morphology and sexual dimorphism.

In this chapter I investigate sexual dimorphism and geographic variation in size and colour in adult North Island rifleman (Acanthisitta chloris granti). Rifleman are a very small (average 6 g), monogamous, native insectivorous species that displays sexual dichromatism and reversed sexual size dimorphism (Higgins et al. 2001). Rifleman plumage colour has been suggested to function in crypsis, as male and female colouration appears to match the different foraging environments used by each sex (Hunt and MacLean 1993). Hunt & MacLean (1993) found that during the breeding season, males spent a higher proportion of their time in the leafy canopy where the green colouration typical of male feathers may serve as an effective camouflage. In contrast, females spent more time on the surface of trunks where the yellow and brown mottled appearance of female feathers may be more effective in crypsis. While this study concluded that sexually dimorphic plumage colouration has evolved in response to natural selection for crypsis, several lines of evidence indicate that this may not be the only selective force affecting the evolution of size and colouration in rifleman. Firstly, the analysis of colour in Hunt and MacLean’s study used a simple Munsell colour chart comparison and therefore only compares information relevant to a human visual system as opposed to the more complex avian visual system (Hart 2001; Cuthill 2006). Secondly, in this study sexual differences in foraging niche use were only found during the breeding season and not during non-breeding periods. If colouration is solely produced in response to natural selection for crypsis, sexes should show consistent foraging niche diversity throughout the year, ensuring survival of the short-lived adults (Sherley 1985). Additionally, while cryptic colouration in females has evolved due to the higher proportion of nest attention females provide in many species (Martin and Badyaev 1996; Burns 1998), male rifleman contribute an equal if not greater proportion of parental care during the breeding season (Sherley 1994). Both sexes provision closed cavity nests continuously and at a rapid rate during nestling periods (Sherley 1985), therefore the green colouration of males would serve as a significant disadvantage for crypsis when visiting the nest cavity. Additionally, while rifleman colouration was observed to match the colour of the dominant tree type in this study area, this population occupies an early succession,
highly modified environment which is atypical of most native habitats occupied by rifleman. Finally, native avian populations in New Zealand have evolved in the absence of mammalian predators, therefore the only natural predators of rifleman are likely to be owls (morepork, *Ninox novaeseelandiae*), and native falcons (*Falco novaeseelandiae*). Visual crypsis is unlikely to be an effective anti-predator mechanism against morepork predation, as they are nocturnal predators (Higgins *et al.* 2001), although they have been known to prey on rifleman. New Zealand falcons have a diet which consists predominantly of birds (Seaton *et al.* 2008), however the extremely small size of the rifleman makes it unlikely that they are a highly desirable food source for falcons and no records of falcon preying on rifleman are known. While natural selection for crypsis may partially explain the evolution of sexual dichromatism in the rifleman, it seems possible that alternative forms of natural selection and/or sexual selection may also be acting on rifleman morphology.

Rifleman are a sedentary, territorial and monogamous species that form long-term pair-bonds (Higgins *et al.* 2001). Females have high reproductive potential but productivity is variable, with pairs producing up to two clutches per season, each containing between 2 and 5 eggs (Gray 1969; Sherley 1985). Eggs are relatively large in proportion to female size and are laid at 48 hour intervals, indicating significant investment in reproduction for females. However, nuptial feeding by males is thought to off-set the energy requirements of reproduction for females (Sherley 1985; Sherley 1993). Previous work has suggested that the larger size of females in this species may be associated with fecundity selection, although the hypothesis has never been directly tested (Sherley 1985; Hunt and MacLean 1993). Both sexes contribute substantially to the breeding effort with males performing an equal or higher proportion of incubation and nestling feeding and the majority of nest building (Sherley 1994). Cooperative breeding occurs in approximately one third of nests and helpers are most often males who are thought to gain direct benefits through access to potential mates or indirect benefits through kin selection (Sherley 1985; Sherley 1990). While rifleman were once found throughout the North Island of New Zealand, populations are now fragmented and isolated from one another (Higgins *et al.* 2001; Robertson *et al.* 2007). Rifleman are considered poor flyers with a low dispersal capability and are therefore unlikely to migrate between isolated habitat fragments (Higgins *et al.* 2001). The species occupies mature forest in all populations, however regions vary in population density (Robertson *et al.* 2007), which is likely to be associated with competition for mates. The influence of sexual selection on rifleman morphology has never been investigated. Given the potential for high levels of pairing and territory competition at high densities, sexual selection on
males may influence the evolution of sexual dimorphism in this species, therefore producing geographic variations in morphology.

This chapter aims to describe general patterns of sexual dimorphism and geographic variation in sexually dimorphic morphological traits in North Island rifleman populations and investigate some potential drivers of geographic variation in terms of both natural and sexual selection.

My objectives included the following:

1) To describe the degree of sexual dimorphism in size and colouration of male and female rifleman using a description of colour relevant to avian visual perception.

2) To describe patterns of geographic variation in size and plumage colouration both within and between the sexes.

3) To investigate potential natural selection drivers of population variation in size. In the absence of detailed habitat data, an analysis of size variation in relation to Bergmann’s rule is conducted.

4) To investigate potential sexual selection drivers of population variation in size and colour, including analysis of how population density (as an indicator of competition for mating opportunities) relates to levels of size and colour both within and between sexes and analysis of potential physiological trade-offs between colour and size.

Methods

Rifleman in native populations (i.e. not resulting from translocation) were targeted for capture at six isolated populations around the North Island of New Zealand. These sites included Little Barrier Island, Taranaki Regional Park, Pureora Forest Park, Boundary Stream Mainland Island, Mohi Bush and the Tararua Ranges. These populations represent Insular, Western, Central, Eastern Ranges, Eastern Coastal and Southern regions of the North Island respectively (hereafter referred to by region name) (Fig. 1). All locations are currently managed by the New Zealand Department of Conservation and the research methods and data collection used in this study were permitted by both the New Zealand Department of Conservation (Banding permit 2010/025; Regional bird handling permits WE-25869-FAU, NO-26310-FAU, AK-27236-FAU, WK-28729-RES, WA-27986-FAU) and The University of Auckland Animal Ethics Committee (R762).
Capture and measurements

Rifleman were located for capture by walking established tracks and following individuals located by both sound and sight. Once target rifleman individuals were sighted, suitable mist-netting locations were selected within the immediate vicinity. Mist-nets were combined with playback of lure calls to catch rifleman within identified territories. Individuals were targeted in non-breeding periods to avoid catching juveniles whose morphology is distinct from adults (Higgins et al. 2001).

Each bird captured was sexed based on plumage (Heather and Robertson 2005), banded and the tarsus length (Sutherland et al. 2004) of the left leg of each bird was measured using electronic Vernier calipers (to the closest 0.1 mm). The weight of each individual was measured using a pesola scale to the nearest 0.5 g. Three pin feathers were removed using sterilized tweezers from the mantle of each bird and were stored in air-tight plastic eppendorf tubes. Feathers were stored in a cold dark location following collection. The mantle of the bird was chosen for feather sampling because the green/yellow plumage colouration exhibited by both male and female rifleman extends across the entire back of the adult bird but not to the underside, where both sexes have white
plumage (Higgins et al. 2001) (Fig. 2). This body region also had pin feathers large enough for adequate colour analysis using spectrophotometry, when compared to the smaller feathers across the head. Removal of body feathers was preferable to removal and analysis of wing feathers so as to ensure that flight ability was not reduced for individuals caught for the analyses.

Fig. 2. Male (left) and female (right) rifleman demonstrating colour plumage dimorphism between the sexes

Spectral Analysis

Spectral reflectance of the pigmented feather tips was measured using an Ocean Optics USB2000+ Fiber Optic Spectrometer with a PX-2 Pulsed Xenon Light Source (Ocean Optics Ltd, Dunedin, Florida, USA). Spectrometer calibration was carried out using a certified white reflectance standard (Model WS-1-SL, Labsphere Inc., New Hampshire, USA) and a dark standard (black felt material). All spectral measurements and analyses were performed by the same observer (SJW). Reflectance was measured for individual feathers by first flattening each plucked feather onto a small square of black felt material. Calibration was repeated prior to measurement of each individual’s feathers. Measurements were taken at a 45° angle to the surface of the feather by inserting the probe into a block holder (Andersson and Prager 2006). The tips of each of the three feathers obtained from each bird were measured (Fig. 3) with a total of three replicate scans per feather, using the SpectraSuite software (Ocean Optics Ltd). Raw spectra were averaged for all three scans to create an average per feather then all three feathers were averaged to produce a single raw spectrum per individual. Raw spectra were then transformed by spectral smoothing using AVICOL Version 6.0 (Gomez 2012), producing spectra in 1 nm bins. Interpolated spectra were used to calculate several measures of feather colouration (Fig. 4). Measurements were calculated based on tristimulus scores which have been demonstrated to be an accurate
means of measuring relative colour, particularly in pigment systems (Butler et al. 2011). Spectral intensity or ‘brightness’ was measured as the average reflectance ($R_{\text{mean}}$) (Andersson and Prager 2006). Saturation or ‘chroma’ was measured by segment classification whereby the bird-visible spectrum was divided into four equal wavelength-region segments corresponding to traditional UV-A (320-415 nm), Blue (415-510 nm), Green-Yellow (510-605 nm) and Orange-Red (605-700 nm) regions. Chroma was calculated for each region separately by dividing the total reflectance for the region of interest by the total reflectance across the entire bird-visible spectrum following (Montgomerie 2006). For example, UV chroma was measured as $(R_{415} - R_{320})/(R_{\text{total}})$. Spectral location or ‘hue’ was measured for two separate spectral locations, the UV region (320-415 nm) and the complete spectrum (320-700 nm). As green and yellow colouration is often a result of carotenoid pigmentation (McGraw 2006) which produces a bimodal reflectance spectrum (Bleiweiss 2004), two measures of Hue were calculated: Hue (UV) was measured as $\lambda(R_{\text{max}(320-415)})$ or the wavelength at which reflectance is at the maximum within the UV range and Hue (Total) was measured as $\lambda(R_{\text{max}(320-700)})$ or the wavelength at which reflectance was maximal for the entire bird-visible spectrum (Table 1) (Andersson and Prager 2006; Montgomerie 2006).

![Feather region used for colour measurement](image)

Fig. 3. Images of male (left) and female (right) feathers used for analysis of colouisation. Arrows indicate the region of each feather used for measurement of plumage colouration.
Table 1. Tristimulus colour variables used in the analysis of rifleman plumage colouration

<table>
<thead>
<tr>
<th>MEASUREMENT</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightness (Total)</td>
<td>$R_{320-700}$</td>
</tr>
<tr>
<td>Brightness (Mean)</td>
<td>$R_{320-700}/n$</td>
</tr>
<tr>
<td>UV Chroma</td>
<td>$R_{320-415}/R_{320-700}$</td>
</tr>
<tr>
<td>Blue Chroma</td>
<td>$R_{415-510}/R_{320-700}$</td>
</tr>
<tr>
<td>Green-Yellow Chroma</td>
<td>$R_{510-605}/R_{320-700}$</td>
</tr>
<tr>
<td>Orange-Red Chroma</td>
<td>$R_{605-700}/R_{320-700}$</td>
</tr>
<tr>
<td>Spectral Saturation</td>
<td>$R_{\text{max}} - R_{\text{min}}/R_{320-700}$</td>
</tr>
<tr>
<td>Hue (UV)</td>
<td>$\lambda_{\text{Rmax}(320-415)}$</td>
</tr>
<tr>
<td>Hue (Total)</td>
<td>$\lambda_{\text{Rmax}(320-700)}$</td>
</tr>
</tbody>
</table>

Fig. 4. Example reflectance spectrum showing variables calculated from spectral data. Variables include Total Brightness as the total reflectance from 320-700 nm, Mean Brightness as the average reflectance from 320-700 nm, Hue (UV) or ($\lambda_{\text{Rmax}(320-415)}$) as the wavelength of maximum reflectance in the UV region, Hue (Total) or ($\lambda_{\text{Rmax}(320-700)}$) as the wavelength of maximum reflectance for the total spectrum, Spectral Saturation as ($R_{\text{max}} - R_{\text{min}}/R_{320-700}$), and four measures of Chroma including UV chroma ($R_{320-415}/R_{320-700}$), Blue Chroma ($R_{415-510}/R_{320-700}$), Green-Yellow Chroma ($R_{510-605}/R_{320-700}$) and Orange-Red Chroma ($R_{605-700}/R_{320-700}$) (Modified from Benites et. al. (2007)).
Statistical Analyses

All measures of size and colour were checked for normality and equality of variances prior to ANOVA analysis using the Levene’s and Shapiro-Wilk’s Tests respectively. Appropriate transformations were performed for any non-normal data or non-parametric alternatives were used where necessary. Analysis of general levels of sexual dimorphism (across all populations) was performed using one way ANOVAs for measures of sexual size dimorphism including both tarsus size and weight. Sexual dimorphism in colour variation was analysed by combining individual measures of colour variation across both sexes into a Principal Components Analysis (PCA).

To analyse general patterns of population variation in size, ANCOVA’s were performed, incorporating sex and population effects. A sex*population interaction term was included to test for population variation in sexual dimorphism in size. One way ANOVAs for each sex were also used for both size variables (tarsus and weight) to describe geographic size variation. Geographic variation in colour was investigated by performing a series of ANCOVA’s incorporating sex and population effects, and a sex*population interaction term. Parameter estimates were then used to investigate population contributions to significant model effects. Separate Principal Components Analyses were then created for each sex, followed by a MANOVA and one-way ANOVAs on original colour variables to describe fine-scale population variations in colouration.

To investigate the effects of Bergmann’s Rule on rifleman size, average population values for both male and female size traits were regressed against measures of both latitude and altitude (as approximations of temperature).

Potential physiological trade-offs were investigated by analysing the co-variation of size and colour traits using a series of linear regression analyses. Regression of tarsus size versus colour was conducted separately for each sex but incorporated individual data from across all populations to indicate general species-level physiological trade-offs. A series of ANCOVA’s were then performed to investigate whether there was any indication of population-specific trade-offs. ANCOVA’s incorporated tarsus and population effects and a tarsus*population interaction term. Any measurements that exhibited significant interaction terms were then subject to further regression analyses on individual populations.
Finally, to investigate whether density levels influenced levels of population variation and sexual dimorphism, an index of sexual dimorphism was calculated for each population (Mean value for larger sex/Mean value for smaller sex - 1) following Lovich and Gibbons (1992). Male biased traits were given a negative sign, whereas female-biased traits were given a positive sign as females exhibited higher values across most traits. Population level sexual dimorphism calculations were then regressed against population density. Population density data was compiled from raw data from the OSNZ Atlas of Bird Distribution in New Zealand (Robertson et al. 2007). Density estimates were gathered during field surveys by volunteer amateur ornithologists, involving compilations of observations using standardised datasheets. Population density data is therefore expressed as a standard percentage of the number of record sheets for each map grid square (10 km²) where rifleman were recorded (% sightings). Significant analyses were then subject to ongoing analyses by investigating the relationship between density and average male or female population values for relevant traits.

**Results**

**General patterns of sexual dimorphism**

Sexual dimorphism in rifleman was evident for measures of both tarsus size and plumage colouration. Females had significantly longer tarsi ($F=70.92, P<0.0001$) and were heavier than males (Kruskal-Wallis, $X^2=35.86$, $P<0.0001$) (Figs 5, 6).

![Graph showing differences in tarsus size between males and females across all populations](image)

Fig 5. Differences in tarsus size between males and females across all populations (Means ± s.e.m., Males n=51, Females n=53)
Female feathers were generally brighter, with a higher proportion of blue and red chroma compared to males (Fig 7). Males generally had higher green-yellow (GY) chroma and had consistently higher spectral saturation compared to females. Females had a higher hue value within the yellow-red region of the spectrum as opposed to males who were characterized by a green hue. Measures of plumage colouration were highly correlated and so a Principal Components Analysis (PCA) was performed to reduce the original variables down to a smaller set of orthogonal variables for analysis of sexual dimorphism. The first four principal components represented 71% of the variation across both sexes. The first principal component (PC1) explained 51% of the variation and was positively associated with brightness, blue and red chroma, and negatively associated with spectral saturation. PC2 explained a further 20% of the variance in the data and was predominantly correlated with GY chroma and a negative relationship with both measures of hue. PC3 explained 14% of the variation and was positively correlated with GY chroma and UV hue, while the final PC (PC4) was explained predominantly by UV hue and total hue (Table 2). When PC1 and PC2 were plotted, sexual dimorphism in plumage colouration was evident (Fig 8). The separation of males and females was predominantly explained by variations in PC2. Male rifleman plumage had higher PC2 scores compared to females, reflecting the high levels of GY chroma and lower hue measurements compared to females.
Fig 7. Reflectance curves of male (blue) and female (green) feathers showing means (lines) ± s.e.m. (error bars) (Males n=61, Females n=59)

Table 2. Eigenvectors resulting from a PCA on male and female plumage colouration variables. The table shows the correlations between the top four PC’s and the original colour variables. Predominant relationships are highlighted in bold.

<table>
<thead>
<tr>
<th></th>
<th>PC 1</th>
<th>PC 2</th>
<th>PC 3</th>
<th>PC 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean brightness</td>
<td>0.46</td>
<td>0.17</td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>UV chroma</td>
<td>0.35</td>
<td>0.33</td>
<td>-0.40</td>
<td>0.29</td>
</tr>
<tr>
<td>Blue chroma</td>
<td>0.47</td>
<td>0.05</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>GY chroma</td>
<td>0.05</td>
<td>0.64</td>
<td>0.51</td>
<td>-0.06</td>
</tr>
<tr>
<td>Red chroma</td>
<td>0.42</td>
<td>0.02</td>
<td>0.38</td>
<td>-0.31</td>
</tr>
<tr>
<td>Spectral sat</td>
<td>-0.42</td>
<td>0.18</td>
<td>0.39</td>
<td>-0.14</td>
</tr>
<tr>
<td>UV hue</td>
<td>0.09</td>
<td>-0.42</td>
<td>0.52</td>
<td>0.72</td>
</tr>
<tr>
<td>Total hue</td>
<td>0.29</td>
<td>-0.49</td>
<td>0.13</td>
<td>-0.53</td>
</tr>
</tbody>
</table>
To investigate the effects of population variation and sex on size and colour measures, an ANCOVA was performed that incorporated population and sex as effects. An interaction term (population*sex) was included in each model to test for population dependent variation in levels of sexual dimorphism. Measures of tarsus length and weight had significant population (Tarsus, $F=8.56, P<0.0001$, df=4; Weight, $F=4.83, P=0.0005$, df=5) and sex (Tarsus, $F=91.12, P<0.0001$, df=1; Weight, $F=42.26, P<0.0001$, df=1) effects but no significant interaction terms (Tarsus, $F=0.78, P=0.5436$; Weight, $F=1.85, P=0.1092$), indicating no significant variation in the level of sexual dimorphism between populations.

To investigate variation across populations, ANOVAs were performed on tarsus and weight separately. Male tarsus length was significantly different between populations ($F = 6.72, P = 0.0002$). Tukey Kramer HSD post-hoc comparisons showed that both Eastern Coastal and Eastern Range males had significantly smaller tarsus size compared to both Southern (ER = $P=0.0096$, EC = $P=0.0128$) and Insular males (ER = $P=0.0026$, EC = $P=0.0086$) (Fig. 9A). Male weight also varied between populations (Welch’s Test, $F=4.60, P=0.0070$).
Southern males were significantly heavier than all other males, while Western males were significantly lighter than all other males (Fig. 10A). Female tarsus length was also significantly different between populations (ANOVA, F = 3.85, P = 0.0086). Eastern Range females were significantly smaller in tarsus size compared to Insular females (P = 0.0050) (Fig. 9B). Central and Eastern Coastal female measurements were excluded from analysis due to small sample sizes (n=4). No other comparisons showed significant differences. Female weight did not differ significantly between populations (Welch’s Test, F=2.74, P=0.0602), although Southern females were larger on average compared to other populations (Fig. 10B).

Fig. 9. Population variation in male (A) and female (B) tarsus length. Values show means with error bars showing s.e.m. Results of post-hoc Tukey Kramer tests are shown (A/B indicate group membership)

Figure 10. Population variation in male (A) and female (B) weight. Values show means with error bars showing s.e.m. Results of post-hoc Tukey Kramer tests are shown (A/B indicate group membership). The Central population for both sexes and the Eastern Coastal population for females were excluded from analyses due to low sample sizes (n<4).
ANCOVAs on colour variables showed different sex and population effects depending on the colour variable (Table 3). Blue chroma had significant population and sex effects but no significant interaction term. UV chroma had a significant population effect and a significant interaction term. Red chroma and UV hue had significant effects of sex and significant interaction terms. Variation in both measures of hue was solely explained by sex. Mean brightness, GY chroma and spectral saturation had significant sex and population effects but also showed a significant interaction term, indicating that sexual dimorphism varies by population.

Table 3. ANCOVA model results showing population, sex and interaction effects including F Ratio and P values. Significant results are highlighted in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Population F</th>
<th>Population P</th>
<th>Sex F</th>
<th>Sex P</th>
<th>Interaction F</th>
<th>Interaction P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightness</td>
<td>2.38</td>
<td>0.0438</td>
<td>20.47</td>
<td>&lt;0.0001</td>
<td>18.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>UV chroma</td>
<td>8.57</td>
<td>&lt;0.0001</td>
<td>0.93</td>
<td>0.3365</td>
<td>3.37</td>
<td>0.0073</td>
</tr>
<tr>
<td>Blue chroma</td>
<td>3.16</td>
<td>0.0107</td>
<td>28.72</td>
<td>&lt;0.0001</td>
<td>1.17</td>
<td>0.3271</td>
</tr>
<tr>
<td>GY chroma</td>
<td>3.49</td>
<td>0.0058</td>
<td>13.43</td>
<td>0.0004</td>
<td>4.30</td>
<td>0.0013</td>
</tr>
<tr>
<td>Red chroma</td>
<td>1.48</td>
<td>0.2038</td>
<td>71.23</td>
<td>&lt;0.0001</td>
<td>3.70</td>
<td>0.0040</td>
</tr>
<tr>
<td>Spec Sat</td>
<td>11.98</td>
<td>&lt;0.0001</td>
<td>46.86</td>
<td>&lt;0.0001</td>
<td>4.41</td>
<td>0.0011</td>
</tr>
<tr>
<td>UV Hue</td>
<td>1.31</td>
<td>0.2670</td>
<td>6.92</td>
<td>0.0098</td>
<td>2.90</td>
<td>0.0172</td>
</tr>
<tr>
<td>Hue</td>
<td>1.87</td>
<td>0.1052</td>
<td>189.67</td>
<td>&lt;0.0001</td>
<td>1.36</td>
<td>0.2460</td>
</tr>
</tbody>
</table>

For mean brightness, the significant interaction term was explained by higher levels of sexual dimorphism in the Central population (t = 2.60, P=0.0106) and a reversed sexual dimorphism in the Eastern Ranges population (t = -4.56, P<0.0001) (Fig 11). These effects were reversed for UV Chroma, with the Eastern Ranges population having a higher level of sexual dimorphism (t = -3.19, P=0.0019) and the Western population demonstrating reversed sexual dimorphism (t = 1.98, P=0.0498) (Fig. 12). For GY chroma, the Western population had significantly higher levels of sexual dimorphism compared to other populations (t = -3.03, P=0.0031), while the Insular population had lower levels of sexual dimorphism (t = -0.19, P=0.0049) (Fig. 13). A significant interaction term for red chroma was explained by a reduction in dimorphism in the Eastern Ranges population (t = -3.87, P=0.0002) and a higher level of dimorphism in the Central population (t = 2.21, P=0.0292) (Fig. 14).
Fig 11. Least Square Means plot showing population variation in male and female brightness. Levels of sexual dimorphism in brightness vary between populations.

Fig. 12. Least Square Means plot showing population variation in male and female UV chroma. Levels of sexual dimorphism in brightness vary between populations.
Fig. 13. Least Square Means plot showing population variation in male and female GY chroma. Levels of sexual dimorphism in brightness vary between populations.

Fig. 14. Least Square Means plot showing population variation of male and female red chroma. Levels of sexual dimorphism in brightness vary between populations.
To investigate general effects of population on colour variation in rifleman, separate principal components analyses were performed for each sex and the top four principal components from each analysis were used in a MANOVA. The PCA for male feathers revealed four primary principal components which described 90% of the variation between individuals across all populations. Principal component 1 (PC1) explained 50% of the variation and was most closely associated with high brightness, UV and blue chroma as well as lower values of spectral saturation (Table 4). PC2 explained 15% of the variation and was highly positively correlated with both UV and total hue. PC3 was associated with GY and red chroma while PC4 was significantly associated with total hue and had a negative association with UV hue. The top four principal components explained 98% of the variation in individual female colour variation. PC1 explained 61% of the variation and was highly correlated with all measures of chroma and brightness. PC2 explained a further 15% of the variation and was predominantly associated with GY chroma, spectral saturation and was negatively associated with total hue. PC3 was highly correlated with both UV and total hue, while PC4 was correlated with spectral saturation and total hue, and negatively associated with UV hue.

Table 4. Eigenvectors resulting from a principal components analysis on male (left) and female (right) plumage colouration variables. The table shows the correlations between the top four PC’s and the original variables for each sex. Predominant correlations are highlighted in bold.

<table>
<thead>
<tr>
<th>Sex</th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Brightness</td>
<td>0.45</td>
<td>-0.13</td>
</tr>
<tr>
<td>UV chroma</td>
<td>0.45</td>
<td>-0.19</td>
</tr>
<tr>
<td>Blue chroma</td>
<td>0.42</td>
<td>-0.29</td>
</tr>
<tr>
<td>G-Y chroma</td>
<td>-0.33</td>
<td>-0.29</td>
</tr>
<tr>
<td>Red chroma</td>
<td>0.24</td>
<td>0.16</td>
</tr>
<tr>
<td>Spectral sat</td>
<td>-0.49</td>
<td>-0.11</td>
</tr>
<tr>
<td>UV Hue</td>
<td>0.06</td>
<td>0.59</td>
</tr>
<tr>
<td>Total Hue</td>
<td>0.06</td>
<td>0.62</td>
</tr>
</tbody>
</table>

The MANOVA results indicated that male plumage colouration was significantly different between populations (Wilks’ $\lambda = 0.17; F_{15, 143} = 8.52; P < 0.0001$). Female colouration was also significantly different between populations (Wilks’ $\lambda = 0.40; F_{15, 138} = 3.65; P < 0.0001$). To investigate which original colour variables were responsible for significant population variations, individuals ANOVAs were performed on all original (i.e. non principal component) colour variables. ANOVAs did not investigate population variation across principal
components due to the fact that multiple PC’s were explained by several relatively evenly weighted colour variables, meaning the power of each PC in explaining population variation was diluted. All measures of male plumage colouration except for total hue were significantly different between populations (Table 5). Post-hoc Tukey Kramer comparisons demonstrated that Eastern Range males were significantly brighter than all other populations (P<0.0162) (Fig. 15). Insular males were also significantly brighter than Western males (P<0.0025). Following Welch’s Test, all measures of chroma were analysed using non-parametric post-hoc comparisons by using individual Student’s t tests. Eastern Range males were found to have significantly higher UV chroma (P<0.0120) and blue chroma (P<0.0046) compared to all other populations. Western males had significantly higher Green-Yellow chroma than Southern, Insular and Eastern Range males (P<0.0225). Eastern Range males had higher red chroma compared to all populations except Southern males (P<0.0028) which had higher red chroma than both Western and Insular males (P<0.0001). Overall spectral saturation was significantly lower for males in the Eastern Ranges compared to all other regions except the Southern population (P<0.0194). Western males had significantly higher overall spectral saturation compared to Insular, Eastern Ranges and Southern males (P<0.0001). Measurements of hue were relatively consistent across all populations, with spectra showing a bimodal reflectance distribution with a peak in the UV region at approximately 330 nm and a secondary peak of reflectance at approximately 541 nm (Fig. 16).

Table 5. Geographic variation in plumage colour variables for adult male rifleman. Values show the mean (s.e.m.). Significant results of ANOVA and non-parametric Welch’s Tests are marked with *
Female colouration was relatively consistent between populations, showing no significant population differences for measures of brightness or any of the four measures of chroma (Table 6). Following a log transform due to non-normality, overall spectral saturation was also non-significant between populations. While both measures of hue were found to be significantly different using Welch’s Test, non-parametric post-hoc comparisons using Student’s t-tests with a Bonferroni correction ($\alpha=0.008$) removed the significance of these colour variables. The hue of female feathers was found to be approximately 647 nm. The UV peak of female feathers was higher than males at approximately 348 nm (Fig. 17).

Table 6. Geographic variation in plumage colour variables for adult female rifleman. Values show the mean (s.e.m.). Significant results of ANOVA and non-parametric Welch’s Tests are marked with *. Central and Eastern Coastal regions are not represented due to inadequate sample sizes (n=4).

<table>
<thead>
<tr>
<th>Colour Variable</th>
<th>Insular (n=18)</th>
<th>Western (n=12)</th>
<th>Eastern Range (n=14)</th>
<th>Southern (n=8)</th>
<th>DF</th>
<th>F</th>
<th>Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightness</td>
<td>3.75 (0.23)</td>
<td>3.81 (0.34)</td>
<td>3.37 (0.27)</td>
<td>4.04 (0.29)</td>
<td>3</td>
<td>0.86</td>
<td>0.4695</td>
</tr>
<tr>
<td>UV Chroma</td>
<td>0.10 (0.01)</td>
<td>0.08 (0.02)</td>
<td>0.10 (0.02)</td>
<td>0.09 (0.01)</td>
<td>3</td>
<td>0.65</td>
<td>0.5890</td>
</tr>
<tr>
<td>Blue Chroma</td>
<td>0.16 (0.01)</td>
<td>0.15 (0.02)</td>
<td>0.17 (0.02)</td>
<td>0.17 (0.01)</td>
<td>3</td>
<td>0.25</td>
<td>0.8616</td>
</tr>
<tr>
<td>GY Chroma</td>
<td>0.43 (0.02)</td>
<td>0.43 (0.03)</td>
<td>0.35 (0.03)</td>
<td>0.45 (0.03)</td>
<td>3</td>
<td>2.66</td>
<td>0.0588</td>
</tr>
<tr>
<td>Red Chroma</td>
<td>0.44 (0.03)</td>
<td>0.49 (0.04)</td>
<td>0.39 (0.03)</td>
<td>0.52 (0.04)</td>
<td>3</td>
<td>2.52</td>
<td>0.0691</td>
</tr>
<tr>
<td>Spec Sat</td>
<td>0.0039 (0.0001)</td>
<td>0.0045 (0.0002)</td>
<td>0.0038 (0.0003)</td>
<td>0.0042 (0.0001)</td>
<td>3</td>
<td>2.59</td>
<td>0.0756</td>
</tr>
<tr>
<td>Hue (UV)</td>
<td>331 (1.58)</td>
<td>359 (12.07)</td>
<td>347 (10.11)</td>
<td>338 (3.48)</td>
<td>3</td>
<td>3.32</td>
<td>0.0421*</td>
</tr>
<tr>
<td>Hue (Total)</td>
<td>610 (10.84)</td>
<td>664 (12.58)</td>
<td>660 (15.56)</td>
<td>653 (13.32)</td>
<td>3</td>
<td>4.34</td>
<td>0.0144*</td>
</tr>
</tbody>
</table>
Fig. 15. Population means (± s.e.m.) for measures of male plumage colouration including brightness, relative measures of chroma and spectral saturation
Fig. 16. Male plumage reflectance spectra (mean ± s.e.m.) showing variation in spectral shape between populations
Fig. 17. Female plumage reflectance spectra (mean ± s.e.m.) showing variation in spectral shape between populations
To visualize population variation and levels of sexual dimorphism in colour variables, population means and standard errors of the first two principal components (see General Sexual Dimorphism section above) were plotted (Fig 18). Males and females were separated predominantly by the consistently higher levels of PC2 (higher GY chroma and lower hue) in males. Males also tended to have lower levels of PC1 (brightness, blue and red chroma) with the Eastern Ranges population being the exception. Population variation in sexual dimorphism is evident, with a higher degree of separation between males and females for the Western and Eastern Ranges populations compared to all other population pairs. Finally, population variations within each sex are obvious from the principal components analysis. For male colouration, Eastern Range males were characterized by higher PC1 values, indicating increased brightness as well as higher blue and red chroma. Western and Eastern Ranges males had higher PC2 values than other populations, representing higher GY chroma. For females, Central females had higher values for both PC1 and PC2 indicating brighter and more chromatic feathers within the UV, blue, GY and red regions of the spectrum. Eastern Ranges females had the lowest values for PC2, indicating lower GY chroma compared to other populations.
Fig. 18. PC1 (brightness, UV, blue and red chroma) versus PC2 (GY chroma and hue) values (means +/- s.e.m.) showing separation of both males and females and of isolated populations. PC1 and PC2 explain 74% of the variance in adult rifleman plumage colour.
Geographic variation in relation to altitude and latitude

Population variations in male and female size did not correspond to Bergmann’s Rule. Male tarsus size and weight did not correlate with altitude (Tarsus F=0.11, P=0.7635, Weight F=0.00, P=0.9664) or latitude (Tarsus F=0.22, P=0.6705, Weight F=0.19, P=0.6946). Female size and weight also did have any relationship with altitude (Tarsus F=0.40, P=0.5706, Weight F=0.52, P=0.5396) or latitude (Tarsus F=0.43, P=0.5599, Weight F=1.32, P=0.3342).

Physiological trade-offs - the co-variation of size and colour

To explore the relationship between size and colour in both male and female rifleman a series of linear regression analyses were performed separately for each sex and combining individuals across all populations. When all populations were combined, no evidence of physiological trade-offs between size and colour traits was evident for either males or females.

To investigate the effects of population variation on these relationships, ANCOVAs were performed on each sex separately using tarsus and population as effects and a tarsus*population interaction term. For males, brightness measurements were not affected by tarsus size (F=2.27, P=0.1399) but the interaction term approached significance (F=2.55, P=0.0536) indicating population variation in the relationship between size and brightness. UV chroma (F=1.06, P=0.3096), blue chroma (F=0.39, P=0.5345), GY chroma (F=2.04, P=0.1066), spectral saturation (F=0.42, P=0.7910) and total hue (F=0.92, P=0.4598) all failed to show significant tarsus*population interaction terms indicating no significant population variation in the relationship between size and colour variables. Both red chroma (F=2.99, P=0.0293) and UV hue (F=5.49, P=0.0012) had a significant tarsus*population interaction term. For females, no colour variables showed a significant interaction term between size and population indicating no evidence for population-specific trade-off effects.

Variables that indicated population variation in interaction terms for males were subject to separate linear regression analyses to investigate population specific relationships between size and colour. The relationship between tarsus size and brightness was non-significant for all populations except the Western population which showed a significantly negative relationship between brightness and size (F=7.69, P=0.0242) (Fig. 19). The relationship between tarsus size and red chroma was also non-significant for all populations, although the
Eastern Ranges population showed a trend toward decreased red chroma with larger tarsus size (F=4.20, P=0.0706) (Fig. 20). Tarsus size and UV hue showed no relationship for all populations except the Western population which had a significantly negative relationship between UV hue and tarsus size (F=34.92, P=0.0004) (Fig. 21).

Fig 19. The relationship between tarsus size and mean brightness in males from the Western population. Larger males show lower levels of feather brightness.
Fig 20. The relationship between tarsus size and red chroma in males from the Eastern Ranges. Larger males have lower levels of red chroma.

\[ y = -0.0806x + 2.1232 \]
\[ R^2 = 0.3184 \]

Fig 21. The relationship between tarsus size and UV hue in males from the Western population. Larger males have lower hue values.

\[ y = -13.06x + 610.91 \]
\[ R^2 = 0.8136 \]
Sexual dimorphism and population density

Population specific measures of sexual dimorphism were regressed against population density as a possible indicator of levels of competition for mating opportunities. Rifleman populations had different population density levels (Fig. 22). Rifleman appeared to show evidence of decreased sexual dimorphism at higher densities for measures of red chroma (F=8.75, P=0.0417, R²=0.686) (Fig. 23). This decrease in sexual dimorphism was explained by an increase in levels of red chroma in males at higher densities (F=27.87, P=0.0062, R²=0.874) (Fig. 24).

Fig. 22. Population density of rifleman (measured as % of sightings per 10 km) in six sample populations across the North Island. The Eastern Ranges population is characterised by the highest population density.

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1. Eastern Ranges population is characterised by the highest population density.
Fig 23. Sexual dimorphism of red chroma feather colouration in relation to population density. Sexual dimorphism in red chroma decreases with increased population density.1

1 Bird Distribution 1999-2004 © Ornithological Society of NZ Inc.

Fig 24. Average male red chroma in relation to population density. Males in high density populations have higher levels of red chroma.1

1 Bird Distribution 1999-2004 © Ornithological Society of NZ Inc.
Discussion

Sexual dimorphism and geographic variation in size in the rifleman

Male and female rifleman displayed significant sexual size dimorphism. While female-biased size dimorphism is common in most animal groups, a higher proportion of bird species show male-biased size dimorphism (Owens and Hartley 1998). This pattern has been associated with an increase in sexual selection on male size as larger males may perform better in physical combat and may be more attractive as potential mates (Fairbairn and Preziosi 1994). Patterns of both intra-sexual competition and inter-sexual mate attraction are therefore considered to exert selection pressure on male size (Lande 1980). The results described in this study indicate that sexual selection for increased male size is unlikely to explain size variation and sexual size dimorphism in the rifleman. Rifleman showed evidence of consistent female-biased size dimorphism with females on average 0.75 g heavier and 0.83 mm larger (tarsus size) than males. While population variation was evident for both tarsus size and weight variables, the significant population effects were predominantly explained by a significantly smaller size in the Eastern Ranges and Eastern Coastal populations. This trend was found in both males and females, resulting in a relatively consistent level of sexual dimorphism in size across populations, indicating that males and females appear to be subject to the same level of selection across populations. An analysis of Bergmann’s rule in relation to geographic variation in size in both sexes failed to find a significant result, indicating that temperature variations across populations do not explain the geographic size variations observed here. Size did not co-vary with density, assumed in this study to be associated with competition for pairing and mating opportunities and therefore representing levels of sexual selection. The behavioural ecology of rifleman indicates that male rifleman are unlikely to be subject to sexual selection for increased size. Male rifleman do not physically defend territory boundaries, predominantly maintaining territories via mutual avoidance (Cameron 1990) and vocalisation. They are rarely involved in physical interactions with rival male conspecifics (Cameron 1990). Sexual selection on male size is presumed to relate to the advantages gained by larger males predominantly in physical intra-sexual combat (Cuervo and Moller 1999; Figuerola and Green 2000). Clearly, larger size does not convey a significant benefit to male rifleman. It is also unlikely that sexual selection for smaller size explains the female-biased size dimorphism observed in this study. While smaller size may be an advantage to male rifleman due to ease of access to small cavities used for nesting, or for insectivorous foraging efficiency due to increased agility (e.g. Andersson and Norberg 1981; Szekely et al.)
While size dimorphism in rifleman appears unrelated to sexual selection on male size, fecundity selection on female size may explain patterns of dimorphism in this species. Fecundity selection is assumed to explain the female-biased size dimorphism found in most invertebrate taxa in particular (Head 1995; Kraushaar and Blanckenhorn 2002; Stephens and Wiens 2008). While larger than males, female rifleman are still very small, representing New Zealand’s smallest birds (Higgins et al. 2001). This small size may mean that female rifleman are potentially reproductively limited as larger size appears to be associated with reproductive output for females of several bird species (Cabana et al. 1982; Lislevand et al. 2009). However, female rifleman have a relatively high potential productivity, producing up to two clutches of between 2 and 5 eggs per season (Gray 1969; Sherley 1985). These eggs represent a considerable proportion of a females body weight and require a high level of reproductive investment (Sherley 1985). Males nuptial feed females prior to egg production and this is considered vital to meeting the high additional energy requirements needed for females to reach their reproductive potential (Sherley 1989). Unfortunately, data on reproductive rates of females could not be gathered in this study therefore any conclusions regarding the relationship between size and female fecundity are purely speculative. Further research investigating the relative reproductive success of females of variable size would enable analysis of the validity of the female-fecundity hypothesis in rifleman.

Sexual dichromatism and geographic variation in colouration in rifleman

Rifleman showed consistent and significant sexual dichromatism. Males were characterized by a consistent ‘green’ hue (approx. 541 nm) and had higher UV and a proportional increase of GY chroma compared to females. Females had a hue within the yellow-red region (approx. 647 nm) and were brighter and had more red chroma than males in the majority of populations. Using a human-based colour classification system, Hunt & MacLean (1993) concluded that rifleman plumage matched each sex’s respective foraging niche and was therefore likely to function in crypsis. While avian visual models were not employed in the current study due to the need for collection and analysis of single feathers, the measurement of rifleman feathers using spectrophotometry has allowed a description of rifleman plumage based on the avian visual spectrum as opposed to the more limited human visual system. The results indicate that the plumage of both male and female rifleman contains a spectral peak in the UV region, which was previously unidentified. While this study does not refute Hunt & MacLean’s assertion that male and female plumage colouration generally matches
background habitat, the presence of a UV peak in the plumage of both sexes means that rifleman are unlikely to be as cryptic as previously thought and that males in particular may in fact be relatively bright and conspicuous to avian predators against some forest backgrounds. Birds contain more cones than humans, allowing them to see into the UV region of the electromagnetic spectrum (Bowmaker et al. 1997). Analyses based on human colour perception may misrepresent avian perception of plumage colouration (Hofmann et al. 2007) which may have functional significance for intra-specific communication (Griggio et al. 2010). In rifleman, Hunt and MacLean (1993) found that plumage colouration only matched the foraging background of each sex during the breeding season which conflicts with expectations of entirely cryptic plumage for rifleman. Male and female rifleman both provision the nest during the breeding season, with the male often providing more parental care than the female (Sherley 1994). The bright green plumage of male rifleman would serve as a distinct disadvantage to crypsis while attending nest cavities which are usually built within branches or trunks. The proportion of time males would spend exposed during nesting is likely to outweigh the advantage of cryptic colouration while foraging, indicating that for males at least, natural selection for crypsis may not be the only selective force influencing colour.

The analysis presented here also indicates that plumage colouration varies on a spatial scale. Significant population effects were demonstrated for measures of sexual dimorphism in UV, GY and red chroma and for brightness and spectral saturation. Males from the Eastern Ranges in particular were brighter and had higher levels of UV, blue and red chroma compared to other populations which resulted in lower levels of sexual dimorphism in this population. Females are ordinarily brighter than males, however the increase in male brightness in the Eastern Ranges population caused reversed male-biased brightness dimorphism in this single population. Sexual dimorphism in red chroma was also drastically reduced in the Eastern Range population. Females had consistently higher red chroma compared to males for all populations except the Eastern Ranges where sexual dimorphism was almost completely absent due to both an increase in male red chroma and a decrease in female red chroma. Geographic variation in plumage colouration may support a cryptic function for rifleman colouration if geographic regions differ significantly in habitat type and therefore background colouration (Endler 1993; Delhey et al. 2010). Hunt and MacLean’s study found that rifleman plumage colouration matches the colour of Kanuka (Kunzea ericoides) trees, the predominant tree type within that study population. However, the population sampled in Hunt and MacLean’s study is highly managed, occupying an atypical habitat type compared to the majority of natural rifleman populations. Unfortunately, a detailed analysis of habitat variation was not possible during the course of the current study, therefore a robust test of the
crypsis hypothesis for rifleman is not possible here. Further research should include a spectrophotometric analysis of population-specific foraging substrates used by both male and female rifleman combined with an analysis of habitat use by each sex in each respective population.

While based on small sample sizes, the results of this study indicate that the hypothesis that sexual selection may be a driver of sexual dimorphism and population variation in colouration in rifleman warrants further investigation. In this study, levels of sexual dimorphism in red chroma were significantly associated with density. This relationship was explained by males increasing in levels of red chroma as density increased. Density is assumed to provide a relative measure of potential competition for male mating opportunities in rifleman, as cooperative breeding tends to occur at high densities (see Chapter Five) and helpers are almost always male (Sherley 1990). As territory spaces become filled and lower numbers of females are available, unpaired males may opt to cooperate to gain access to potential mates or indirect benefits (Sherley 1990). While long-term monogamous species are considered subject to lower levels of sexual selection as they do not compete for multiple females (Scott and Clutton-Brock 1990), monogamous males may still experience high levels of competition in attempting to compete for more fecund females (Kirkpatrick et al. 1990) or in maintenance of territories and pair-bonds (Scott and Clutton-Brock 1990). These conditions may put pressure on males to invest in traits which increase their likelihood of pairing successfully. Relative measures of red chroma resulting from the deposition of carotenoid pigments may provide information to prospective females on the quality and condition of males (Pryke et al. 2001; Pryke and Andersson 2003; Senar et al. 2005; Griffith and Pryke 2006; Hill 2006). Physiological trade-offs between colour display and body size are often provided as evidence of sexual selection on colour traits (Nijhout and Emlen 1998; Moczek and Nijhout 2004; Tomkins et al. 2005; Bonduriansky 2007). While this study found a general trend toward decreased size with increased red chroma in male rifleman at the high density Eastern Ranges site, this result was not significant, therefore no robust evidence of physiological trade-offs was found for male rifleman.

Implications of colour variation in the rifleman

This study provides evidence of geographic variations in both the size and colouration of male and female rifleman. The geographic variation in sexual dichromatism observed in this study may have significant implications for theories on the evolution of plumage variation in cryptically coloured species. Species with supposedly cryptic colouration have been the subject of very few investigations quantifying intra-specific variation in colour and I know of no other study that has looked at geographic variation in the green plumage of
forest birds. While green plumage is assumed to function exclusively in crypsis (Bortolotti 2006; Delhey et al. 2010), and therefore to be affected by natural selection rather than sexual selection, the combination of structural and pigmentary mechanisms responsible for green colouration mean that these feathers may convey more information to receivers than previously thought. Both structural and pigment-based colours have been shown to be condition dependent or to provide some measure of quality in some species (Hill 2000; McGraw et al. 2002; Siefferman and Hill 2003; McGraw et al. 2005; Hill 2006; Griggio et al. 2010). Carotenoid colouration in particular, often responsible for ‘green’ colouration, may provide extensive information on individual health and quality, due to the costs associated with carotenoid metabolism (Hill 2000; Arriero and Fargallo 2006; McGraw 2006; del Cerro et al. 2010; Chui et al. 2011). Most investigations into the role of sexual or social selection in the evolution of plumage colour variation have focused on obvious and brightly coloured display traits, as expression of these traits in males has been extensively linked to both male competitive interactions and female mate choice (Hill 2006; Senar 2006). However, the assumption of greater condition-dependence in obvious signalling traits compared to non-signaling traits (Cotton et al. 2004) has been shown to under-estimate the role of condition and quality on cryptic plumage colouration (Delhey et al. 2010). While based on small sample sizes and providing only preliminary analyses, this study indicates that males occupying populations at higher densities have higher red chroma in their feathers. Previous explanations of sexual dichromatism in rifleman have proposed that natural selection for crypsis is likely to explain patterns of colour variation in this species. However, this study indicates that rifleman plumage is more conspicuous than previously described and patterns of habitat use do not follow predictions based on an entirely cryptic function for rifleman colouration. Further research on mechanisms of mate choice in rifleman is needed to investigate the hypothesis that rifleman plumage variation is driven by sexual selection.

The results presented in this chapter are interesting to compare to the molecular analysis presented in Chapter Two. While Eastern Range males appear to have distinct morphology in terms of both size and colour when compared to other populations across the North Island, this finding is not concordant with the molecular analysis. The Eastern Range population was found to occupy a Phylogenetic position with relatively recent connectivity to other mainland North Island populations including the Western and Central populations. While high molecular divergence estimates characterise separations between the Insular and South-Eastern populations when compared to the remainder of the mainland populations, the morphological data presented here show no indication that this marked temporal separation between populations has resulted in the evolution of
morphological variation in size and colouration. Given the lack of concordance between molecular and morphological datasets, it seems unlikely that long-term drift is a sufficient explanation for the patterns of geographic morphological variation observed in this study. The marked divergence in the morphology of the Eastern Ranges individuals appears to have evolved in a relatively rapid timeframe, perhaps supporting a selective mechanism for population divergence.

Despite the lack of concordance between datasets, if male plumage colouration is affected by sexual selection for increased red chroma, the geographic variation observed in this study may have significant consequences for conservation management of the sub-species. If density variations across sites result in variations in colouration, the movement of individuals between sites showing morphological variations or the re-establishment of gene flow between variable populations may impact on the ability of transferred individuals to successfully gain mates, affecting the viability of managed populations. Additionally, assortative mating associated with display traits that signal phenotypic quality such as colour may drive divergence between populations in isolation and therefore have significant implications for gene flow between populations, and ultimately speciation (e.g. Saetre et al. 1997; Price 1998; Patten and Pruett 2009; Pryke and Griffith 2009; Pryke 2010).

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CHAPTER FIVE: Geographic variation in breeding behaviour: the effect of density and cavity availability on cooperation in the North Island rifleman
**Introduction**

The mating system used by individual avian species has been the subject of a vast amount of research due to the extreme diversity of breeding strategies utilised by different species. This diversity of breeding strategies can evolve due to variations in levels of both sexual and natural selection and are therefore influenced by population density, population sex ratio and resource limitation (Andersson 1994; Alcock 1998). For this reason, variations in the population dynamics of geographically separated populations may result in intra-specific geographic variation in breeding behaviour. As the breeding strategy utilised by separated populations can impact on life-history factors such as mortality and fecundity, the characterisation of geographic variations in breeding behaviour is an important field of study.

Cooperative breeding is a breeding strategy that has received a lot of attention in the literature as the apparent choice to forego independent breeding in order to assist in the breeding efforts of other individuals appeared at first to represent altruistic behaviour. Numerous investigations into the evolution of cooperation have demonstrated that helping behaviour in fact incurs benefits to both helpers and breeders, and therefore represents a stable breeding strategy that has evolved in numerous avian groups (Koenig *et al.* 1992; Komdeur *et al.* 2008). Cooperation can result in direct benefits to the helper such as gaining experience for future breeding attempts as parents (Komdeur 1996a; Komdeur 2003a), the possibility of accessing breeding opportunities within the group (Komdeur *et al.* 1995; Richardson *et al.* 2002; Du and Lu 2009), initiating reciprocal helping behaviour in the future (Ligon and Ligon 1983), and positive effects on survival or reproductive success through access to established territories (Emlen and Wrege 1991; Komdeur 1996a). Indirect benefits may also be significant for helpers, as most will increase their inclusive fitness by provisioning genetically related offspring (Emlen and Wrege 1989; Dickinson *et al.* 1996; Richardson *et al.* 2002; Covas *et al.* 2006). Helping can also benefit both the current breeders’ survival and reproductive success as well as the survival and condition of offspring (Heinsohn 1992; Richardson *et al.* 2002; Ridley 2007; Cockburn *et al.* 2008; Kingma *et al.* 2011).
However, cooperative breeding can also incur costs related to the lack of direct breeding by the helper, or to food shortage in the presence of too many helpers in the breeders’ territory (Komdeur et al. 1995; Dickinson et al. 1996; Komdeur 1996b; Brouwer et al. 2006). The decision to help or to retain helpers therefore depends on a variety of ecological and social factors. The ecological constraints hypothesis (Emlen 1982a) states that cooperative breeding may evolve where independent breeding is constrained due to limitations in resources, which may be represented by food or other requirements for successful breeding (Emlen 1982a; Covas et al. 2004; Atterberry-Jones and Peer 2010; Breininger et al. 2010). One form of the ecological constraints hypothesis, the habitat saturation hypothesis, maintains that high densities of territorial individuals is a primary factor in the development of helping behaviour. Under the habitat saturation hypothesis, subordinate individuals are expected to gain more from remaining in natal territories as helpers if the habitat is currently saturated (Brown 1974; Emlen 1982a; Komdeur et al. 1995; Komdeur 2003b) making independent breeding unlikely. This is especially so in sedentary species where density is typically high (Brown 1974; Arnold and Owens 1999). Thus, helping may be seen in some systems as a ‘best of a bad job’ scenario. Because of the interaction between resource or space limitation and cooperative breeding, population variation in the level and type of cooperative breeding seen in different populations is likely to have evolved in response to geographically variable resource levels. This variability in breeding behaviour may have significant consequences for the ongoing viability of individual populations as levels of cooperation may affect reproductive success (Sydeman et al. 1988). Additionally, geographic variation in levels of cooperation is significant for the evolutionary potential of the species as a whole because geographically (and culturally and/or genetically) isolated populations exhibiting different breeding behaviour may have separate evolutionary trajectories (e.g. Komdeur et al. 2008).

In this chapter I investigate geographic variation in both general breeding behaviour and levels of cooperation in populations of the North Island rifleman (*Acanthisitta chloris granti*). The relationship between habitat saturation, ecological constraints and the extent of cooperative breeding across rifleman populations is also investigated as a driver of geographic variation in cooperative breeding. Rifleman have been the subject of very little study, however a comprehensive breeding behaviour analysis of a single population of the South Island sub-species (*Acanthisitta chloris chloris*) has indicated that while breeding units typically consist of a single male and a single female, some
territories contain helpers which may contribute to the breeding effort by provisioning young and assisting with nest maintenance (Sherley 1990). Adult helpers, which are usually male, may be either casual helpers who sporadically assist at multiple nests of mostly unrelated breeders, or may be regular helpers that assist reliably at mostly unrelated nests (Sherley 1990). Some adult helpers are thought to gain mating opportunities by pairing with offspring or abandoned females from the nest at which they help. Male breeders benefit from having adult helpers as their contribution allows the smaller male breeders (relative to larger females) to decrease energy expenditure on offspring provisioning, while female breeders appear to survive better when helped (Sherley 1990). Juveniles from the first clutch have also been reported to occasionally help at the second clutch (Sherley 1990), a behaviour which is thought to result in learning opportunities for helpers and opportunistic helping benefits for breeders. This indicates that helpers may benefit from kin selection in some cases. Cooperative breeding therefore appears to be driven primarily by a shortage of females combined with kin selection in the South Island population.

The research carried out in the South Island indicates that multiple mechanisms may be driving the evolution of cooperation in rifleman. However, the ecological constraints hypothesis has never been investigated for the species. Rifleman use cavities for nesting and therefore cavity availability is likely to be a limiting resource and may represent an ecological constraint on independent breeding. The population in the South Island occupies a highly modified and managed habitat, with most pairs breeding in artificial nest-boxes (Sherley 1985). Cavity availability is therefore unlikely to be substantially limiting for this population. In the North Island however, most populations are unmanaged in terms of nest site provisioning. Rifleman in these native populations rely on natural cavities for nesting, building spherical nests within small tree cavities (Gray 1969; Higgins et al. 2001). While rifleman build small nests and are therefore likely to be able to use a range of cavity sizes, territorial year-round sedentary individuals are likely to experience constraints in terms of the number of available cavities, particularly in habitats where cavities are scarce. While the avifaunal assemblages found within natural rifleman populations include cavity nesters, rifleman typically use much smaller cavities than those used by other cavity nesting species (Higgins et al. 2001). Therefore, the only constraints on cavity availability are likely to be from intraspecific competitors and from natural limitations in cavity numbers for sedentary territorial individuals. The North Island sub-species has not been the subject of any published studies, therefore the extent to which they use cooperation is unknown.
North Island populations of rifleman have a fragmented distribution, being restricted largely to isolated mountain ranges across the mainland and a single off-shore island (Robertson et al. 2007). Because of this fragmented distribution, conservation initiatives have recently created two populations as a result of translocation. Translocation has been used extensively to reintroduce species into areas from which they have become regionally extinct (Armstrong and McLean 1995). Founding populations following translocation are typically low density (Armstrong and Wittmer 2011) and therefore not at carrying capacity, therefore translocation source and recipient sites represent a natural experiment where the effects of density and resource limitation on aspects of a species behaviour can be tested without the confounding effect of genetic divergence. Translocation sites have been used in the past in analysis of drivers of cooperative breeding as changes in population dynamics and behaviour may change over time as populations approach carrying capacity (e.g. Komdeur et al. 1995).

The aim of this chapter is to describe population variation in general breeding behaviour (i.e. male and female contributions to parental care) and to investigate the relationship between cooperation and ecological constraints, measured in terms of a) territory space and b) cavity availability. Using the paired source and recipient sites for both of the translocations carried out with rifleman in the North Island, this study addressed the following objectives:

1) To investigate whether North Island rifleman exhibit geographic variation in nest provisioning behaviour and levels of cooperation
2) To test whether levels of cooperation are associated with population density, indicating that territory saturation/availability is limiting in rifleman
3) To test whether levels of cooperation are associated with breeding resource availability, measured in terms of cavity availability
4) To investigate whether the types of cavities available are dictated by differences in habitat type between populations by describing the floristic composition and the predominant cavity-providing vegetative species of each site
5) To investigate whether rifleman are in fact limited by true cavities, by describing nest site characteristics between different habitats and testing whether the use of nest sites is influenced by cavity availability
Methods

Study sites

Data was collected at four sites across the North Island including the source and recipient sites of both North Island rifleman translocations.

Boundary Stream Mainland Island (BS) is located within the Maungaharuru Range, within the eastern ranges of the North Island of New Zealand (39º 06’S, 176º 48’E) (Fig. 1). BS is an 800 ha block of native forest that has been managed as a ‘mainland island’ site by the New Zealand Department of Conservation (DoC) since 1996. The site is actively managed to reduce mammalian predator densities and contains a high density of rifleman (see Chapter Four), which are native to the region (Robertson et al. 2007). BS has been subject to historical logging impacts, however the site contains mature native forest and rifleman are completely unmanaged at the site. No artificial nest boxes are provided for rifleman, therefore individuals build nests within natural cavities.

The Cape Kidnappers and Ocean Beach Wildlife Preserve (CK) is located on the Cape Kidnappers Peninsula in Hawkes Bay, North island of New Zealand (39º 40’S, 177º 02’E) (Fig. 1). CK is a privately-funded conservation initiative on private land and has a mixed habitat composed of bare farm-land and a dry river-bed surrounded by regenerating native bush. The forested ridges surrounding the river bed are composed of fairly monotypic early succession vegetation species. However, the river gullies contain remnant mature native trees and regenerating scrub. The rifleman at CK were translocated from Boundary Stream Mainland Island (BS) over a series of successive transfers between 2008 and 2010. The population is at a low density as a result of high post-release mortality and/or high levels of dispersal following release (unpublished data). CK is managed by extensive trapping and poisoning of invasive mammalian pests and nest-boxes are provided to assist with nesting.

Little Barrier Island (LBI) is a closed sanctuary located in the Hauraki Gulf of the North Island (36º, 12’S, 175º, 04’E) (Fig. 1). The island is considered to host the most complete assemblage of North Island endemic avifauna in New Zealand and rifleman are native to the island (Turbott 1961). While logging was carried out in the 19th century and invasive species occupied the island at this time (Watson 1961), it has been managed as a wildlife sanctuary.
since 1895. Invasive species were completely eradicated in 2004 (Rayner et al. 2007). Despite historical logging, the island is covered with mature native forest which varies in composition with altitude (Hamilton and Atkinson 1961). The island is considered to host medium densities of rifleman compared to other areas through the mainland (Robertson et al. 2007).

Tiritiri Matangi Island (TM) is a sanctuary located in the Hauraki Gulf on the east coast of the upper North Island (36° 24’S, 175° 04’E) (Fig. 1). The island consists of patches of regenerating native forest varying in diversity and composition but most containing some remnant mature trees. Rifleman were translocated from Little Barrier Island in three transfers between 2008 to 2010. The island is mammalian predator-free and rifleman are provided with nest-boxes to assist with breeding. As a result of recent translocation, the island is considered to be low density as the population is still establishing.

Fig. 1. Source and recipient sites for the two translocations carried out on North Island rifleman to date, including Little Barrier Island to Tiritiri Matangi Island (left) and Boundary Stream Mainland Island (BSMI) to Cape Kidnappers and Ocean Beach Wildlife Preserve (CKOBWP).
Cooperative breeding and parental care

Nests were defined as cooperative if more than a single male and single female individual were observed provisioning the nest within nest-watch periods. Levels of cooperative breeding at each site were determined by locating breeding pairs using both playback of rifleman calls and observations. Surveys were conducted throughout the 2009-2010 and 2010-2011 breeding seasons (September to February). Once pairs were located by sight or sound, they were observed periodically for signs of nesting behaviour, including nest-building, incubation and nest provisioning (Gray 1969; Sherley 1985). Focal animal observations during periodic site visits allowed me to identify the general phase of breeding, and the timing of the chick-rearing phase could be determined based on previous breeding data for the species (Gray 1969; Sherley 1985). Nest watches were carried out at each nest during the ‘late-nestling’ phase, characterized as being when chicks have reached five days old or later. The timing of this phase was selected based on previous studies demonstrating that helpers are unlikely to be permitted to contribute to the nesting effort prior to five days (Sherley 1985). Nest watches consisted of a single 60 minute observation period during which all visits to the nest were recorded and the sex and identity of the visitor noted. Individual identities of most birds at BS, TM and CK could be determined by observation of unique combinations of coloured plastic leg bands. Birds at LBI were not banded due to potential impacts on the success of catching during ongoing translocations taking place from LBI during the study period, meaning individuals could not be identified using leg bands at this site. Additionally, nest-watches at this site were restricted to a single 30 minute interval due to time restraints related to translocation procedures. The presence of helpers was possible by identifying individuals provisioning each nest. If nest contributors were un-banded, the presence of helpers was identified by observation of two individuals of the same sex provisioning the nest in rapid succession (i.e. visual identification of two provisioning individuals of the same sex at the same time). The presence of un-banded individuals in the study and the low frequency at which both helpers and breeders provisioned at the same time means that helping is likely to be under-estimated in all populations, however was necessary due to the inability to catch and band any un-banded birds once breeding had commenced.
Population density estimates

Rifleman are inconspicuous birds with quiet, high frequency calls (Higgins et al. 2001; Krull et al. 2009), so distance sampling was deemed an inadequate methodology for accurate density measurements. Instead, density surveys were conducted using playbacks of North Island rifleman calls, known to cause a response in territorial birds. Areas containing rifleman were extensively surveyed within the BS, TM and CK sites using established pathways or transect lines that ensured coverage of all areas of bush. Rifleman calls recorded from North Island sites were broadcast at 50-100 m intervals and the number, sex and (where available) identity of responsive individuals was recorded. GPS positions for all playback points were taken to allow calculation of the area surveyed. It was assumed that rifleman were able to hear and respond to calls from 50 m away, which is likely to be a conservative estimate (Mitchell 2004). Due to the translocation series that was being carried out on LBI during this study, density estimates at this site did not use playback as it can result in habituation and thus reduce catching ability for future translocations. Instead, a team of eight individuals was used to survey an area of 83 ha using observations based on sightings and calls. Tracks were re-sampled six to seven times each by different observers over the course of five days and GPS points noted for each observation. Sightings were deemed repeats if observations were noted at approximately the same GPS location (within 100 m). Surveys were repeated at the same time of year (November) for two years to ensure density estimates did not vary significantly between years and allowing an average across both years to be calculated. Density was found to be almost identical across both years, despite translocation removing up to 30 territorial individuals per year. Population densities were measured as the total number of birds identified across the surveyed area and then a relative measure of birds per hectare was calculated. Polygons were created by projecting a 50 m radius around GPS points recorded for playback sites. For the LBI site, polygons were created around tracks used for surveying as playbacks were not used. The total area surveyed was then calculated for each site and a measure of density estimated as the number of birds per hectare of surveyed land.
Habitat type and cavity availability

Habitat type and cavity availability at each site was assessed by using the point centred quartile method (PCQM) (Pollard 1971) which allows a standardised estimate of cavity density by point sampling. Sample points were selected by creating transect lines parallel to walking tracks and riverbeds at a distance of 10 - 15 m to ensure each sample point was surrounded by natural forest. These features were chosen as a basis for transect lines as they provided good coverage of forested areas within all regions and allowed access to areas with difficult terrain. Along each transect, sample points were selected at randomly generated intervals, with a minimum distance between each point of 50 m. At each sample point a quartile stick was used to identify radial sampling quarters. Within each quarter the area within a 25 m radius from the sample point was searched for the nearest tree over 10 cm DBH (diameter at breast height) (deemed an adequate size to contain a rifleman nest) containing a cavity. Both the distance to the tree and the species of tree were then recorded. Cavities were defined as any tree hole over 2 cm diameter, following a previous study on small passerine cavity nesters (Remm et al. 2008).

An estimate of cavity density derived from data was calculated using the formula:

\[ \lambda = \frac{4(4n - 1)}{\pi \sum_{i=1}^{n} \sum_{j=1}^{4} R_{ij}^2} \]

Where \( \lambda \) equals an unbiased population estimate of cavity density, \( n \) is the number of sample points along the transect, \( 4n \) represents the number of samples or observations (one for each quarter at each point), \( i \) is a particular transect point (where \( i = 1; \ldots; n \)), \( j \) represents a quarter at a transect point (where \( j = 1; \ldots; 4 \)) and \( R_{ij}^2 \) is the point to tree distance at point \( i \) in quarter \( j \) (Pollard 1971). Confidence intervals at the \( (1 - \alpha) \) 100% level were then calculated as:

Lower endpoint:

\[ \lambda = \left( \frac{Z_\alpha + \sqrt{16n - 1}}{\pi \sum_{i=1}^{n} \sum_{j=1}^{4} R_{ij}^2} \right)^2 \]
Upper endpoint:

\[ \lambda = \left( \frac{Z_{1-\frac{\alpha}{2}} + \sqrt{16n - 1}}{2\pi \sum_{i=1}^{n} \sum_{j=1}^{4} R_{ij}^2} \right)^2 \]

Where \( Z_{\beta} \) is the standard normal \( Z \)-value corresponding to probability \( \beta \).

The PCQM procedure also allowed a general description of the general composition of each habitat. Within each sample point, the species of the nearest tree over 10 cm DBH was recorded regardless of the presence of cavities.

*Cavity selection by nesting rifleman*

Nests were located by following birds exhibiting breeding behaviours until they entered the cavity. Nest characteristics were then recorded including the species of tree used, the nature of the cavity (enclosed, semi-enclosed, burrow, open, nest-box), the part of the tree containing the cavity (i.e. trunk, limb), the cavity height and the aspect of the cavity. Height was estimated using a Suunto clinometer (Suunto, Finland). Note that cavity height and aspect were not recorded for nests from the CK site. Nest boxes within both recipient sites were created and placed by conservation managers at each location and were designed based on specifications contained in Gray (1969). Nest boxes were nailed to mature tree trunks at between 1-2 m height and were placed throughout the habitat surrounding release sites.

*Statistical analysis*

Relative parental care for males, females and helpers was measured as both the total number of visits to the nest and by calculating the proportion of the total visits to the nest by each sex or individual. Following tests for normality and equality of variances using the Levene and Shapiro-Wilks tests, differences in the level of parental care between the sexes and between cooperative and non-cooperative nests were analysed using ANOVAs.
variation in the levels of parental care provided by both males and females were also analysed using ANOVA. The level of cooperation per site was calculated as the proportion of nests demonstrating cooperation (nest visitation by helper individuals) during nest-watch periods. Relationships between cooperation and both density and cavity availability were not analysed statistically due to a lack of variation and low sample sizes. To analyse geographic variation in the use of different cavity types, population differences in the proportion of nests built in closed cavities was analysed using a Chi Squared test. The relationship between cavity availability and the use of enclosed nests was measured using a Spearman's rank correlation. Spearman's rank correlation was also used to test the relationship between the floristic diversity of cavity-providing species and the choice of species rifleman use for nest sites within each location. Finally, Welch’s Test and ANOVA were used to investigate population variations in the height and aspect of rifleman nest cavities respectively.

**Results**

*Geographic variation in parental care and cooperative breeding*

Across all nests, males (social fathers) and females differed in the amount of parental care provided to chicks during the late nestling phase. Males provisioned the nest 12 times per hour on average compared to 10 times per hour for females. To provide a relative measure of the contribution of males and females, the proportion of the total parental care (provisioning visits) provided to the nest by each sex was calculated. Males contributed significantly more parental care than females (ANOVA, df=1, F=7.0148, P=0.0094), with males providing approximately 55% of care (Fig. 2)
Fig. 2. The proportion of the parental effort provided by male and female rifleman (n=49 nests). Social fathers provide significantly more parental care than females.

There was no evidence that either the total visitation rate or the relative proportion of parental care varied geographically for either males (F=0.9372, P=0.4306) or females (F=1.1190, P=0.3514). When nests containing only a single breeding pair (i.e. no helpers) were analysed, population variation remained non-significant (F=1.3330, P=0.2815) (Figs 3-6).
Fig 3. The mean number of provisioning visits per hour by social fathers across the four populations. Graphs show means ± s.e.m. Male parental care did not differ significantly between populations (LBI=Little Barrier Island, BS=Boundary Stream, CK=Cape Kidnappers, TM=Tiritiri Matangi)

Fig 4. The mean number of provisioning visits per hour by females across the four populations. Graphs show means ± s.e.m. Female parental care did not differ significantly between populations (LBI=Little Barrier Island, BS=Boundary Stream, CK=Cape Kidnappers, TM=Tiritiri Matangi)
Fig 5. The mean proportion of male parental care across the four populations. Graphs show means ± s.e.m. Male parental care did not differ significantly between populations (LBI=Little Barrier Island, BS=Boundary Stream, CK=Cape Kidnappers, TM=Tiritiri Matangi).

Fig 6. The mean proportion of female parental care across the four populations. Graphs show means ± s.e.m. Female parental care did not differ significantly between populations (LBI=Little Barrier Island, BS=Boundary Stream, CK=Cape Kidnappers, TM=Tiritiri Matangi).
To analyse cooperative breeding, analyses were restricted to nest watches during years where most individuals were
individually banded. The proportion of nests at which individuals exhibited cooperative breeding behaviour differed
between populations. No helpers during the nestling phase were witnessed during nest-watches at LBI, TM or CK.
Cooperative breeding was evident at the BS site, where five of the 17 nests observed (29%) had helpers who
provisioned the nest during the late-nestling phase. Two of the five cooperative nests had two helpers, one of either
sex. All of the remaining cooperative nests had a single male helper. Interestingly, two of the helpers were known,
paired males; at least one of which had nested successfully and was raising fledglings in a neighbouring territory at
the time of helping. Two of the five cooperative nests had both a male breeder and a male helper that were un-
banded meaning it was not possible to identify the helper’s contribution to the breeding effort.

Across all populations, the proportion of parental care (provisioning visits) did not vary between nests with or
without helpers for either males (F=2.0618, P=0.1590) or females (F=0.1811, P=0.6728). Overall, helping
therefore did not result in net benefits in terms of decreased parental provisioning for females, and effects of helper
effort on male breeder effort could not be detected (due to an inability to identify individual males). This was also
the case when analyses were restricted only to BS (F=0.1712, P=0.6864). However, while small sample sizes
prohibited a statistical analysis, there was some evidence of sex-specific advantages for retaining helpers within the
three nests for which data on helper identity was present. Two of the three nests with identifiable helpers had male
helpers who contributed between 20% and 43% of the total provisioning effort. This helping effort appeared to
result in a decrease in parental care for males: both female breeders still contributed a proportion of parental care
comparable to the average across all populations (41-45%), while male parental care dropped from the across-
population average of 53% to between 16-35%. Interestingly, in the single nest with an identified female helper, the
situation was reversed. While male care remained near the across-population average at 50%, female care dropped
to 33% for this nest while the female helper contributed 17% of the provisioning. While no cooperation was
witnessed at the LBI, TM and CK sites during nest-watch periods, a single nest visitation from an apparent helper
(identified via individual band combination) was witnessed on one occasion at the TM site. At the CK site, one nest
was entered by an extra-pair female, although no provisioning (food delivery) was witnessed. Additionally, a helper
male was observed feeding fledglings, despite continuous aggression from both parents.
Density and cooperative breeding

Population density varied between sites (Fig 7). A total of 83 hectares was sampled at LBI, in which 67 adult individuals were identified, producing a density estimate of 0.81 birds/ha. BS had a high population density compared to other sites; in an area of only 11 hectares 53 territorial adults were identified, resulting in a density estimate of 4.82 birds/ha. CK had low population density with the 23 hectare area containing 0.91 birds/ha. Finally, TM had a surveyed area of 22 hectares in which 24 birds were found, resulting in a population density of 1.09 birds/ha.

![Graph showing population density (birds/ha) across four populations: LBI, BS, CK, TM. BS has the highest density, followed by TM, CK, LBI.](image)

Fig 7. Population density (birds/ha) across the four populations. The BS site has a higher population density compared to all other sites (LBI=Little Barrier Island, BS=Boundary Stream, CK=Cape Kidnappers, TM=Tiritiri Matangi)

Only a single population was found to exhibit cooperative breeding during nest-watch periods (BS). Unfortunately, due to the complete lack of observed cooperative breeding in all but this site, a statistical analysis testing the relationship between observed cooperation and density was not possible. Possible evidence in support of a relationship between density and cooperation comes from the fact that the only observed cooperation during nest-watch periods occurred in 27% of nests at the BS site which also contains the highest density of birds.
Cavity Availability and Cooperative Breeding

Cavity availability varied between the four sites. The natural source sites LBI and BS had high cavity availability, containing 417 cavities/ha (95% CI: 357-485) and 304 cavities/ha (95% CI: 254-362), respectively. TM contained 263 cavities/ha (95% CI: 184-371). CK had low cavity availability, with only 71 cavities/ha (95% CI: 57-88). The 95% CI indicated statistically significant differences between CK and the other three sites.

Due to the lack of cooperation during nest-watches at three of the four sites, a statistical analysis of the relationship between cooperation and cavity availability was not performed. There was no indication that cooperation increased with decreasing cavity availability as the only site with limited cavity availability (CK) had no cooperative nests.

Habitat type and cavity availability

The dominant vegetative species varied between locations. BS had the highest floristic diversity with 17 species recorded (Fig. 8). BS was dominated by Weinmannia (19%), Pseudowintera (9%), Beilschmiedia (9%) and contained a high proportion of Cyathea tree ferns (30%) (Fig. 9). Lower levels of Melicytus (7%) and Nothofagus (5%) also featured. LBI also contained high overall species diversity with 12 different tree species characterised in sampling (Fig. 8). The dominant tree species was Kunzea, which was the nearest tree in 31% of the sample quarters. However, Dead Wood (25%), Nothofagus trees (10%) and Cyathea tree-ferns (11%) were also found to occur regularly (Fig. 9). CK was characterised by a lower diversity of trees species overall (Fig. 8). Kunzea (58%) featured highly at this site with species such as Melicytus (16%), Corynocarpus (8%), and Alectryon (11%) making up most of the remainder (Fig. 9). TM also had low floristic diversity with Dysoxylum (44%) and Cyathea tree-ferns (38%) making up the large majority of the tree species sampled (Figs. 8-9).
Fig 8. Floristic diversity (number of species) of trees sampled as the nearest tree and as the nearest tree containing cavities within the four sites
Fig 9. The proportion of each vegetation species identified as the nearest tree (>10 cm dbh) within sample quarters using the PCQ method across the four study sites.
The diversity of trees containing cavities was generally in line with the diversity of overall trees found at each site. The source sites, BS and LBI, contained a higher diversity of cavity-containing trees compared to the translocation recipient sites (Fig. 8). The *Kunzea* trees which dominate LBI also appear to host a high percentage of the available cavities (48%) (Fig. 10). Dead Wood also contained a high percentage of the available cavities (19%) and 7% of cavities were hosted by either *Nothofagus* or *Coprosma*. While LBI contained a high number of *Cyathea* tree-ferns, these did not often contain cavities. A similar result was found for the BS population. While *Cyathea* tree-ferns were dominant at BS, they provided no resources in terms of cavity availability. However, *Weinmannia* trees were the dominant cavity provider (35%), while *Melicytus* (16%) and Dead Wood (15%) also provided cavities. Interestingly, while CK was characterized by low overall floristic diversity (Fig. 8), cavities were found in a slightly higher number of different tree species, including *Kunzea* (30%), *Corynocarpus* (16%), *Melicytus* (24%), *Hedycarya* (9%) and *Alectryon* (19%) (Fig. 10). Cavity availability on TM was provided predominantly by *Dysoxylum* (31%) and *Metrosideros* (22%). Dead *Cyathea* also provided cavities in the form of dead stumps (13%) (Fig 10).
Fig. 10. The proportion of dominant vegetation species identified as the nearest tree (>10 cm dbh) containing a cavity within sample quarters identified using the PCQ method across the four study sites.
Cavity selection by nesting rifleman

Rifleman were found to be relatively flexible in their use of cavities for nesting. While the majority of nesting attempts were contained within conventional cavities (i.e. enclosed nests inside tree holes) at most sites, birds also built nests which were only semi-enclosed (Fig. 11). For example, nests were located within dead *Cyathea* stumps and dead *Cyathea* fronds, or in the forks of trees with only partial cover. Several individuals chose to build nests within small burrows in the ground, either within banks or under fallen logs. Additionally, a number of birds built nests that were not enclosed within any kind of cavity, instead being built as a spherical nest within the fine branches of trees or dead branchlets and foliage of fallen trees. Finally, several individuals chose to nest inside artificial nest-boxes which are provided at the translocation recipient sites. The proportion of nests built within enclosed natural cavities varied between sites ($X^2=38.67$, df=3, Prob>ChiSq<0.0001).

![Graph showing the percentage of each nest type used by rifleman across the four sites. Populations vary significantly in the proportion of nest sites built in enclosed natural cavities.](image)

**Fig 11.** The percentage of each nest type used by rifleman across the four sites. Populations vary significantly in the proportion of nest sites built in enclosed natural cavities.

To investigate whether the use of flexible non-enclosed cavities resulted from a lack of cavity availability, a regression analysis between the proportion of nests built in enclosed natural cavities and cavity availability was performed using populations as samples. No statistical relationship between cavity availability and enclosed natural cavities varied between sites ($X^2=38.67$, df=3, Prob>ChiSq<0.0001).
The results of this study indicate that while males generally contribute more parental care during the nestling phase in North Island rifleman, there is no evidence of geographic variation in provisioning allocation between the sexes across these populations. In this study North Island rifleman were found to breed cooperatively in some cases, and levels of cooperation varied between populations. Although small sample sizes regarding units of biological analysis (i.e. populations/geographic localities) and a lack of variability meant that a statistical analysis of the relationship between density and cooperation was not possible, there was some evidence that population variation in cooperation may be related to population density, as the high density population exhibited the only cooperation observed within nest-watch periods. The fact that cooperation was used at a higher proportion of nests at the high density site may indicate preliminary support for the habitat saturation hypothesis, although further research with higher sample sizes and repeated nest-watches should be used to confirm this. In contrast, I found no support for the ecological constraints hypothesis where constraints were represented by cavity availability. Support for this
hypothesis has been found in numerous studies and has predominantly focused on ecological variables such as food (e.g. Ford et al. 1988; Covas et al. 2004) and breeding sites (e.g. Kokko and Lundberg 2001). As a territorial cavity nester, rifleman have a natural limitation on reproductive success as they are dependent on nest sites which may be scarce in certain environments. I therefore predicted that cooperation in rifleman may relate to density through limitation of breeding sites (i.e. cavities) available for nesting. I found no evidence that cavity availability is related to levels of cooperative breeding, indicating that if ecological constraints are acting to promote cooperative breeding in rifleman, cavities are not the limiting resource.

An increase in cooperation did not appear to result in a decrease in parental effort for either sex. Female breeders contributed similar levels of parental care at nests with or without helpers, and no population variation in the relative proportion of parental care provided by each sex was detected. However, an inability to identify helpers compared to breeding males meant that this could not be investigated in full. In the three nests with identified helpers, there was preliminary evidence that helping resulted in sex-specific benefits in terms of decreased reproductive effort. However, further research will be required to confirm this pattern. Overall, males contributed significantly more to chick rearing during the late-nestling phase compared to females, however this was reversed in the two nests with male helpers. Previous research on the South Island sub-species of rifleman has found that males consistently provide more parental care during chick rearing than females (Sherley 1994). Males also appear to benefit most from the presence of helpers, with male parental provisioning effort decreasing when helpers assist with breeding. Sherley (1990) hypothesized that unrelated male helpers gained access to potential mates by helping. While my study did not provide any direct evidence of this, observations provide some support for this hypothesis in North Island rifleman. Male rifleman at several locations were observed assisting with raising offspring that were sired by different males, particularly when their mate was lost or when pairing opportunities arose (e.g. when a neighbouring female lost her partner) through the breeding season. In one location, a breeding female lost four partners in a single breeding season and replacement males rapidly filled the breeding position, provisioning chicks that were sired by the previous male. These observations, combined with the fact that the majority of helpers were male, indicates that cooperative populations may have excess males who have to compete for opportunities to pair with females. Given their short life-span (Sherley 1985) the cost of raising another male’s offspring may be outweighed by the future
benefits of accruing a female for future breeding attempts. In this way, females are the limiting resource as opposed to cavities. Mate limitation has been found to be a driver of cooperation in other species, indicating that constraints in terms of mating opportunities may predominate over competition for space or territory resources in some populations (e.g. Pruett-Jones et al. 2010).

The habitat occupied by rifleman varied between locations, with different vegetative species dominating each site. The natural source sites were both characterized by a higher overall floristic diversity, and an increased diversity of cavity-providing trees compared to the recipient translocation sites. Habitat variation across the distribution of many species may have significant consequences for population divergence. As both foraging and breeding behaviours are often modified in relation to environmental heterogeneity, population variation in habitat type can result in divergent niche use if populations are separated for significant periods of time or are highly differentiated in terms of environment (e.g. Laerm 1973; Herrel et al. 2008). Additionally, ongoing divergence in use of habitat may lead to differentiation of morphology if species are physiologically adapted to a particular foraging niche (e.g. Grenier and Greenberg 2005; Herrel et al. 2008). The fact that rifleman occupy habitats with different dominant vegetative species and different levels of availability of resources may have consequences for separated populations of the species. Rifleman morphology is uniquely adapted for their foraging niche, with large hind claws and long upturned beaks for successful gleaning of invertebrates under the bark of trunks and branches (Hunt and MacLean 1993). The sites included in this study were highly variable in terms of both the density and the floristic diversity of mature trees. Variations in habitat features such as these may result in behavioural variations between separated populations over time, as individuals adapt to utilize resources within each location.

The locations used in this analysis had varying levels of vegetative diversity, particularly of species containing cavities for nesting. However, despite the fact that the natural source sites contained a higher diversity of cavity-containing trees, a higher diversity of potential cavity locations did not correspond to a higher diversity of cavity selection for nest sites. These results indicate that rifleman are flexible in their use of the habitat, particularly for nest site selection. Population variation in cavity availability could lead to flexibility in breeding behaviour,
territoriality and nest site selection in order to allow rifleman to successfully nest despite resource limitations, causing populations to differ behaviourally. However, rifleman appear well adapted to coping with variations in the level of cavity availability between sites. While rifleman occupy territories, they do not patrol or actively defend territory boundaries, and territories are largely maintained via mutual avoidance (Cameron 1990). Intrusions are often tolerated without aggression and neighbours are often observed occupying nests within a very short distance of one another indicating that territoriality is not likely to exert pressure on neighbouring individuals in terms of cavity competition. Additionally, this study showed that rifleman are highly flexible in their choice of cavity site. Rifleman built successful nests at a range of different heights of the forest and in a range of different vegetation species. Additionally, rifleman appeared to not be restricted to conventional enclosed cavities, building nests in ground burrows, partially enclosed stumps or loose tree-fern fronds and even built closed spherical nests in the open. This type of plasticity was not a response to varying levels of cavity availability so does not appear to be the result of rifleman compromising on nest requirements in response to cavity limitation across sites. This type of plasticity may explain why rifleman successfully occupy a range of different habitat types and climatic conditions (Robertson et al. 2007), allowing them to build insulated nests in a variety of environments. While the modification of nest site selection in response to local resource limitation may have significant consequences for population divergence, the sites selected for this analysis provide evidence that behavioural modification of nest site selection between sites is likely to be a result of behavioural plasticity as opposed to an adaptive shift. Firstly, rifleman built non-enclosed nests in all locations, regardless of cavity availability, indicating that this flexibility may be a response to localized cavity options within the territory as opposed to broad-scale behavioural shifts due to different environments. Secondly, individuals located at translocation recipient sites are of the same genetic lineage as individuals at the source sites, meaning that any shifts in behaviour do not correspond to inherited behavioural adaptations in distinct genetic lineages.

Population differences in the level of cooperative breeding observed in this study have implications for the evolutionary potential of the species. Cooperative breeding has been shown to result in benefits to both helpers and breeders in terms of reproductive success and survival (Heinsohn 1992; Komdeur 1996; Richardson et al. 2002; Ridley 2007; Kingma et al. 2010). If lower densities result in lower levels of cooperation in isolated populations,
the loss of cooperative breeding may influence fecundity and therefore population dynamics. Additionally, population differences in the use of cooperative breeding may result in differential evolutionary trajectories through the interaction of cooperation and density dependent demographic characteristics (Komdeur et al. 2008), particularly for populations separated by long term isolation. For example, increased natal philopatry in cooperative species has been associated with decreased speciation due to a lack of dispersal across new habitats (Cockburn 2003).

Conclusion

This study provided preliminary evidence supporting the habitat saturation hypothesis but demonstrated no support for a specific form of the ecological constraints hypothesis: cavity limitation. While no geographic variation in general patterns of male and female parental care was observed, geographic variation in levels of cooperation was found and may relate to population density levels. Rifleman appear to be flexible cavity users, demonstrating a remarkable plasticity in nest site choice as opposed to being specifically adapted to changes in habitat type. Decreased cavity availability did not result in higher levels of cooperation. Male rifleman may instead by constrained by limitations in the number of available females, resulting in cooperation at high densities.

References


CHAPTER SIX: Summary and conservation implications
Rifleman conservation

The aim of this thesis was to investigate levels of genotypic and phenotypic diversity across isolated populations of the North Island rifleman (*Acanthistta chloris granti*). Rifleman have been classified as regionally common by the IUCN (IUCN 2012), and therefore have been the subject of very little discussion regarding conservation management. However, their distribution is highly fragmented with populations isolated to discontinuous mountain ranges across the North Island, separated by large areas of local extinction due in large part to habitat clearance (Robertson *et al.* 2007). While some populations retain high densities of rifleman (Robertson *et al.* 2007), North Island populations have been recognized as ‘at risk, declining’ (IUCN 2012) and noted declines have been documented in several regions (Smith and Westbrooke 2004; Elliott *et al.* 2010). The discovery of an isolated and low density population in the far north (Pierce 1994), which represents the sole mainland population north of the central North Island (Robertson *et al.* 2007), has further emphasized the need for discussion on future conservation approaches for the sub-species. Reflecting the increasing awareness of the need for intervention in rifleman conservation, two recent North Island initiatives have translocated small groups of rifleman from local source sites to sanctuaries in the hope of establishing self-sustaining populations of rifleman (unpublished data). Given the likelihood of further use of translocation in rifleman conservation management, this investigation into population variation comes at an opportune time. As translocation may result in the re-connection of previously isolated populations, or in the re-establishment of populations which have been locally absent for potentially extended periods of time, information on population variability is vital in guiding appropriate management decisions for threatened species.

Summary of results

This research aimed to analyse levels of variability in genotypic and phenotypic traits which were particularly likely to influence the relative success of translocation in the re-establishment of extinct populations or the mixing of previously separated populations.
Chapter Two aimed to analyse levels of genotypic variation both within and between isolated populations of North Island rifleman. Using both coding and non-coding regions of the mitochondrial genome, I analysed sequence variation between six isolated populations across the current range of the North Island sub-species. The results demonstrated significant genetic divergence between three key areas within the North Island distribution. As expected, the sole insular site (Little Barrier Island) was characterized by marked divergence from all mainland populations. Tentative dating estimates of this separation from the mainland coincide with the creation of the island as a result of volcanism, following which isolation from the mainland appears to have represented a significant barrier to ongoing dispersal and gene flow of rifleman from the mainland. Across the mainland of the North Island, two significant patterns of population connectivity were uncovered. Firstly, an unexpected lack of connectivity between the south-eastern populations (including the Southern and Eastern Coastal populations) and the remaining mainland populations was described. Despite the close proximity of Eastern Ranges and Eastern Coastal populations, these populations showed the most marked divergence of all population comparisons, indicating long-term historical isolation. The dating of this divergence appears to coincide with Pleistocene sea-level changes which effectively isolated southern and south-eastern refugia from the upper North Island. Interestingly, despite this historical lack of gene flow and the lack of current population connectivity, there is evidence of low levels of relatively recent re-connection between south-eastern and other mainland populations. Finally, while the remaining mainland populations (including Western, Central and Eastern Ranges populations) show a lack of recent gene flow, levels of haplotype and nucleotide sharing indicate historical gene flow was ongoing in the late Pleistocene. Several populations were characterised by low genetic diversity. These estimates therefore provide evidence of a dynamic history of connectivity between mainland rifleman populations, influenced by both natural vicariance events and recent anthropogenic impacts.

In Chapter Three I investigated population variation in a behavioural trait: vocalisation. My analysis indicated that rifleman populations vary significantly in fine-scale acoustic characteristics of call vocalisations. While the Southern population in particular appeared to consistently vary from other populations in fine-scale spectral features of calls, playback experiments indicated that territorial male rifleman responded equally to all playback treatments,
regardless of the source population, indicating that fine-scale population variation may be the result of phenotypic
drift and has little consequences for conspecific recognition.

Chapter Four analysed levels of phenotypic morphological variation between North Island populations. Using
colour reflectance spectrophotometry of rifleman feathers combined with measures of size, I investigated whether
sexual dichromatism and sexual size dimorphism varied between populations. Previous research on rifleman used a
colour analysis based on a human visual system to conclude that rifleman colouration functions predominantly for
crypsis in a forested environment (Hunt and MacLean 1993). Using an extended analysis of rifleman feather colour
based on the more complex avian visual system I found that rifleman feathers are characterised by significant UV
reflectance. I hypothesized that the bright green colouration of male feathers would serve as a distinct disadvantage
to males who provide high levels of parental care during the breeding season (Sherley 1994), indicating that crypsis
may be an inadequate explanation for the evolution of sexual dichromatism in rifleman. While restricted to
preliminary descriptive data, my analysis indicates that populations vary in colouration and size both within and
between the sexes. The Eastern Ranges population in particular had significantly brighter feathers and had higher
proportions of UV, blue and red chroma in males which resulted in reduced sexual dichromatism in this population.
Levels of red chroma were significantly correlated with population density, assumed in this study to represent
competition for mating opportunities. Although the results of this study should be interpreted with caution due to
small sample sizes, these findings may indicate that male colouration may be affected by sexual selection in
response to competition for mating opportunities at high population densities, an hypothesis that warrants further
attention.

Finally, in Chapter Five I analysed geographic variation in breeding behaviour across four representative sites. The
recent establishment of two populations as a result of translocation was seen as an opportunity to investigate the
habitat saturation and ecological constraints hypotheses in two sets of populations for which genetic lineage was
controlled for. Unfortunately, geographic variation in both density and cooperation measures were not variable
enough to allow an effective statistical analysis across the four sites. However, there was some evidence that
cooperation was used predominantly when population density was high. Cavity availability did not appear to influence cooperative breeding, largely due to the fact that rifleman were highly flexible in their use of ‘cavities’. The four sites varied in habitat composition and cavity availability, however rifleman nest site use appeared to be largely unaffected by variations in the types and number of cavities available. This flexibility in the use of breeding site may explain the successful occupation of different habitat types and climatic conditions by rifleman.

**Conservation implications**

*Designation of ESUs and MUs*

The analysis of levels of variation between populations of a threatened species is seen as an important pre-requisite to effective conservation management planning (Moritz 1994; Crandall et al. 2000). The description and definition of key units of diversity, including Evolutionarily Significant Units (ESUs) and Management Units (MUs) within species, allows conservation managers to ensure that adaptive diversity is maintained and that evolutionary processes are preserved (Crandall et al. 2000). Despite little consensus on the appropriate way to classify ESU’s and MU’s, most definitions agree that measures of both genotypic and phenotypic or ecological variation should guide designation of conservation units within species (Waples 1991; Bowen 1999; Crandall et al. 2000; Fraser and Bernatchez 2001). The preservation or re-establishment of historical levels of genetic diversity and genetic connectivity between isolated populations ensures that threatened species retain key adaptive variation that allows populations to adapt to changing environmental conditions (Saccheri et al. 1998; Bakker et al. 2010). A focus on the maintenance of natural levels of population connectivity is also assumed to preserve important patterns of gene flow that significantly impact the evolutionary potential of populations (Crandall et al. 2000). While the management of genetic diversity is typically seen as pertaining to long term conservation goals as opposed to short term management of populations, decisions regarding translocation are seen as the exception to this rule (Moritz 1994b). As translocation results in the re-establishment of previously extinct populations or in the reconnection or mixing of previously separated populations, levels of genetic diversity and genetic connectivity should be considered when making decisions on appropriate source and recipient sites for translocation (Frankham et al. 1999).
Phenotypic and ecological variations may also have consequences for the successful establishment of new populations via translocation, and particularly for the reconnection or mixing of previously separated populations. Habitat-dependent phenotypic variations such as levels of morphological crypsis, breeding site selection, feeding morphology and behaviour, or communication behaviours dependent on site-specific environmental variations may be significant for the successful survival and reproduction of translocated individuals. The successful establishment of translocated populations requires both ongoing survival and successful reproduction of translocated individuals. Behavioural and morphological variations may influence the effective population size of founding populations by acting as pre-mating reproductive barriers or by affecting an individual’s ability to adequately defend required resources (Anthony and Blumstein 2000). Variations in traits used in intra-sexual competitive interactions as well as inter-sexual courtship displays may therefore have significant consequences for population establishment.

The results of this study provide several key findings that have significance for conservation management decisions for rifleman. Genetic analyses indicate that three significantly divergent lineages of North Island rifleman are found across the North Island distribution, indicating an historical lack of gene flow. These three divergent lineages include the insular population, the south-eastern populations (Southern and Eastern Coastal populations) and all remaining mainland populations. Interestingly, measures of phenotypic and behavioural variation analysed in this study generally do not conform to these same genetic clades, therefore this investigation found little evidence of ecological non-exchangeability between these three genetic lineages (Table 1). The results of the vocal variation analysis showed some concordance with the genetic data in that the genetically divergent Southern population also appeared to consistently vary in fine-scale vocal features when compared to other populations. However, other population comparisons did not follow expectations from genetic analyses. Additionally, geographically variable vocal signals did not result in reduced recognition between conspecifics, indicating no evidence for a lack of ecological exchangeability in relation to behavioural traits. The morphological analysis carried out here also showed a lack of concordance to genetic data with the sole divergent population being the Eastern Ranges population, which shows high levels of genetic connectivity with both the Western and Central populations.
Table 1. A summary of the study findings including geographic variation in vocalisations, size, colour, cooperative breeding and genetic variation. The results demonstrate little concordance between datasets, indicating no evidence for ecological non-exchangeability.

<table>
<thead>
<tr>
<th>Population</th>
<th>Vocalisations</th>
<th>Size</th>
<th>Colour</th>
<th>Cooperation</th>
<th>Genetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warawara (Northern)</td>
<td>Fine-scale</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>LBI (Insular)</td>
<td>Fine-scale</td>
<td>Non-distinct</td>
<td>Non-distinct</td>
<td>Non-cooperative</td>
<td>Insular clade</td>
</tr>
<tr>
<td>Taranaki (Western)</td>
<td>Fine-scale</td>
<td>Non-distinct</td>
<td>Non-distinct</td>
<td>NA</td>
<td>Mainland clade</td>
</tr>
<tr>
<td>Pureora (Central)</td>
<td>Fine-scale</td>
<td>NA</td>
<td>Non-distinct</td>
<td>NA</td>
<td>Mainland clade</td>
</tr>
<tr>
<td>Boundary Stream (Eastern Ranges)</td>
<td>Distinct</td>
<td>Distinct</td>
<td>Distinct</td>
<td>Cooperative</td>
<td>Mainland clade</td>
</tr>
<tr>
<td>Mohi Bush (Eastern Coastal)</td>
<td>NA</td>
<td>Non-distinct</td>
<td>Non-distinct</td>
<td>NA</td>
<td>South-Eastern clade</td>
</tr>
<tr>
<td>Tararuas (Southern)</td>
<td>Distinct</td>
<td>Non-distinct</td>
<td>Non-distinct</td>
<td>NA</td>
<td>South-Eastern clade</td>
</tr>
</tbody>
</table>
Under most proposed definitions, evidence of substantial and historical genetic divergence warrants the classification of separate lineages as ESUs (Ryder 1986; Waples 1991; Bowen 1999; Fraser and Bernatchez 2001). However Moritz (1994) proposes that ESU status should be afforded only to populations which are found to be reciprocally monophyletic at mitochondrial markers and show significant divergence at nuclear markers. This study found evidence of reciprocal monophyly at the COI coding region of the mitochondrial genome for insular and mainland comparisons. While South-Eastern populations were highly significantly divergent from other mainland populations, three individuals from the Southern and Eastern Coastal populations show evidence of relatively recent migration between these regions and central mainland populations. Samples from these individuals are the only exception to a general pattern of reciprocal monophyly between South-Eastern and the remaining mainland populations. Nuclear markers were not used in this analysis. However, the primary concern with mitochondrial markers is that they will represent biased estimates of gene flow of maternal lines and are not representative in cases of sex-biased dispersal (Watanabe et al. 1985). While the analysis of nuclear markers will permit a more extensive analysis of population divergence, rifleman are unlikely to be affected by sex-biased dispersal as rifleman have reduced dispersal ability due to restricted flight morphology and there is no evidence to suggest that females have a lower dispersal capability than males (Higgins et al. 2001).

Moritz’s (1994) classification of ESUs has been criticized as being overly restrictive in that a very small number of individuals representing evidence of recent gene flow or migration can override general patterns of historical divergence (Fraser and Bernatchez 2001), as is found in this study. Under Crandall et al.’s (2000) framework, both historical and current levels of genetic and ecological exchangeability are emphasized in the designation of species, ESUs and MUs. The three divergent rifleman lineages described in this study appear to demonstrate a lack of historical and current genetic exchangeability, yet show no evidence of ecological non-exchangeability. Under Crandall et al.’s definition, rifleman populations should therefore be treated as a single unit for conservation management, with current sub-population structure retained (Crandall et al. 2000). However Crandall’s definition has been criticized as over-emphasizing the role of ecological non-exchangeability, with Fraser and Bernatchez (2001) arguing that genetic non-exchangeability should be afforded the same importance as ecological non-exchangeability in reflecting significant divergence of separated lineages.
Overall, it appears as though there is evidence for treating the three divergent North Island rifleman lineages as separate MUs until further nuclear data can be analysed. Rifleman fit the criteria for separate MUs under most criteria including Moritz’s stringent genetic framework (Moritz 1994) as they show significant genetic divergence at mitochondrial loci. The three regions represent historically divergent lineages, isolated for several million years as a result of natural vicariant processes as opposed to recent anthropogenic impacts. Dimmick *et al.* (1999) maintain that the designation of conservation units over-emphasizes the role of gene flow and adaptation, while under-emphasizing the impact of vicariance events on population divergence. The three divergent lineages identified in this study contain unique haplotypes and a high degree of sequence divergence, indicating that each lineage has been subject to distinct evolutionary processes over substantial timeframes and now represents a distinct component of the overall diversity of the sub-species. I suggest that the separate management of these regions as MUs is a prudent approach to ensure that local adaptation and diversity is not diluted by the mixing of significantly divergent lineages. However, the analysis of nuclear markers and further exploration of ecological non-exchangeability is warranted. If further analysis indicates extensive divergence of nuclear genetic markers across locations, there may be evidence for identifying these three genetic lineages as separate ESUs.

**Recommended source and recipient sites for translocation**

As this study has found evidence of three divergent genetic lineages across the North Island, it is recommended that these MU’s are maintained as discrete conservation units until further research using nuclear loci can be carried out. Translocation between these units should be avoided until this research is performed.

While Western, Central and Eastern Range populations demonstrated significantly more recent gene flow and population connectivity, these populations are characterized by the presence of unique haplotypes and significant sequence divergence in some cases and currently share no gene flow. Additionally, the Eastern Ranges population appears to show significantly divergent size and colour morphology compared to the other mainland populations. The region is characterized by a higher population density than all other mainland sites. This study provided preliminary evidence that high density (assumed to represent competition for mating opportunities) may result in
sexual selection for increased investment in male colour traits (red chroma). The assumption that density results in increased pairing competition is supported by the finding that in this population, cooperative breeding is found in a significantly higher proportion of nests compared to other low-density populations. While adaptive changes in colour investment and breeding behaviour may represent density-dependent proximate responses, the translocation of individuals to the Eastern Ranges site from other mainland locations may result in decreased competitive ability or decreased pairing success for translocated males. I therefore recommend that the Eastern Ranges population is not considered as a recipient site for translocation, although the establishment of new populations sourced solely from the Eastern Ranges may result in successful establishment and therefore should not be prohibited. Aside from the restriction on the use of the Eastern Ranges as a recipient site for translocation, there appears to be no reason to limit gene flow between the Western, Central and Eastern Ranges populations. The Central population is characterised by relatively low genetic diversity, however it shows recent genetic connectivity with both the Western and Eastern Ranges population, indicating that gene flow should be permitted between these populations. The Western population in particular has high genetic diversity and should be considered as an appropriate source site for translocations attempting to re-establish regionally extinct populations in the Western region.

Finally, two genetically depauperate populations were identified in the analysis of genetic diversity carried out in this study. The Insular and Eastern Coastal populations are both characterized by completely unique haplotypes and extremely low levels of genetic diversity. Both populations show evidence of recent genetic bottlenecks which are likely due to anthropogenic impacts including habitat restriction and mammalian predator invasions. Populations subject to genetic bottlenecks and showing low levels of genetic diversity should rarely be considered as source sites for translocation (Frankham et al. 1999) as low genetic diversity is associated with increased extinction risk due to the effects of inbreeding (Frankham 1998), loss of adaptive potential (Lande and Shannon 1996; Bakker et al. 2010) and declines in fitness (Reed and Frankham 2003). These populations are therefore not considered to be ideal source sites for further translocations.
Reflections on past translocations based on current knowledge

This investigation was carried out at an opportune time in the history of conservation management of the rifleman. While many species are subject to translocation without any prior information on levels and patterns of intra-specific diversity, this study provides valuable information which can be used to guide further management decisions for the sub-species. The results of this study emphasize the importance of analysis of population diversity prior to translocation. Two translocations of rifleman have been carried out in the North Island to date. The first translocation of North Island rifleman transferred a founding population from the Eastern Ranges population to the Cape Kidnappers and Ocean Beach Wildlife Preserve (CK), located on the Cape Kidnappers peninsula. The choice of the Eastern Ranges population as a source site was seen as appropriate due to the proximity of the location (66 km) combined with the high density of individuals available at the Eastern Ranges site. While the population at CK is establishing and breeding successfully, and represents an important step in the re-colonisation of rifleman into the Hawkes Bay area, the results of this study indicate that the Eastern Ranges population may have been an inappropriate choice of source site for the translocation. The Eastern Coastal population is found only 24 km from the newly established CK site. However, this population represents a distinct and highly divergent genetic lineage of rifleman which has been identified as a separate MU and may even represent a different ESU. While rifleman are currently unlikely to migrate between these sites due to habitat fragmentation, the potential mixing of two separate and divergent ESUs now has a much higher likelihood as a result of human-mediated translocation.

The second translocation carried out in the North Island transferred a small number of founders from Little Barrier Island (the insular site) to a nearby island sanctuary, Tiritiri Matangi Island. This choice of source site was deemed appropriate as the insular site was considered likely to host a distinct lineage of rifleman and was the only potential source site in close proximity to Tiritiri Matangi Island. The establishment of an additional rifleman population in the region was seen as a safe-guarding mechanism to protect the lineage in the case of any detrimental impacts that may cause the decline of the insular population. While a lack of alternative source sites in the region means that the insular population was the most appropriate choice of source site at the time, and the translocation appears to have resulted in the successful establishment of a growing and reproducing population, the results of this study indicate that the rifleman translocated to Tiritiri Matangi from the insular site are almost genetically identical. This
extremely low level of genetic diversity indicates that the Insular site may not represent an appropriate source site for any translocation of rifleman. While the effects of inbreeding are debated in the literature (Ardern and Lambert 1997; Jamieson et al. 2006), there is ample evidence of detrimental effects of inbreeding on the evolutionary potential and fitness of populations (Keller and Waller 2002; Hale and Briskie 2007; Jamieson et al. 2007). The analysis of levels of population genetic diversity prior to translocation is likely to have resulted in the recommendation that alternative source sites were considered before translocation from the insular population. Despite the absence of alternative local source sites for further translocations around the Auckland region, it is recommended that the insular and Tiritiri Matangi Island populations are not used as source sites until further options are explored. Plans to re-introduce rifleman into the mainland Auckland region should consider alternative mainland populations as source sites for translocation.

**Future work**

While the results of this study provide clear evidence of genetic divergence between key regions within the North Island rifleman distribution, further work is needed before ESU status is addressed. While numerous definitions of ESUs emphasize evidence of substantial historical isolation or adaptive change, the most conservative definitions outline a clear requirement for reciprocal monophyly at mitochondrial genetic loci combined with significant divergence at nuclear genetic loci (Moritz 1994). Further research should therefore be carried out investigating patterns of population connectivity and diversity using nuclear loci. Additional sampling from areas representing intermediate regions to the sites used in this study may also provide further information regarding patterns of gene flow on a finer spatial scale. Finally, further analysis into ecological traits which may vary on a spatial scale will allow an extended investigation into potential ecological non-exchangeability. Some suggested traits include fine scale habitat use, diet and feeding morphology (e.g. beak and claw shape).
References


