Ecological immunology and genetic diversity of the endangered Mauritius parakeet

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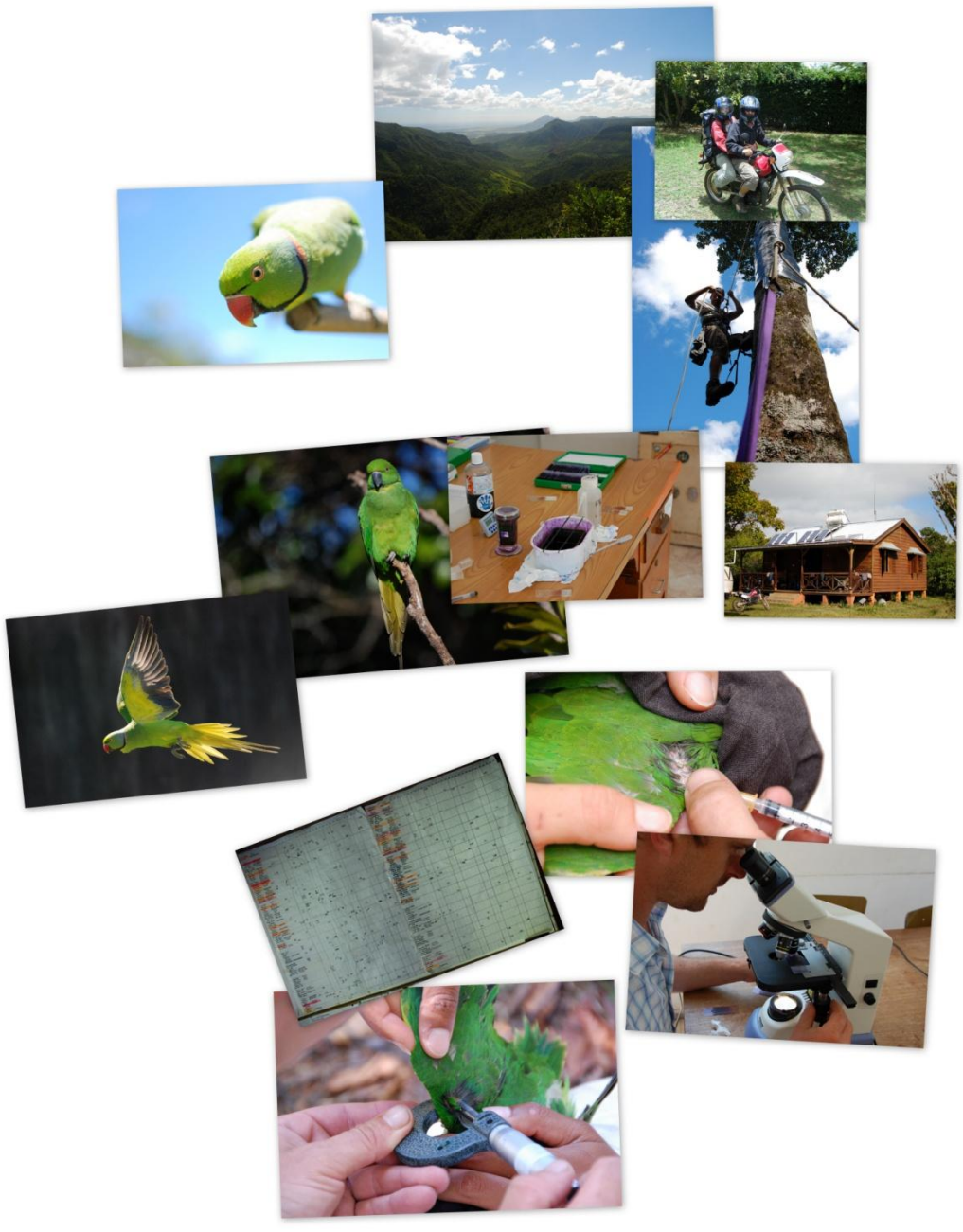

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Abstract

Studies of avian ecological immunology attempt to describe the biotic and abiotic factors which explain natural variation in immune function within and among free-living bird species. Understanding this variation and the trade-offs associated with maintaining appropriate immune defences and individual life history variables has important implications for the conservation of endangered species, many of which are characterised by small population size and reduced genetic diversity. Such species often display increased susceptibility to infectious diseases as a result of inbreeding depression and are prone to the effects of novel parasites and pathogens.

This thesis aims to explain the variation in immune function in the endangered, island-endemic Mauritius parakeet (*Psittacula echo*), a species which has passed through a considerable population bottleneck but now thrives by virtue of ongoing conservation management despite the presence of a highly infectious disease. Identifying the ecological, environmental and genetic elements which define individual immunity offers the potential to predict the survival probability of juvenile individuals in a disease landscape thereby representing an exciting prospect for the field of conservation reintroduction biology.

Interactions among indices of immune function are investigated at the individual level for Mauritius parakeets and also at the species level with the sympatrically occurring and non-native Indian ringneck parakeet (*Psittacula krameri*). Patterns of species-level genetic diversity of the Mauritius parakeet spanning two decades are examined and interspecies variation in immune function and genetic diversity is explored. Productivity and survival of Mauritius parakeets is summarised during and after a disease outbreak and an in depth analysis of the predictors of infection status and immunocompetence in this species is offered.

This study highlights the complexity of the immune system and the challenges faced when trying to characterise it among individuals in an ecological context. I reveal a declining trend in species-level genetic diversity among Mauritius parakeets due to low natural dispersal demonstrating the importance of adaptively managing endangered species. I illustrate how, as a result of population bottlenecks or contrasting evolutionary histories that the Mauritius parakeet displays an attenuated immune function when compared to the Indian ringneck parakeet. I find no evidence to suggest that genetic diversity or inbreeding predicts disease infection in Mauritius parakeet nestlings and finally I use long-term monitoring data to cherry pick suitable individuals for reintroduction.

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Chapter 1. Introduction

1.1 Background

Parasite defences and immune responses can be costly to an organism and are known to be associated with reduced levels of individual fitness and potential trade-offs with other life history variables and physiological functions such as reproductive effort, or the expression of sexual ornaments which compete for the allocation of finite resources (Hamilton and Zuk, 1982, Folstad and Karter, 1992, Sheldon and Verhulst, 1996, Norris and Evans, 2000). Therefore, within an ecological context where various biotic and abiotic factors combine to effect individual fitness or condition, immune responses and susceptibility to infectious agents are likely to vary across species, populations and individuals (Sheldon and Verhulst, 1996, Schulenburg et al., 2009). The emerging field of 'ecoimmunology' aims to describe and explain the forces that drive this variation in natural, free-living populations.

Characterising individual-level immune function within natural populations whilst incorporating heterogeneous environmental and ecological stressors provides a vital contribution to explaining host life history strategies and their evolution. However, the vertebrate immune system is recognised as highly complex and immune responses are the products of multifaceted physiological, genetic and behavioural interactions, rendering them inherently difficult to quantify and interpret (Norris and Evans, 2000, Lazzaro and Little, 2009, Schulenburg et al., 2009, Boughton et al., 2011, Graham et al., 2011).

Difficulties arise when attempting to quantify immune responsiveness (or immunocompetence) because many estimates are based upon indices derived from a single capture event; these measures may involve counting immunologically important blood cells and determining parasite density for example. Ecological immunologists now recognise that measuring a specific element of immunity in order to define an individual's immunological phenotype is inadequate and that multiple factors involving both the cellular and humoral

components of innate and adaptive immune function must be considered (Westneat and Birkhead, 1998, Adamo, 2004, Viney et al., 2005, Demas et al., 2011). In addition, the interpretation of these measurements of immunity must be considered in the context of simultaneous parasite pressures, for example, an immune assay may provide information about general immune function but also reflect the current infection status of that individual (Owens and Wilson, 1999, Norris and Evans, 2000, Zuk and Stoehr, 2002, Owen and Clayton, 2007). To further complicate the situation, one cannot assume that the fittest individual is that which exhibits the maximal immune response or the fewest parasites (Zuk and Stoehr, 2002, Graham et al., 2011) because these assumptions negate considerations of, for example, pathogen-specific responses, autoimmunity and maintenance of optimal parasite densities in relation to overall host fitness (Adamo, 2004, Viney et al., 2005, Graham et al., 2005, Graham et al., 2011).

Despite these complications in interpreting ecoimmunological data, a wide variety of techniques have been developed (reviewed by: Salvante, 2006, Boughton et al., 2011) in an effort to elucidate the patterns of ecological variation in immunity. The majority of associated studies focus on avian systems because of the relative ease with which birds can be humanely captured, manipulated and released.

Commonly used methods to assess immune function in birds include: (i) determining individual leukocyte profile via differential counts of immunologically important blood cells from blood films (e.g. Davis et al., 2008); (ii) the response to experimental injection with phytohaemagglutinin (PHA) measured by tissue swelling after intra-dermal exposure (e.g. Smits et al., 1999), and (iii) assays designed to evaluate the level and efficacy of general or specific antibodies found within the non-cellular component of whole blood (e.g. Matson et al., 2005). Numerous studies have demonstrated that these and other similar techniques can be used to provide evidence that elements of the immune system are associated with, and inextricably linked to, a wide range of fitness related life history traits in free-living wild bird populations. For example, among studies of birds it has been demonstrated that the

response to PHA challenge is positively associated with nestling survival (e.g. Saino et al., 2003, Christe et al., 1998), increased nutritional status (e.g. Hasselquist and Nilsson, 2012), plumage colouration (e.g. Saks et al., 2003) and establishment success following introduction (Møller and Cassey, 2004). Survival and recruitment has also been predicted by counts of white blood cells (e.g. Lobato et al., 2005), whilst assessment of the humoral component of the avian immune function has revealed associations between levels of natural antibodies (NAbs) and inbreeding indicating that populations with low levels of genetic diversity display reduced levels of constitutive innate antibodies (Whiteman et al., 2006).

1.2 Genetic effects

The influence that inbreeding and genetic diversity have on individual immune function and disease susceptibility has become a key research focus in the study of avian ecoimmunology. Loss of genetic diversity through random drift as a result of consanguineous matings is a common feature of small populations (Frankham, 1997, Keller and Waller, 2002) and is associated with fitness costs including decreased immunity and increased susceptibility to pathogens (Acevedo-Whitehouse et al., 2003, Reid et al., 2003, Rijks et al., 2008). Such heterozygosity fitness correlations (HFCs) have received much attention in the literature and have been used to invoke inbreeding depression in wild populations (e.g. Keller, 1998, Slate et al., 2000). However, more recently it has been recognised that an increase in homozygosity is not necessarily a result of inbreeding (Balloux et al., 2004, Slate et al., 2004, Szulkin et al., 2010) and that measures of multilocus heterozygosity (MLH) are imprecise predictors of pedigree-derived inbreeding coefficients especially when levels of inbreeding are relatively low (Balloux et al., 2004, Pemberton, 2004, Slate et al., 2004). Furthermore, Slate et al., (2004) demonstrate that the magnitude of the correlation between MLH and inbreeding depends upon both the number of loci used to derive MLH and the mean and variance of the level of inbreeding. Pemberton (2004, 2008) advocates the construction of reliable pedigrees in order that individual inbreeding coefficients can be directly calculated. Whilst this method is recognised as a more accurate assessment of

quantifying individual-level inbreeding, it is reliant upon pedigree depth and an assumption that founding individuals are not related (Keller and Waller, 2002, Szulkin et al., 2010). Furthermore, detailed pedigrees are rare, logistically challenging to construct for large populations of long-lived, free-ranging organisms and therefore only exist for a few well studied species. Nevertheless, as Szulkin et al., (2010) postulate, pedigree-derived inbreeding coefficient and MLH offer complementary methods to study both direct and indirect effects of inbreeding on individual fitness.

Knowledge of both individual pedigrees and MLH are available for a small number of well studied free-living bird populations, often comprising island populations or those of conservation concern where individuals have been marked and closely monitored over time (e.g. Taylor et al., 2010, Grueber et al., 2011b). Populations such as these provide rare opportunities to study free-living populations in a natural laboratory. By combining long term individual life history data with measures of individual inbreeding coefficient, genetic diversity and immune function in the presence of a highly infectious disease we can identify the interactions, trade-offs and costs associated with maintaining an effective immune system, thereby potentially enabling the identification of individuals and populations most likely to persist.

1.3 Implications for conservation

The ability to identify individuals within a population which are more likely to successfully establish new populations has important implications for the conservation of endangered species and reintroduction biology. Re-establishing viable, self-sustaining populations of species with little or no long-term intervention in areas where they have been extirpated is the aim of conservation reintroductions (IUCN, 1987, Seddon, 1999). Whilst these initiatives can be successful they are costly and success rates are generally poor (Griffith et al., 1989, Beck et al., 1994, Fischer and Lindenmayer, 2000, Armstrong and Seddon, 2008). By integrating elements of genetic diversity and immune function into reintroduction planning

conservation managers have the opportunity to 'cherry-pick' individuals which are more likely to survive as part of a release cohort in a new environment, thereby increasing the chance of establishing a persistent and viable population.

1.4 Study population

The Mauritius (or echo) parakeet (*Psittacula echo*) is the last remaining Psittaciforme species native to the Mascarene Islands, an archipelago in the South-Western Indian Ocean (Figure 1) which has witnessed around thirty avian extinctions including six Psittaciformes since human discovery of the islands in the 16th century (Cheke and Hume, 2009). The Western Indian Ocean Islands are considered important stepping stones in the adaptive radiation of Old-World parrots and have been implicated in 'seeding' the continents of Africa and Asia rather than *vice-versa* (Kundu et al., 2012). Once distributed throughout Mauritius (Jones and Duffy, 1993), the Mauritius parakeet is now restricted to the Black River Gorges National Park, a 67km^2 area of degraded forest with few remaining fragments of native flora. In the 1980s the species was described as the rarest parrot in the world when fewer than ten individuals were thought to remain (Jones, 1980). However, following a 30-year-long successful conservation programme, the Mauritius parakeet was the only bird species to be down-listed on the IUCN Red List from 'critically endangered' to 'endangered' in 2007 (IUCN, 2012). In 2012 the population consists of approximately 100 known breeding pairs with a total population size of approximately 500 individuals (MWF, 1996-2012). This remarkable recovery is the result of an intensive conservation management effort from 1997 to 2004 which included captive breeding and release programmes, management of wild nest sites, supplemental feeding and predator control (Jones and Duffy, 1993, Jones, 2004, Jones and Merton, 2012). Preventing this species from becoming extinct was only possible because of the dedication of many individuals and collaborative partners such as The World Parrot Trust, Durrell Wildlife Conservation Trust, The Mauritius Wildlife Foundation (MWF) and The National Parks and Conservation Service Mauritius (NPCS).

The reasons for the decline of this parakeet species are in common with many endangered species, particularly those inhabiting islands that have relatively recently been colonised by people and include habitat loss, introduced predators and non-native competitors (Jones, 1987, Jones and Duffy, 1993). Population size continues to increase however, owing to the ongoing provision of supplemental food, predator protection and artificial nestboxes by a dedicated team of volunteers and experienced field biologists directed by MWF (MWF, 1996-2012).

Efforts are being made to reduce invasive, non-native vegetation and to encourage recolonisation by native flora species in the hope of increasing the area of native mature forest but the process is a lengthy one. It is unlikely that invasive predators and competitors will ever be eradicated from Mauritius and therefore ongoing control and management is the only available option. Further reintroductions of this species into appropriate areas of good quality forest are planned and the possibility of introducing it to the neighbouring island of La Réunion as an ecological replacement or taxon substitute have also been discussed (Temple, 1981, Jones and Duffy, 1993, Hansen, 2010).

Importantly, this conservation recovery success, besides saving a species from extinction, has resulted in the establishment and ongoing collection of detailed life history data for almost every individual produced since conservation efforts began over 25 years ago. The breeding attempts of each known pair are closely monitored throughout each season and each one is carefully documented from when the first egg is laid until fledging or failure. This close monitoring allows each individual to be uniquely marked with colour rings, and facilitates the collection of blood samples from every known fledgling and the majority of adults. Furthermore, the monitoring has enabled researchers to identify each nesting adult and has therefore facilitated the construction of an observational pedigree which features over 1500 individual records and has been genetically confirmed with a suite of species-specific microsatellite markers (Raisin et al., 2009, Raisin, 2010). This pedigree has been used to demonstrate the existence of extra-pair paternity (Taylor and Parkin, 2009) albeit at

relatively low (~11%) overall levels (Raisin, 2010) and to highlight the effects of conservation management on population genetic structure as a result of the translocation of individuals (Groombridge et al., 2012, Raisin et al., 2012).

Long-term monitoring is pivotal to understanding the evolutionary, ecological and environmental processes which influence population dynamics. Continued monitoring of the Mauritius parakeet after its initial recovery proved invaluable when, in 2005 it became apparent that an outbreak of beak and feather disease virus (BFDV) had affected the population and once again threatened the species' existence (Kundu et al., 2012). Beak and feather disease virus is a single-stranded DNA circovirus and is one of the most common infections of parrots (Ritchie et al., 1989). It is transmitted both horizontally and vertically and infection can lead to psittacine beak and feather disease (PBFD), characterised by feather dystrophy and immunosuppression (Ritchie et al., 1989, Todd et al., 2000 Ritchie et al., 2003).

Tracking the evolution of this virus before, during and after the outbreak was only possible due to the existence of blood samples collected as part of this long-term monitoring strategy. Fears of another avian extinction on Mauritius were, however, allayed as it soon became evident that the Mauritius parakeet population continued to grow, albeit at a reduced rate due to a decrease in juvenile survival (Jones and Merton, 2012). The irruption of the virus prompted researchers to begin a more intensive blood sampling programme in order to establish prevalence and since 2005 a sample has been taken from every known nestling as part of the field monitoring project resulting in an archive of over 900 individual samples spanning the period 1992 to 2012.

The Mauritius parakeet is no longer under the immediate threat of extinction but has given researchers an unparalleled opportunity to study the dynamics of a recovering, bottlenecked population by investigating at an individual level, how genetic diversity, inbreeding,

immune function and parasite infection interact with other life history variables in a wild free-living population of birds.

1.5 Aims and objectives

The aim of this thesis is to improve current knowledge concerning the effects of inbreeding on immune function in small populations by applying molecular techniques to characterise individual-level genetic diversity and a variety of field and laboratory-based assays to quantify individual-level immune function whilst integrating available life history and pedigree data collected as part of a long term monitoring programme.

To place the results obtained for the endemic island species in a wider context, I also apply the same suite of laboratory and field techniques to evaluate population-level genetic diversity and immune function in a sympatrically occurring, non-native and invasive congeneric species, the Indian ringneck (or rose-ringed) parakeet (*P. krameri*). This species was introduced to Mauritius in the 1880s from the Indian sub-continent via the trade in caged birds (Cheke and Hume, 2009) and it is estimated that the current population numbers in the tens of thousands (C. Jones, pers. comm.). It represents a potential reservoir of infectious disease and direct competition with the Mauritius parakeet for nesting resources.

Moreover, I provide evidence to inform the field of reintroduction biology by attempting to use these data to identify individuals within a population which, if released as part of a conservation reintroduction, would represent the greatest chance of successful establishment and long-term population persistence.

Specific objectives contributing to the aims of this thesis are as follows:

- to investigate the relationships among numerous indices of immune function
- to identify long-term patterns in genetic variation of an endangered parakeet

- to identify and compare predictors of immune function between an endemic island parakeet species and an introduced continentally-evolved congeneric species

- to identify patterns of productivity and recruitment in an endangered parakeet and how this may be influenced by infectious disease and immune function

1.6 Thesis outline

The thesis is structured as follows:

Chapter 2 investigates the collinearity of various different indices of innate immunity derived from recognised methods of assessing individual-level immune function in birds. The complexity of the vertebrate immune system dictates that different components of it are fundamentally interconnected, which presents a challenge for most studies of ecoimmunology. I use common principal components analysis in order to identify consistent patterns of collinearity at different organisational levels both within and among two parakeet species on Mauritius. The identification of dependant variables of immune function has implications for improving the interpretation of immune function assays and immune challenge techniques.

Chapter 3 documents a fine-scale spatial and temporal analysis of genetic diversity in the Mauritius parakeet, spanning a period of more than 25 years across the conservation programme and the species subsequent population recovery. Reintroduction projects often overlook (or are unable to characterise) the long-term genetic effects of population management. I use microsatellite markers to identify trends in genetic diversity associated with population fragmentation and historical and contemporary management regimes, resulting in recommendations for future management directions.

Chapter 4 investigates the differences in immune function variables and levels of genetic diversity between two similar species of parakeet with contrasting evolutionary histories. I assess the physiological responses of each species to experimental immune function

challenge and use generalised linear mixed models to predict a number of immune function indices incorporating parameters of genetic diversity and nesting environment. Variation among species is evaluated in the context of an island-endemic bottlenecked species in relation to a sympatrically occurring and morphologically similar non-native invasive congeneric species.

Chapter 5 summarises productivity and recruitment probability among Mauritius parakeets during and after an outbreak of a highly infectious disease. I use generalised linear mixed models incorporating elements of disease infection, inbreeding, immune function, genetic diversity and other life history variables to identify significant predictors of recruitment probability. I also investigate the effects of disease infection on individual immune function indices and attempt to identify individuals which would be suitable as a founding population for a proposed future reintroduction.

Chapter 6 offers a discussion of the key findings and conclusions of this thesis and proposes future areas of research in avian ecological immunology and implications for the ongoing management of the Mauritius parakeet.

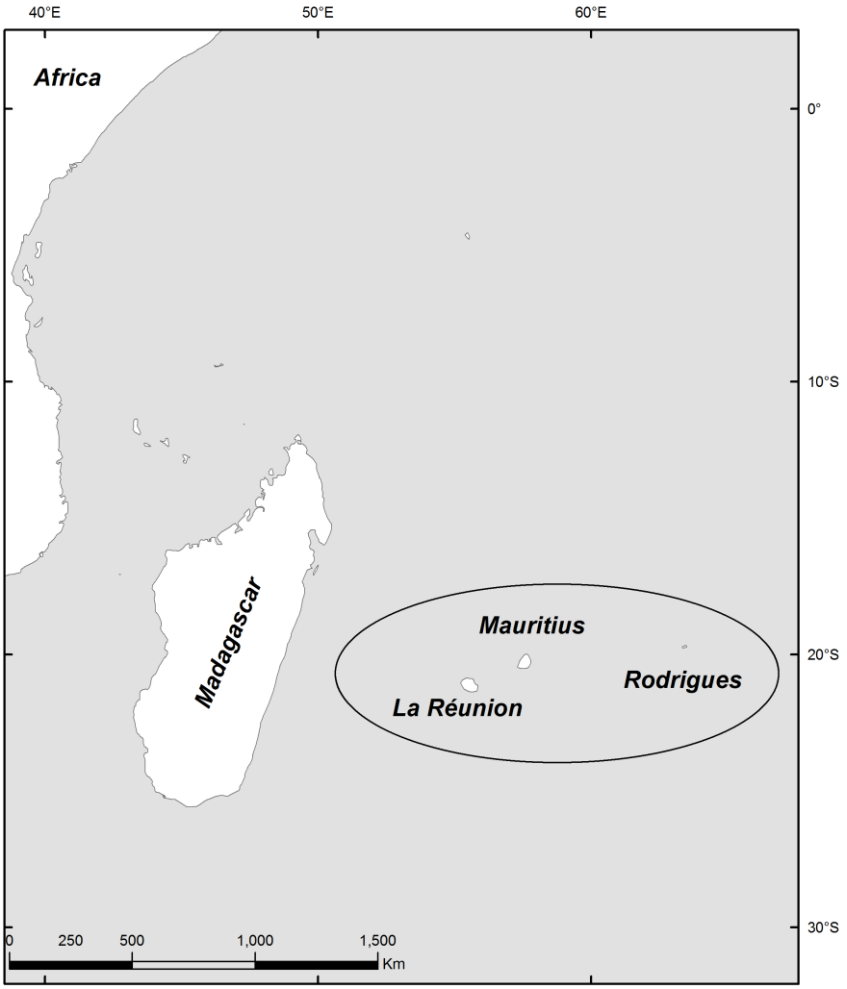


Figure 1 The major islands of The Mascarenes.

Chapter 2. Correlates of immune function measures within and between two parakeet species

Abstract

Characterising individual variation in immune function under natural ecological processes is the goal of ecological immunologists. Measuring and interpreting this variation in the context of life history trade-offs represents a major challenge to this discipline. Often, indices of immune function are not independent and simple analytical tools such as principal components analysis (PCA) are used to determine the correlative relationships between these indices. Such tools can assist in identifying whether immune function measures are correlated and can therefore be used as single variables. In this chapter, three of the most commonly used measures of immune function are applied to free-living populations of parakeets and results analysed for congruence among immune response measurements at different organisational groups using common principal components analysis (CPCA), correlation circles and pairwise correlations. Results reveal that relationships among immune response measurements after exposure to phytohaemagglutinin (PHA) vary between two very similar species indicating differing immune function arrangement. Likewise, matrices of correlation coefficients of immune response measurements were found to be significantly different among individuals of the same species as a result of PHA exposure. Common principal components analysis can be a useful tool in identifying structural patterns between organisational groups however, the simultaneous use of correlation matrices is advised to aid interpretation.

2.1 Introduction

The field of ecological immunology or 'ecoimmunology' attempts to describe natural variation in immune function (Sheldon and Verhulst, 1996). This objective is facilitated by considering the evolutionary and ecological factors which combine to determine the fitness

consequences of such variation in free-living individuals, populations and species over time and across environments (Norris and Evans, 2000, Martin et al., 2011).

The complexity of the vertebrate immune system and the numerous biotic and abiotic factors which interact to forge an individual's immunity and specific responses to pathogens and parasites have presented many challenges to the eco-immunologist. Interest in this 'physiological black box' (Schmid-Hempel, 2003, Martin et al., 2011) has lead researchers to formulate numerous methods (reviewed by Boughton et al., 2011) intended to describe one or more elements of an individual's immune function. Traditionally, immunologists have studied model organisms in carefully controlled laboratory conditions in order to further understand the mechanisms of an organism's immune system (Delves et al., 2006), however, in the last two decades there has been an increase in efforts to describe these mechanisms in natural systems (Sheldon and Verhulst, 1996, Norris and Evans, 2000, Sadd and Schmid-Hempel, 2009). Laboratory conditions, although useful in such studies, cannot realistically represent the complex interactions between the many different variables responsible for determining an individual's response to pathogen exposure and the 'decisions' and trade-offs it must make if it is to tolerate, avoid or clear pathogen infection (Pedersen and Babayan, 2011). Avian systems have been the attention of much of this research owing to the relative ease with which individual birds can be captured, identified and manipulated to collect appropriate data and samples. However, deciding which samples to collect, which immunological assays to employ and how the resulting data are interpreted represent major challenges for evolutionary biologists interested in ecoimmunology.

2.1.1 Measuring immune function

Much progress has been made over the last two decades in an effort to devise methods which characterise avian 'immunocompetence', (defined here as the general physiological capacity of an individual to produce immune function-related responses), or 'immune function response' (the magnitude of an individual's response to a specific challenge)

(Vinkler and Albrecht, 2011). Reviews of many of these methods providing critical assessments of feasibility and suitability have been offered (e.g. Norris and Evans, 2000, Adamo, 2004, Salvante, 2006, Sadd and Schmid-Hempel, 2009, Boughton et al., 2011) and ecologists recognise that using a single measure of immune function cannot possibly be used to describe the individual variation encompassed within such a complex and multi-faceted system (Adamo, 2004, Lee, 2006, Martin et al., 2006c, Matson et al., 2006, Graham et al., 2011). Likewise, it would be impractical to attempt to characterise every single dimension of immune function and each ecological and evolutionary factor which influences it. Researchers therefore, need to carefully select which methods are best suited to answer their specific questions (Boughton et al., 2011) based on the logistical challenges associated with collecting such information, knowledge of a particular study system and by using reasonable assumptions supported by biological, ecological and evolutionary theory. Such questions, discussed by Buehler et al (2011), are generally associated with; (i) the life history trade-offs associated with mounting an immune response (Norris and Evans, 2000), (ii) the optimum number of assays required to characterise immune defences and if these assays are independent (e.g. Matson et al., 2006), and (iii) if relationships can be generalised across taxa and across spatio-temporal gradients (e.g. Matson, 2006, Hasselquist, 2007).

2.1.2 Interpreting measures of immune function

Ecological immunologists increasingly recognise the difficulties in interpreting immune response measurements (Norris and Evans, 2000, Adamo, 2004, Owen and Clayton, 2007, Graham et al., 2011, Buehler et al., 2011). Recent studies illustrate that an increase in magnitude of a specific immune response may not necessarily represent an increase in fitness (Viney et al., 2005, Vinkler et al., 2011) and similarly it cannot be assumed that an individual displaying an increased parasite load is less fit (Graham et al., 2011). A reduced immune response measurement may in fact indicate immunosuppression or a re-organisation of immune function priorities as a result of an infection which may or may not be independent of the immune response being measured. Organisms exist in 'immunobiomes'

(Horrocks et al., 2011) where immune systems are characterised over ecological and evolutionary timescales by everything they encounter whether commensal or pathogenic. Furthermore, host tolerance varies among individuals; what is pathogenic to one individual may be inconsequential to another; viral infection in one individual may lead to disease whilst infection may be tolerated in another (Martin et al., 2011). The cost of this tolerance may be the defining factor in parasite density; if the cost of resistance or avoidance is high then the host may tolerate some level of parasitism in order that overall fitness is maximised under current environmental conditions (Stjernman et al., 2008).

Graham et al., (2011) recommend therefore, that trade-offs in host fitness should be quantified by both parasite density and immune response magnitude with a combination of controlled experiments and appropriate statistical analyses. Designing a controlled experiment within natural systems, whilst not impossible can be logistically difficult however, and parasite densities can only be quantified for those known and targeted.

2.1.3 Relationships among immune function indices

A potential complication of using numerous immune response measurements is that the relationships between different elements of the immune system are relatively poorly understood for free-living non-model organisms. Researchers therefore, rarely know *a priori* which of their chosen measures are dependent and are to be included in analyses as single variables, and which have to be treated separately (Matson et al., 2006). Avoiding the simultaneous use of multiple dependent measurements as independent explanatory variables is, perhaps, the first step in the analysis of any ecoimmunology dataset which uses numerous measures of immunity. In such a complex physiological arrangement the problem of collinearity among different measures of the immune system is well recognised (Matson et al., 2006, Martin et al., 2007). Numerous attempts have been made to characterise these relationships by using relatively simple correlation coefficients (e.g. Hõrak et al., 2006, Mendes et al., 2006) and regression (Parejo et al., 2007). Matson et al., (2006) attempted to

characterise relationships associated with 13 variables of immune function both within and among captive waterfowl species using principal components analysis (PCA). Results suggested a lack of correlation between constitutive immune function variables from different assays at the individual level, perhaps suggesting a lack of trade-offs between different elements of the immune system. Correlations were found among variables of the same assay type at the individual level but these relationships were not apparent among species. Matson et al., (2006) therefore conclude that employing just a small number of immune response measures could be used to characterise immunity within single species systems and suggest that inclusion of an experimentally induced immune response in a similar study would add to current knowledge. Other similar studies have discovered some correlations between innate cellular and humoral immune indices in both captive (Buehler et al., 2008a) and a small number (n = 15) of free-living individuals (Buehler et al., 2008b) of the same species.

Buehler et al., (2011) recognise that identifying relationships among immune indices in this way often requires the amalgamation of results from different organisational groups represented by species or individuals sampled across different spatio-temporal gradients. An assumption of PCA, common to many similar discriminant analyses is that covariance matrices are the same for all groups (Jolliffe, 2002). Buehler et al., (2011) identify that this assumption ignores group affiliation; relationships among immune indices are unlikely to covary among different species owing to variation in evolutionary life histories. If the ultimate goal of this approach is to characterise individual level variation in immune function measurements then organisational groups must also be defined within species with respect to factors known to effect immune function parameters. Sex, age, time of day, and geographic location are all known to affect haematological parameters of birds (Clark et al., 2009) suggesting that correlations between immune function variables (many of which are haematologically derived) will also be impacted upon. Therefore investigating such

correlations among appropriate organisational groups within species is equally as relevant as comparisons among species.

Box1. Summary of abbreviations and acronyms used in the text, figures and tables.

Agg	Agglutination titre
AIC	Akaike's information criterion
Baso	Basophil count
CPCA	Common principal components analysis
Eosin	Eosinophil count
H:L	Heterophil to lymphocyte ratio
HL-HA	Haemolysis haemagglutination
Lys	Complement mediated lysis
Mono	Monocyte count
MP	Mauritius parakeet
PBS	Phosphate buffered saline
PC	Principal components
PCA	Principal components analysis
PCPC	Partial common principal components
PCV	Packed cell volume
PHA	Phytohaemagglutinin
Proport	Proportionality
RNP	Ringneck parakeet
RRBC	Rabbit red blood cells

2.2 Statistical approach

By using common principal components analysis (CPCA, see box 1 for a summary of abbreviations), correlation circles and pairwise correlations Buehler et al., (2011) illustrated that disregarding the aforementioned assumption concerning group affiliation can result in the homogenisation of differential group structure and suggested that this method has the potential for a meta-analyses approach.

For CPCA, matrices are constructed using correlation coefficients of each pair of immune response variables measured. The shared structure of two or more matrices is then derived using a hierarchy of comparisons which correspond to a range of potential relationships among matrices. These relationships are then described according to their hierarchical complexity, from 'equality' where all matrices are effectively identical to 'unrelated' where matrices share no principal components. Between these two extremes lie a variety of partial structural relationships or partial common principal components (PCPC) models whereby matrices can share from one to *p-2* principal components (where *p* represents the total number of possible principal components as defined by the matrix dimensions). Alternatively matrices can share all principal components but display eigenvalues that differ by a proportional constant; known as the full common principal component (CPC) model.

(Phillips and Arnold, 1999). The CPCA output offers two methods for selecting the most appropriate model. The step up approach begins with testing the null hypothesis that the matrices are unrelated and at each step adds a level of hierarchy and calculates the probability that the 'higher' model is more valid than the 'lower' model. A low p value indicates low probability that the higher model is more suitable than the lower model and therefore the lower model is selected as the most appropriate. Alternatively, the model building approach uses Akaike's information criterion (AIC) whereby the lowest value indicates that the higher model is the most appropriate (see Phillips and Arnold, 1999, Houle et al., 2002 for a more detailed summary).

Buehler et al., (2011) recommend the simultaneous use of pairwise correlation coefficients to visualise the relationships among these matrices and to assess their independence; recognising an assumption of CPCA that PCs are orthogonal as highlighted by Houle et al., (2002). In this study, where only two matrices are evaluated for shared structure simple, pairwise T-tests were used to test the difference between matching correlation coefficients for each matrix. Coefficients were transformed before pairwise comparisons using Fisher's z transformation according to Revell (2011). Where more than two matrices are compared boxplots of pairwise correlation coefficients were produced and significance of the mean values from zero were calculated by using one sample T-tests. The use of correlation circles is the final recommendation of Buehler et al., (2011) in such analyses providing the user with a graphical representation of all pairwise relationships simultaneously. Correlation circles are derived from a PCA of the separate correlation matrices and vectors are presented according to their direction and relative loadings on each PC axis. Bivariate relationships between pairwise correlations are interpreted according to the angle separating them, for example; an angle of 0º indicates a very strong positive correlation, an angle of 90º indicates no correlation and an angle of 180º represents two variables very strongly negatively correlated.

This study aims to assist in filling the 'analytical gap in summarizing multiple measures of immune function' identified by Buehler et al., (2011) by investigating the relationships among measures of immune indices of free-living nestlings of two sympatrically occurring and morphologically similar parakeet species at varying organisational levels, both within and among species. This approach attempts to inform the discipline of ecoimmunology by benefitting from the existence of long-term monitoring data which results from the restoration of a critically endangered bird population. The existence of this data provides an ideal opportunity to investigate the relationships between immune function variables in a 'natural laboratory' thereby blurring the distinction between carefully controlled laboratory studies and those of wild populations under natural ecological processes. There are increasing examples of long-term monitoring studies, often as a result of conservation initiatives where vast amounts of individual level life history data are available. These systems have been recognised as crucial tools in evolutionary biology for understanding population dynamics in the context of genetic (e.g. Pemberton, 2008) and environmental (e.g. Burgess et al., 2011) processes for example, and also therefore, offer a unique opportunity to open the physiological black box of immunity in wild populations. This study therefore combines a novel statistical approach to investigating the collinearity among immune function indices with detailed, individual-level life history data concerning two wild and free-living populations of birds.

Immune function indices were collected via several methods; the phytohaemagglutinin (PHA) challenge technique was used to experimentally induce an immune response (Smits et al., 1999, Vinkler et al., 2011), differential leukocyte counts were made from blood smears (Campbell and Ellis, 2006) and constitutive innate humoral immunity was assessed using the haemolysis-haemagglutination assay (HL-HA) (Matson et al., 2005). Common principal components analysis potentially provides a useful and novel method of investigating relationships among these indices and this study aims to evaluate its suitability

when simultaneously combined with other simple methods for data collected from wild, free-living populations.

2.3 Study species

The Mauritius parakeet (*Psittacula echo*) is an endangered species endemic to the island of Mauritius in the Mascarene archipelago. Recovered from around 20 known individuals in the 1990s (Jones and Duffy, 1993) the population now numbers in excess of 500 individuals as a result of an intensive conservation programme (Jones and Merton, 2012). The nature of the recovery of this species has resulted in the collection of a vast amount of individual life history data; more than 95% of the population are individually marked and a genetically confirmed pedigree exists (Raisin, 2010) which spans more than 20 years (approximately 7 generations). A modest level of management and monitoring continues to maintain population growth; around two thirds of the population use artificial nest sites and supplementary food is regularly taken by over half of all known individuals (MWF, 1996-2012). Nevertheless, this population is free-living and subject to the same ecological processes found within a similar, unmanaged population.

The ringneck (or Indian rose-ringed) parakeet (*Psittacula krameri*) is a non-native, invasive species in Mauritius and was introduced via the pet trade in the late 19th Century (Cheke and Hume, 2009). Superficially similar to its endemic and insular congeneric, this highly adaptable, continental species numbers in the tens of thousands in Mauritius and represents direct competition for food and nesting resources (Jones, 1987). The similarity between these species is such that breeding strategies are comparable, making this an ideal study system with which to investigate species level immune function relationships in free-living populations of an endangered, endemic species and a globally successful invasive species. Furthermore, this system offers the opportunity to increase current knowledge concerning the potential reorganisation of immune function in species evolved on islands in comparison

to phylogenetically similar continental species as a result of contrasting parasite communities (Matson, 2006, Matson and Beadell, 2010).

2.4 Materials and methods

2.4.1 Field methods

Mauritius parakeet (MP) nestlings were studied during two breeding seasons as part of an ongoing management and monitoring programme in The Black River Gorges National Park in South-West Mauritius. A total of 258 nestlings were monitored representing 79 breeding females and 122 separate broods spanning two consecutive breeding seasons. All nest sites are either natural cavities which have been modified for access or purposely built nestboxes supplied by the Mauritian Wildlife Foundation. Equal amounts of nesting material dusted with a carbamate insecticide (5% carbaryl) were provided before egg-laying commenced. Brood age was calculated using hatch day of the eldest chick, known to within 2 days and determined by hatch checks as part of an established protocol. All known nest–sites (2009/10, n = 61 20010/11 n = 61) were accessed when the eldest nestling was believed to be 45 days old (approximately 10 days before fledging). At approximately half of these sites (selected randomly) each individual in the brood was challenged with PHA. A second visit was made to all of these sites after 24 hours (+/- 2 hrs) to complete the PHA response measurements and to collect post-challenge blood samples from each individual. At those sites where PHA was not administered, each individual in the brood was sampled for blood during a single nest site visit. Body mass, wing and tail measurements were also collected from each individual during these visits.

Each individual in every known, successful brood (where at least one chick was fledged) was sampled for blood and around half of these individuals were experimentally challenged with PHA to induce an immune response before blood was collected. Each individual was genetically sexed using the sexually dimorphic primer set Z002b (Dawson, 2007) as part of a parallel research study (Chapter 3). Individuals were grouped in the final analysis by sex,

hatch order, hatch day, breeding year, female age and whether PHA-challenged or not. This sampling effort encompasses the entire known breeding population of this species for two consecutive breeding periods.

Ringneck parakeets (RNP) were sampled in a similar fashion during the same two years. A total of 61 nestlings were monitored representing 29 broods. Once again, nest sites were either in purpose built boxes or natural cavities and insecticide treated nesting material was added before egg-laying commenced. Sample size is smaller for this species owing to the difficulties of finding, reaching and accessing natural nest sites and reflects the effort made by the recovery program in concentrating on the endemic species. All individuals were intradermally challenged with PHA and sampled for blood 24 hours later (+/- 2 hrs). Sites were repeatedly visited to record date of egg-laying and hatch day of each chick. Each individual was subsequently genetically sexed and morphometric details were recorded as previously.

PHA assay

The PHA challenge technique was performed following the protocol of Smits et al., (1999). Twenty-five milligrams of PHA-P (Sigma-Aldrich L8754) was diluted in 5ml of phosphate buffered saline solution (PBS; Oxoid, Dulbecco A – BR0014G) to make a 5:1 dilution. This solution was then divided into 60µl aliquots and frozen at -20ºC. Aliquots were thawed thoroughly before use and never refrozen.

An area on the right patagium of each individual was swabbed with 70% alcohol in order to expose a patch of skin clear of any feathers. Tissue thickness was then measured (to the nearest 0.01mm) a minimum of three times using a pressure sensitive micrometer (Silverline 282378) and 20µl of the 5:1 PHA solution was injected (all measurements taken and injections administered by S.J.T.). The area was marked post-injection with waterproof, permanent ink. After a period of 24 hours (+/-2 hrs) a second measurement was made using the same protocol. Pre and post PHA measurements per individual were averaged to attain a

single pre and post-injection measurement for each nestling and the difference between these two measurements was used as an index of the immune response induced by PHA.

Sample collection

Blood samples were collected from all nestlings as part of an ongoing disease monitoring effort. Approximately 0.8ml of blood was drawn from the jugular vein of each individual using a 25 gauge needle attached to a 1.0ml, non-anticoagulant treated syringe. Immediately after collection blood films were made with whole blood using the wedge method (Clark et al., 2009) and air dried. The syringe was then removed from the needle and the remaining blood was dispensed into 3 different containers; (i) 0.5ml was transferred into heparinised collection tubes (Teklab H1230) and carefully agitated to prevent coagulation thereby enabling plasma formation, (ii) two to three drops of blood were stored in absolute ethanol for subsequent genetic and viral analyses, (iii) and the remainder was transferred into an empty microcentrifuge tube and allowed to coagulate so that serum could be extracted.

All samples were transported back to the field site within 2 hours of collection for further processing. The blood films were then fixed in 100% methanol and subsequently stained with Leishman's stain solution (Fisher PB05) following the manufacturer's guide. A small amount of well mixed heparinised blood was used to determine each individual's Packed Cell Volume (PCV) using a haematocrit centrifuge. Samples intended for plasma or serum extraction were refrigerated overnight. A small centrifuge was then used to separate these liquid products from the cellular portion of the whole blood which were then extracted and subsequently stored at -80ºC before being transported to the UK on dry ice.

2.4.2 Laboratory methods

General haemolysis-haemagglutination assay

The haemolysis-haemagglutination assay was performed following Matson et al., (2005) with the following modifications. Assays were carried out in U-bottom, 96-well microplates

(Sterilin 612U96). Twenty-five microlitres of 1.0 M PBS (Oxoid, Dulbecco A – BR0014G) were added to all wells in columns 2-12. Twelve and a half microlitres of 8 individual plasma samples were then added to columns 1 and 2. A multi-channel pipette was then used to serially dilute the plasma solution between columns 2 and 11 resulting in dilutions ranging from 1:3 in column 2 to 1:59049 in column 11. Column 1 was used as a positive control as it only contained plasma, and column 12 was used a negative control given that it contained PBS only and no plasma. A control of chicken plasma was included to every third microplate to ensure assay consistency.

To initiate the assay a solution of rabbit red blood cells was prepared. Whole rabbit blood in Alsever's (Harlan Laboratories UK) was first centrifuged and the red blood cells isolated and washed four times in PBS before being diluted to a final concentration of 1% rabbit red blood cells (RRBC) in 1.0 M PBS. Fresh rabbit blood suspensions were prepared each day.

To each well of the microplate, 12.5µl of rabbit red blood cell suspension was added. The plates were then sealed with plastic film before fitting the lid and were incubated in an oven at 37ºC for 90 minutes. Plates were then tilted at an angle of 45º for 20 minutes before being scanned using a positive transparency flatbed scanner. The resulting image is used to visualise agglutination. The plates were then scanned a second time after being kept flat at room temperature for a further 70 minutes in order to visualise the lysis reaction of the assay. Agglutination and lysis was recorded by identifying the last well in which each part of the reaction occurred as in Figure 1.

Cell counts

Blood films were assessed with a microscope at x1000 magnification and the first 100 leukocytes were identified according to Campbell and Ellis (2006) and Ritchie et al., (1997). Lymphocytes, heterophils, basophils, monocytes and eosinophils were differentiated and the ratio of heterophils to lymphocytes (H:L ratio) was calculated. Eosinophil, basophil and monocyte counts were calculated as percentage of total leukocytes.

2.4.3 Statistical analyses

Using the previously described methods, the following eight variables were derived as indices of immune function: (i) PHA increase, (ii) PCV, (iii) agglutination score, (iv) lysis score, (v) H:L ratio, (vi) basophil concentration, (vii) eosinophil concentration and (viii) monocyte concentration. Pearson's product-moment correlation coefficients were calculated for each pair of relationships to construct correlation matrices for each organisational group for CPCA. The same matrices were used for separate PCAs and resulting correlation circles both executed using the 'ade4' package in R (Dray and Dufour, 2007). Correlation coefficients were then transformed within the R package 'psych' (Revell, 2011) which uses Fisher's 'z' transformation for non-normal data to facilitate T-tests to assess significant differences between pairwise correlation coefficients using the 95% confidence intervals of the transformed indices. Where more than two matrices were included in the same analysis pairwise correlation coefficients among matrices were presented as box and whisker plots enabling visualisation of the consistency of correlation coefficients. Deviation of the mean from zero was calculated for each pairwise relationship among matrices using one-sample T-tests. Significance ($P < 0.05$) therefore indicates that a paired relationship consistently correlates despite group affiliation whilst the range of correlation coefficients can be visualised as the width of the box and whiskers.

Common principal components analysis was carried out using the Common Principal Components Analysis Program (Phillips and Arnold, 1999) and all other statistical procedures were carried out within R (R Development Core Team, 2012).

2.5 Results

2.5.1 Relationships between parakeet species

Only those individuals that were experimentally challenged with PHA were included in the between-species analysis. Due to the limitations of PCA only those individuals from which a full suite of immune indices were collected could be included resulting in sample sizes of n

= 47 for RNPs and n = 99 for MPs. Immune function indices derived from the methods discussed above were abbreviated to (summarised in Box 1.); PHA increase (PHA), packed cell volume (PCV), eosinophil concentration (eosin), basophil concentration (baso), monocyte concentration (mono), heterophil:lymphocyte ratio (H:L), lysis of rabbit red blood cells (lys) and agglutination of rabbit red blood cells (agg).

Common principal components analysis

Results of a between-species CPCA indicate a lack of complete principal component structure suggesting significant differences between the two correlation matrices (Table 1).

Correlation circles and principal components analysis

Figure 2 illustrates the consistency of pairwise relationships of immune function measurements between the two species. Three consistently positive correlations are obvious; (i) lysis and agglutination (ii) monocyte concentration and response to PHA (iii) agglutination and PHA. Five correlations are similarly negative in both species; (i) basophil concentration and PHA, (ii) basophil concentration and monocyte concentration, (iii) lysis and H:L, (iv) PCV and agglutination, (v) PHA and PCV. Eosinophil concentration has a very weak loading on the first two PCs in both species indicated by the short vector length. The H:L ratio represents a very weak loading on the first two PCs in Mauritius parakeets but has a very strong influence on those for ringneck parakeets, whilst some correlations are positive in one species but negative in the other, e.g. (i) PCV and basophil concentration, (ii) PCV and monocyte concentration and (iii) lysis and PHA. It is apparent from correlation circles that the immune response measurements which are likely causing the significant differences reported by CPCA from correlation matrices are PCV and H:L. These contrasts are also apparent from the principal component data in Table 2.

In both species, PHA response and basophil concentration are responsible for the vast amount of variation in the first PC representing a similar vector load and direction. The H:L

ratio has very small loadings on all of the first three PCs within the Mauritius parakeet but a very high loading on PC2 within ringneck parakeets. The relative direction of loading for PCV between the two species for the first two PCs supports the contrast illustrated in the correlation circles.

Significance tests between pairs of correlation coefficients

Table 3 shows the matrices of Pearson's correlation coefficients for both species and highlights where significant differences occur between pairs of immune response variables. Once again the contrasts in relationships involving PCV and H:L are apparent. Other significant differences involve relationships with components of the HL-HA assay (lys:mono and agg:PHA).

2.5.2 Relationships within species

Effects of PHA challenge

I explored the data for Mauritius parakeets to investigate relationships between immune response variables as a result of PHA exposure. Mauritius parakeet individuals were separated into two groups; those which received the PHA challenge (n = 99) and those which did not (n = 94). Correlation matrices were calculated as described above using Pearson's correlation coefficients between the measured immune response variables common to both groups (seven variables, omitting PHA). Results of a CPCA are presented in Table 4.

Correlation circles and separate principal components analysis

Figure 3 illustrates that the correlation between lysis and agglutination scores is once again consistently positive, reflecting the pattern seen in the among-species analysis. The contrast in direction of vectors between the two circles is reflected in the relative loadings of each vector on the first two PCs as seen in Table 5. Concentrations of basophils and monocytes are negatively correlated in both groups as are correlations between lysis and basophils.

Eosinophil concentration has very weak (but opposite) loadings in both groups. PCV and monocyte concentration is positively correlated in challenged individuals and negatively correlated in non-challenged. H:L has a very weak loading among those individuals which received the PHA challenge but is a very strong PC among those which did not receive the challenge thus affecting many pairwise relationships. Basophils have also had a considerable effect upon the loadings of the first two PCs. The other considerable contrast is the relationship between PCV and monocytes; a positive correlation among individuals who received the PHA challenge and negative among those which did not.

Basophil concentration represents much of the variation explained by the first two PCs in those individuals exposed to the PHA challenge. The components of the HL-HA assay display similar PC loadings in both groups. The weak but opposite loading effect of eosinophil concentration on the first two PCs is apparent. The contrast in direction and strength of H:L ratio is apparent across all three PCs.

As seen in Table 6 the significant differences between groups involve relationships which include H:L ratio, eosinophil concentration and agglutination scores. Even though eosinophil concentration has very weak loadings for the first two PCs it results in significantly different relationships with H:L and agglutination. Contrastingly, basophils have a very strong opposite loading on PC2 between species yet this does not produce any significant differences in pairwise correlation coefficients.

Annual variation within species

Annual variation within the Mauritius parakeet was assessed using non-PHA challenged individuals split into separate years; A (n = 48) and B (n = 46) using seven variables of immune function (omitting PHA increase) measurements as above.

CPC analysis revealed no significant variation in structure among immune response variables within Mauritius parakeets that can be attributed to annual variation; principal

components are common to both matrices but the eigenvalues which represent relative loadings on these directional vectors differ.

Even though correlation circles indicate negative correlations in both years between basophils and eosinophils there is a significant difference between the two correlation coefficients illustrated in Table 9; a positive correlation between them in year B and a significantly different negative correlation in year A. These contrasts are illustrated by their relative vector loadings in Table 8. Other contrasting relationships illustrated by the correlation circles are not supported by T-tests on pairwise correlations, e.g. PCV and basophils, despite noticeable trends. Those relationships common to both groups are: (i) agglutination and lysis, (ii) H:L and agglutination and (iii) PCV and lysis.

Within species grouped by sample time of day

Leukocyte profiles have been shown to vary according to the time of day at which the sample was collected due to metabolic energy allocation (Ots et al., 1998). Daytime temperature ranges may also have influence upon proliferating blood cells and their activity, (Clark et al., 2009). Accordingly Mauritius parakeet individuals which were not PHA challenged were divided into five groups according to the time of day at which blood was collected. Five correlation matrices were calculated based on the following time groupings; 0800-1000 (n = 18), 1000-1200 (n = 21), 1200-1400 (n = 10), 1400-1600 (n = 24) and 1600-1800 (n = 11).

Results of CPCA analysis (Table 9) indicate that matrices share common principal components, i.e. a common structure is found among the groups. Correlation circles (Figure 5) reveal several patterns among pairs of correlation coefficients when individuals are segregated into groups according to sample time, going some way to support the conclusion made by CPC that matrices share common structure; lysis and agglutination are positively correlated in all but one group (T3); monocyte concentration and lysis are positively correlated in all but one group and eosinophil concentration and PCV are positively

correlated in all but one group. However, other pairwise relationships vary among groups e.g. the relationship between H:L and lysis which varies between a negative correlation in T1 and T5, zero correlation in T4 and positive in T2 and T3. Boxplots of pairwise correlation coefficients (Figure 6) reveal that only one of these relationships (monocyte:lysis) reflects a mean correlation coefficient which is significantly different from zero indicating a constantly similar relationship. Boxes and whiskers diagrams represent variation in the pairwise relationships; those with narrow variation are those relationships which tend to be consistent among groups.

2.6 Discussion

2.6.1 Relationships among species

One would expect that proportional or equal CPC would result in few or zero significantly different relationships between pairs of immune indices and that more differences would be apparent as the CPC model approaches unrelated Common principal components analysis of the two correlation matrices representing endemic Mauritius and introduced ringneck parakeets suggested partial common structure (CPC3). These two groups therefore share some but not all principal components. Correlation circles and PCA supports these results; some pairwise relationships are constant whereas others are inconsistent. Pairwise T-tests of correlation coefficients also provide evidence to suggest that these two matrices are neither proportionally related nor completely unrelated. Six of these pairwise relationships reflect correlation coefficients that are significantly different between groups (see Table 10) and these relationships are reflected in the contrasting bivariate relationships and relative loadings on the first two PCs as seen in the correlation circles.

2.6.2 Relationships among organisational groups within species

When the data for Mauritius parakeet individuals are split into two groups where the distinction is those which were PHA challenged and those which were not, a pattern of results emerges that is similar to those observed between the two species. The matrices share

some but not all principal components (CPC5). Once again, some pairwise relationships between immune function variables are consistent between the two groups whilst others are not. Those which are not consistent upon visualisation in correlation circles, for example H:L:PCV, are found to have significantly different correlation coefficients. Four of the pairwise correlation coefficients show significant differences, supporting the CPCA results. Correlation circles also appear to support the conclusions of CPCA and pairwise correlation coefficients.

2.6.3 Annual variation within species

Between years CPCA indicated full common structure between the matrices suggesting that all principal components are shared among annual groups but display differing eigenvalues. Correlation circles do not appear to suggest that all pairwise relationships are common to both groups. The relationships between PCV and H:L, and lysis and agglutination are consistent between the two groups. Those which are inconsistent tend to include basophils, eosinophils and monocytes. The fact that only one significant difference in between pairwise correlation coefficients was found (that of eosinophils and basophils) gives support to the conclusion of CPCA that structure is not significantly different. The contrast in this relationship between years was not evident upon examination of the correlation circles. This may have resulted from weaker loadings for both of these variables in the PCA for year B and very high influence among the first two PCs for year A, indicated by the relative vector lengths and the PC values in table 8. The exact biological role of basophils and eosinophils in vertebrates is unclear (Claver and Quaglia, 2009, Sullivan and Locksley, 2009, Voehringer, 2009), however peripheral numbers of basophils are known to increase during pathological conditions indicating their potential involvement in mediating allergic diseases and immunity to pathogens (Karasuyama et al., 2009). Eosinophils have also been associated with allergy, parasitism and exposure to foreign antigens (Campbell and Ellis, 2006) and it is therefore plausible that an interaction between these two rare granulocytes is associated with a hypersensitive reaction or parasite infection. Indeed both cell types have been implicated

in proinflammatory responses (Kim et al., 2010, Locksley, 2010). The apparent annual variation in the interaction of these cells in this study is perhaps reflective of contrasting parasite and pathogen pressures attributable to different environmental conditions between years. For example, average daily rainfall between the months of September and December was significantly different between study years (mean 2009: 7.72mm ±1.66, mean 2010: 2.44mm ±0.52; $t = 2.99$, d.f. = 242, $P < 0.01$).

2.6.4 Time of day

When individuals are grouped according to sample time of day CPCA once again suggests similar structure among the matrices. Evidence from correlation circles tends to support this conclusion; many relationships are common to most matrices, for example: lysis:agglutination, mono:baso, mono:lys, PCV:eosin, PCV:lys. The relationship between monocyte concentration and lysis is the only relationship with a significantly consistent directional loading across groups. Those relationships which appear to be common among correlation circles tend to be reflected in Figure 6 where box and whisker plots indicate a relatively small interquartile range, although the presence of outliers and the small sample size mean that this figure should be interpreted with a degree of caution.

2.6.5 Consistency of signal

Common principal components analysis suggests that the immunological reaction of the endemic and introduced species to experimental immune response provocation is different. This conclusion is supported both by examination of correlation circles derived by separate PCAs and by the results of T-tests on pairwise correlation coefficients. Similarly it is evident (and not unexpected) that individuals within the same species, who have been exposed to the PHA challenge display significantly different pairwise relationships between immune response variables to those who have not been challenged. Separate PCA analyses and T-tests support this conclusion.

In this study, where CPCA results indicate a common structure among matrices the equivalent relationships within correlation circles are less evident. Inconsistencies arise in graphical representation of the pairwise relationships but examination of pairwise correlations or boxplots and individual eigenvalues can assist in providing clarity to these diagrams. These inconsistencies tend to involve the cellular immune indices of rare white blood cell counts and likely arise from the complexity of the system under investigation, individual variation in immune response variables, effects of sample size and methodological consistency. Matrices can only be arranged by a single factor containing many levels and therefore CPCA cannot possibly incorporate the endless list of biotic and abiotic factors which combine to produce an individuals' immune function. However, this analysis has shown that statistically significant differences among clearly defined groups can be identified and this represents an important consideration for further, more exploratory analysis where collinearity among immune function variables needs to be considered. One would not, for example, now assume that the PHA challenge provokes a similar response in both parakeet species. Therefore, each dataset needs to be considered separately if immune response variables were to be assessed with PCA.

Some pairwise relationships appear relatively constant regardless of how matrices are calculated or groups organised. The relationship between lysis and agglutination, for example is relatively consistently positive in all analyses suggesting that a single variable derived from PCA which incorporates both of these variables would be a suitable and valid alternative to including both measurements as explanatory variables in a linear mixed model.

Ultimately however, very few pairwise relationships are common across multiple organisational levels within and among species owing to the complexity of the immune system and the responses of various individually specific immunological pathways. However, CPCA has helped to highlight where differences or similarities in these relationships show a simple general pattern, but it cannot be relied upon alone to identify specific relationships and therefore the simultaneous use of correlation matrices and

associated pairwise comparisons is advised. To enable a more robust interpretation of these results one would need to quantify individual-level infection and exposure to a range of parasites and pathogens in order to identify those individuals which are reacting to an immune challenge. Evidence from this study appears to suggest that the more complexity (in terms of number of matrices) a CPCA comprises the more difficult it is to interpret the subsequent results by using correlation circles and boxplots to represent biological phenomena and interactions between variables that are already poorly understood. Unless, that is there are very strong, consistent relationships between immune response measurements across matrices.

The conclusions of this study are broadly congruent with those of Buehler et al., (2011) and illustrate the complexity of relationships between variables of immune response at different organisational levels. This complexity is further complicated by the functional uncertainty associated with some of the cellular indices and the difficulty in accurately interpreting individual counts of inherently rare white blood cells. Nevertheless, the issue of collinearity of immune function variables in studies of ecoimmunology is an important one, and methods of assessing those relationships such as this can help to identify patterns and potentially uninformative variables. This exploratory investigation can assist in subsequent analyses such as multiple regression when decisions have to be made about which variables are included in statistical models and the value and interpretation of results. Matson et al., (2006) suggest that characterising an individual's immune function in single-species studies can be achieved by the combination of a single humoral and cellular component which may assist in reducing the complexity of multiple, potentially dependant variables. This is perhaps a more realistic alternative of characterising individual-level immune function in studies of free-living bird populations.

2.7 Figures and tables

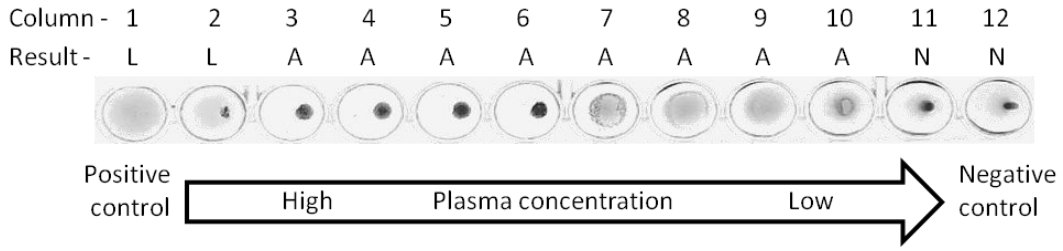


Figure 1 An example of the HL-HA assay for a single individual. Columns 1 and 2 show lysis (L) of rabbit red blood cells. Columns 3-10 exhibit agglutination (A) whilst columns 11 and 12 show no reaction (N). This example would be given an agglutination score of 10 and a lysis score of 2.

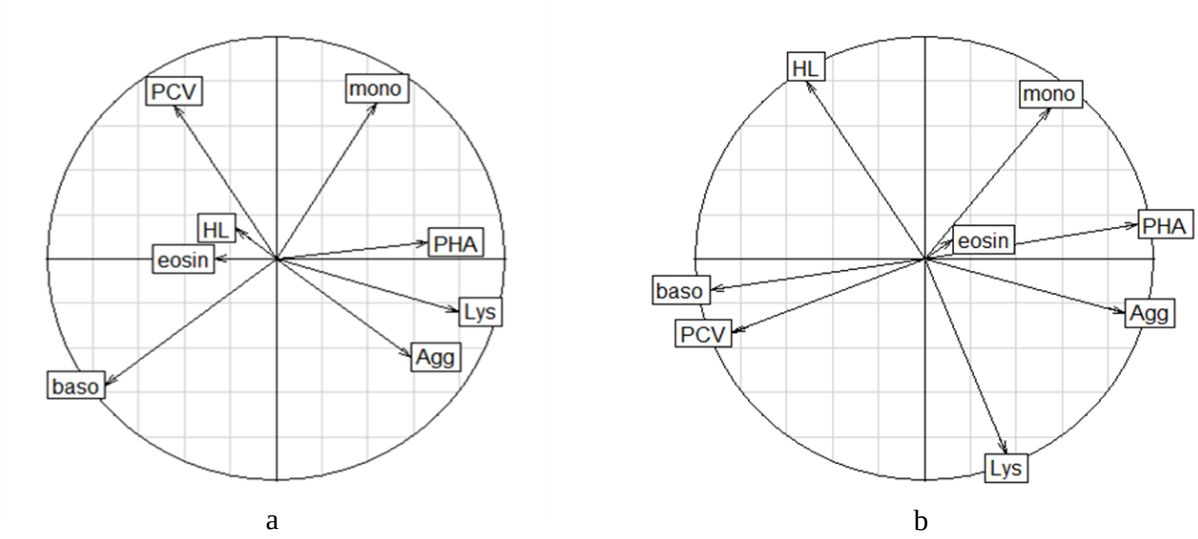
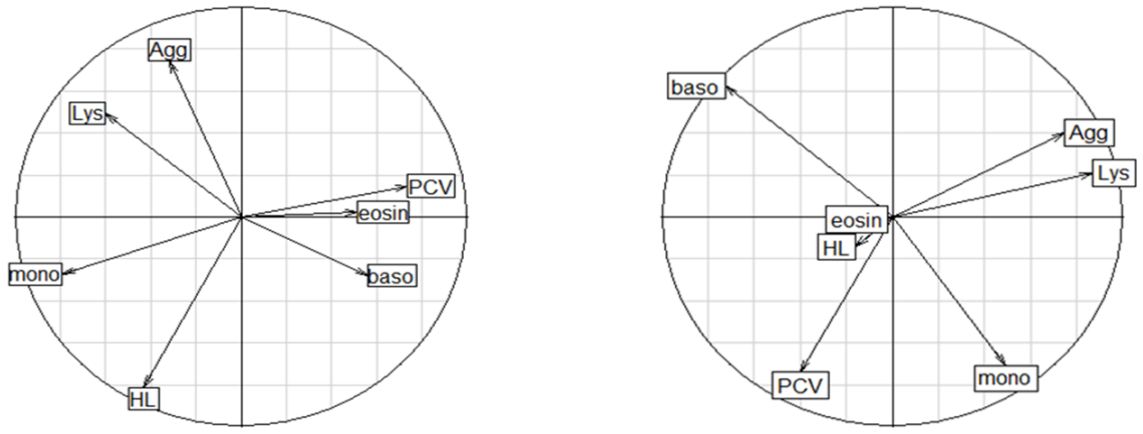
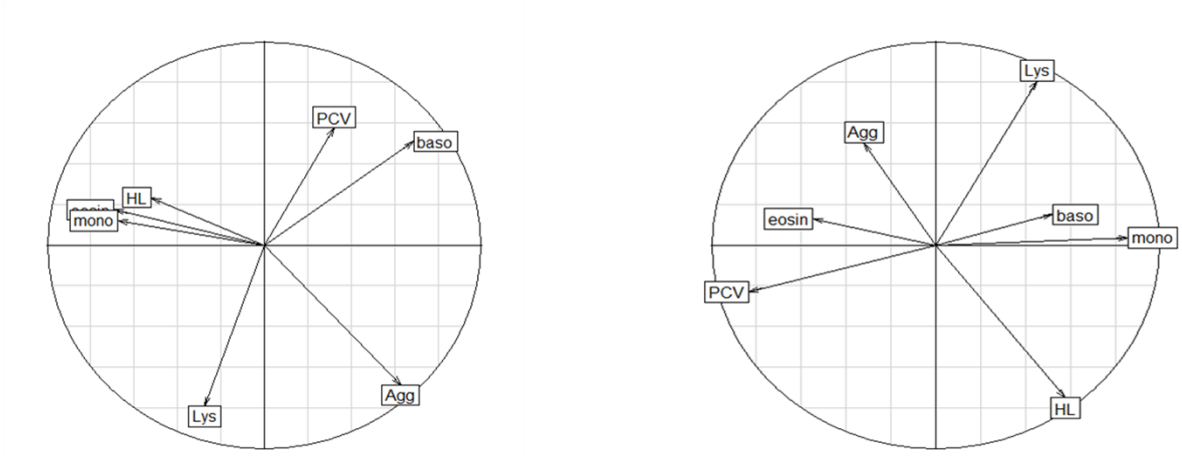


Figure 2 Correlation circles produced from separate principal components analyses representing pairwise correlations among immune response measurements for Mauritius parakeets (a) and ringneck parakeets (b). Axes are represented by the first two principal components PC1 and PC2. Arrow lengths indicate vector strength whilst angles between vectors indicate degree of correlation.



a b

Figure 3 Correlation circles produced from separate principal components analyses representing pairwise correlations among immune response measurements for Mauritius parakeets which did not receive the PHA challenge (a) and those which did (b).



a b

Figure 4 Correlation circles produced from separate principal components analyses representing pairwise correlations among immune response measurements for Mauritius parakeets sampled in season A (a) and season B (b). Eosin is concealed by mono in season A.

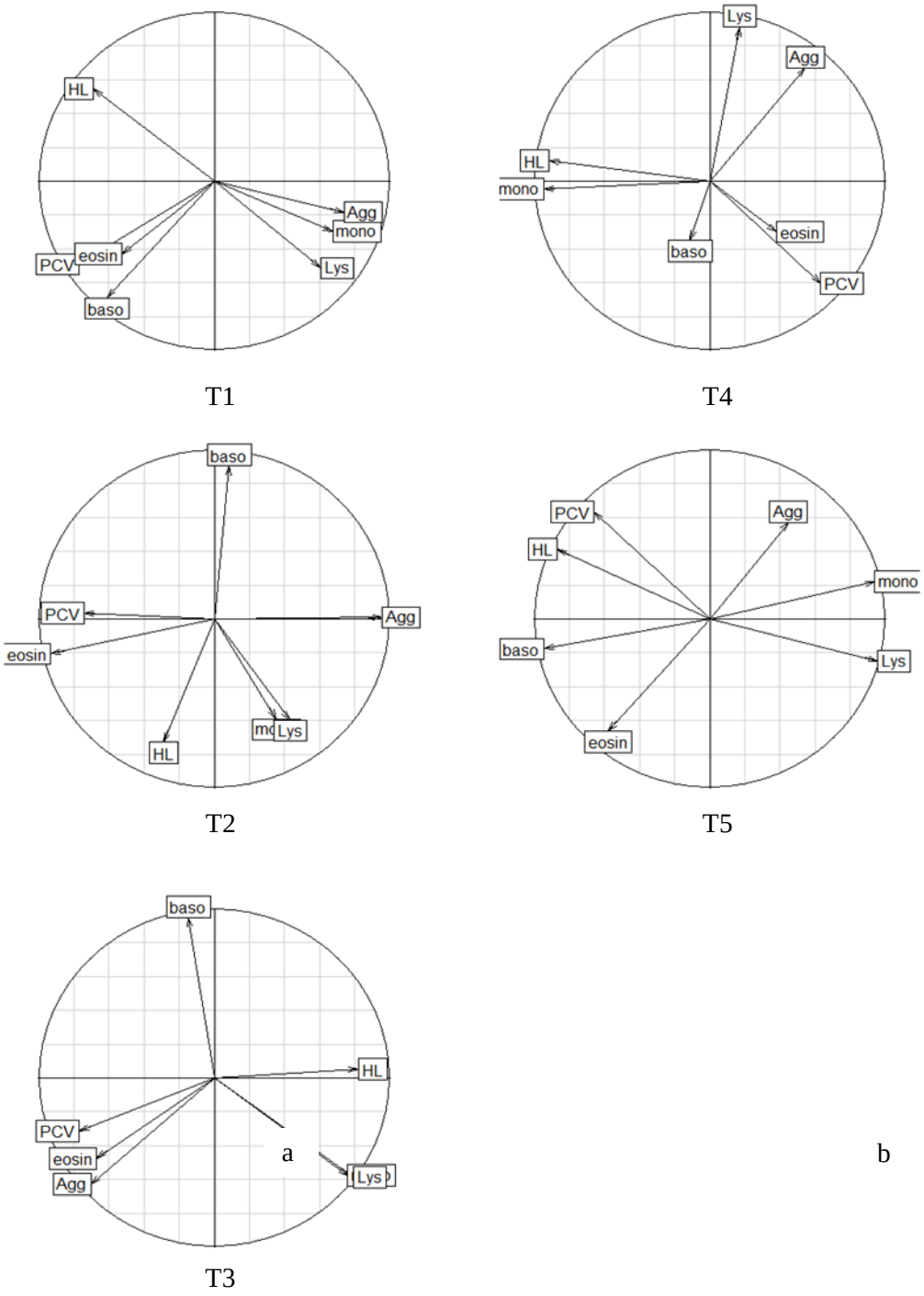


Figure 5 Correlation circles representing pairwise correlation coefficients of immune response measurements divided by time of sample (T1 – T5). Mono obscured by Lys in T3 and T2.

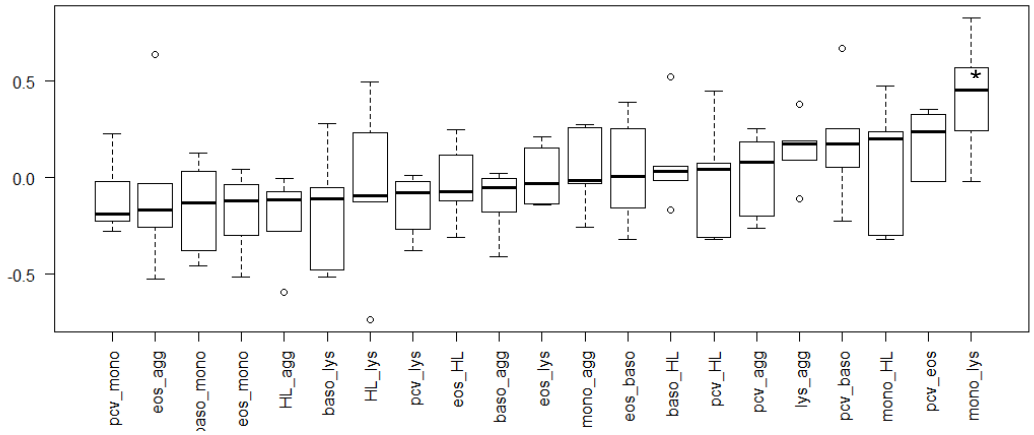


Figure 6 Boxplots of all pairwise correlation coefficients for all 5 time groups. The mean of only one relationship (*mono:lysis) approached a significant deviation from zero (P = 0.05) Width of boxes and whiskers indicate degree of consistency in pairwise relationships among time groups.

Table 1 Common principal components analysis between two species. Both the step up approach and the model building approach suggest CPC(3) as the best model. The matrices share 3 principal components in common.

Higher	Lower	$\chi 2$	df	p	AIC
Equality	Proport	0.041	1	0.84	66.33
Proport	CPC	31.828	7	0.00	68.28
CPC	CPC(6)	1.957	1	0.16	50.46
CPC(6)	CPC(5)	0.413	2	0.81	50.50
CPC(5)	CPC(4)	5.622	3	0.13	54.10
CPC(4)	**CPC(3)**	**12.041**	**4**	**0.02**	54.46
CPC(3)	CPC(2)	5.207	5	0.39	**50.42**
CPC(2)	CPC(1)	2.775	6	0.84	55.22
CPC(1)	Unrelated	6.44	7	0.49	64.44
Unrelated					72

Table 2 The relative loadings of immune variables on the first three principal components for both species. The three variables representing the strongest loadings on the first two principal components are in bold.

	Mauritius parakeet			Indian rose-ringed parakeet		
	PC1	PC2	PC3	PC1	PC2	PC3
PHA	**-0.42**	-0.06	0.54	**-0.47**	-0.11	-0.07
PCV	0.28	**-0.55**	-0.04	0.42	0.23	-0.19
eosin	0.17	0.00	-0.69	-0.06	-0.06	0.95
baso	**0.47**	**0.45**	0.11	**0.47**	0.10	0.07
mono	-0.28	**-0.56**	-0.22	-0.28	**-0.47**	-0.06
Lys	**-0.51**	0.19	-0.24	-0.18	**0.61**	0.10
Agg	-0.38	0.35	-0.33	**-0.44**	0.17	-0.21
HL	0.11	-0.11	-0.03	0.26	**-0.55**	-0.06

Table 3 Correlation matrix. RNP is above diagonal, MP is below. Bold text indicates significant differences (P < 0.05) between pairwise correlation coefficients after transformation using Fisher's z transformation for non-normal data.

	PHA	PCV	eosin	baso	mono	Lys	Agg	HL
PHA		-0.37	0.12	-0.37	0.32	0.07	**0.55**	-0.09
PCV	-0.12		-0.13	**0.41**	**-0.23**	0.01	-0.30	-0.02
eosin	-0.15	0.08		0.06	0.08	0.08	-0.05	0.01
baso	-0.25	**-0.04**	0.07		-0.33	-0.02	-0.32	0.28
mono	0.10	**0.17**	0.16	-0.28		**-0.15**	0.06	0.16
Lys	0.22	-0.05	0.14	-0.07	**0.27**		0.26	**-0.52**
Agg	**0.04**	-0.04	0.11	-0.13	0.01	0.43		**-0.26**
HL	0.07	0.19	0.18	0.02	0.03	**-0.17**	**0.08**	

Table 4 Results of common principal components analysis among groups separated by exposure to PHA. Both the step up approach and the model building approach suggest CPC(5) as the best model. This indicates that the matrices share 5 principal components in common thereby illustrating significant differences in matrix structure between the two groups.

Higher	Lower	χ^2	df	p	AIC
Equality	Proport	0.215	1	0.64	36.75
Proport	CPC	18.955	6	0.04	38.53
CPC	**CPC(5)**	5.313	1	**0.02**	31.58
CPC(5)	CPC(4)	1.753	2	0.42	**28.26**
CPC(4)	CPC(3)	1.795	3	0.62	30.51
CPC(3)	CPC(2)	1.769	4	0.78	34.72
CPC(2)	CPC(1)	4.059	5	0.54	40.95
CPC(1)	Unrelated	2.888	6	0.82	46.89
Unrelated					56

Table 5 The relative loadings of immune variables on the first three principal components for both species. The three variables representing the strongest loadings on the first two principal components are in bold.

	PHA challenged			Not PHA challenged		
	PC1	PC2	PC3	PC1	PC2	PC3
PCV	-0.27	**-0.57**	0.24	**-0.48**	0.12	-0.09
eosin	-0.01	-0.01	-0.64	-0.33	0.02	0.73
baso	**-0.48**	**0.49**	-0.11	-0.36	-0.22	-0.42
mono	0.33	**-0.55**	-0.31	**0.51**	-0.21	0.10
HL	-0.11	-0.11	0.61	0.28	**-0.64**	-0.10
Lys	**0.58**	0.16	0.11	**0.39**	**0.39**	0.28
Agg	**0.50**	0.31	0.21	0.20	**0.58**	-0.43

Table 6 Correlation matrix. Not challenged positioned above the diagonal, below is challenged. Bold text indicates significant differences ($P < 0.05$) between correlation coefficients after transformation using Fisher's z transformation for non-normal data.

	PCV	eosin	baso	mono	HL	Lys	Agg
PCV		0.12	0.14	-0.09	**-0.12**	-0.09	0.00
eosin	0.08		-0.01	-0.09	**-0.11**	0.05	**-0.19**
baso	-0.04	0.07		-0.09	0.02	-0.07	-0.07
mono	0.17	0.16	-0.28		0.27	0.27	0.02
HL	**0.19**	**0.18**	0.02	0.03		-0.05	-0.13
Lys	-0.05	0.14	-0.07	0.27	0.17		**0.18**
Agg	-0.04	**0.11**	-0.13	0.01	0.08	**0.43**	

Table 7 Results of common principal components analysis among individuals separated by year. Both the step up approach and the model building approach suggest CPC as the best model. This indicates that the matrices share all principal components in common.

Higher	Lower	χ^2	df	p	AIC
Equality	Proport	0.013	1	0.91	30.71
Proport	**CPC**	22.436	6	**0.001**	32.69
CPC	CPC(5)	0	1	0.99	**22.26**
CPC(5)	CPC(4)	1.153	2	0.56	24.26
CPC(4)	CPC(3)	1.37	3	0.71	27.10
CPC(3)	CPC(2)	1.725	4	0.79	31.73
CPC(2)	CPC(1)	2.785	5	0.73	38.01
CPC(1)	Unrelated	1.224	6	0.98	45.22
Unrelated					56

Table 8 The relative loadings of immune variables on the first three principal components for both years. The three variables representing the strongest loadings on the first two principal components are in bold.

Year A				Year B		
	PC1	PC2	PC3	PC1	PC2	PC3
PCV	-0.21	**0.43**	-0.30	**-0.51**	0.18	-0.13
eosin	**0.46**	0.13	-0.60	-0.34	-0.10	**0.65**
baso	**-0.46**	0.38	0.24	0.32	-0.12	0.57
mono	**0.45**	0.09	0.16	**0.53**	-0.03	-0.21
HL	0.35	0.17	**0.69**	**0.35**	**0.60**	-0.03
Lys	0.18	**-0.59**	0.04	0.28	**-0.65**	0.00
Agg	-0.42	**-0.51**	-0.01	-0.20	**-0.40**	-0.44

Table 9 Correlation matrix. Above diagonal year A, below year B. Bold text indicates significant differences ($P < 0.05$) between correlation coefficients after transformation using Fisher's z transformation for non-normal data.

	PCV	eosin	baso	mono	HL	Lys	Agg
PCV		0.05	0.21	0.14	0.01	-0.01	0.01
eosin	0.16		**-0.24**	0.19	-0.06	0.06	-0.32
baso	-0.16	**0.25**		-0.02	-0.02	-0.14	0.00
mono	-0.15	-0.20	0.23		0.26	0.19	-0.05
HL	-0.18	-0.12	0.17	0.25		0.10	-0.24
Lys	-0.17	0.05	0.23	0.34	-0.17		0.20
Agg	0.05	-0.01	0.02	-0.03	-0.12	0.13	

Table 9 Results of common principal components analysis among individuals separated by sample time. Both the step up approach and the model building approach suggest CPC as the best model.

Higher	Lower	χ^2	df	p	AIC
Equality	Proport	0.326	4	0.99	140.79
Proport	**CPC**	69.41	24	0.00	148.46
CPC	CPC(5)	3.841	4	0.43	**127.01**
CPC(5)	CPC(4)	4.692	8	0.79	131.21
CPC(4)	CPC(3)	8.91	12	0.71	142.52
CPC(3)	CPC(2)	21.811	16	0.15	157.61
CPC(2)	CPC(1)	6.144	20	0.99	167.80
CPC(1)	Unrelated	25.655	24	0.37	201.66
Unrelated					224

Table 10 Pairwise correlation coefficients found to be significantly different between Mauritius parakeets (MP) and ringneck parakeets (RNP).

	MP	RNP
agg:PHA	0.04	0.55
baso:PCV	-0.04	0.41
mono:PCV	0.17	-0.23
Lys:mono	0.27	-0.15
HL:Lys	-0.17	-0.52
HL:Agg	0.08	-0.26

Chapter 3. Detection of fine-scale spatial and temporal genetic structure in the restored Mauritius parakeet population: implications for long-term genetic monitoring strategies and reintroduction programmes

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Abstract

Threatened populations of birds are often restored after bottleneck events by using reintroduction techniques. Whilst population numbers are often increased by using such measures, the long-term genetic effects of reintroductions and post-release management of the resulting populations are frequently overlooked. By using a fine-scale approach to study long-term temporal and spatial patterns of genetic variation in the restored population of the endangered Mauritius parakeet (*Psittacula echo*), I identify significant genetic structure among isolated population fragments due to a lack of gene flow between subpopulations despite a historical regime of managed translocations. I also identify an overall declining trend in population-wide estimates of genetic diversity across almost two decades since the initial recovery of the population from the most severe part of this species' bottleneck. Additionally, recruitment of fledglings into the breeding population was not predicted by indices of genetic diversity and average internal relatedness was found to be highest in subpopulations where artificial nestboxes are provided. I conclude by offering a number of key recommendations relating to post-recovery management of reintroduced bird populations which support the encouragement of individual dispersal using established management techniques such as artificial nest site provisioning.

3.1 Introduction

Reintroductions, translocations and reinforcements (IUCN, 1998, Seddon et al., 2012) are techniques frequently used in the conservation of endangered species, to re-establish populations within their historic range (Griffith et al., 1989, IUCN, 1998, Seddon, 1999). However, the success of such attempts has been variable (Armstrong and Seddon, 2008, Beck et al., 1994) and many have failed. A multitude of putative reasons for failure have been put forward, such as a lack of post-release monitoring, failure to identify *a priori* targets concerned with assessing success or failure, as well as a variety of genetic issues such as inbreeding and loss of genetic diversity (Fischer and Lindenmayer, 2000, Nichols and Williams, 2006, Seddon et al., 2007).

Armstrong and Seddon (2008) recognise that much of the reintroduction literature is concerned with retrospective analyses of unfocussed and potentially inefficient monitoring of reintroduced populations and therefore promotes an approach to reintroduction biology whereby research questions are first identified and then used to define appropriate data collection incorporated within long-term monitoring programmes. However, identifying appropriate research questions at the outset of a reintroduction project ideally requires an assessment of lessons learned regarding the success and failure of previous reintroduction attempts. This perspective has therefore led scientists to advocate a more standardised approach to monitoring the outcomes of reintroductions (Sutherland et al., 2010), particularly in view of the fact that managed populations are subject to the same stochastic events and evolutionary processes as any natural population. Furthermore, wildlife managers are rarely able to predict the longer-term impacts of initial decisions made at the onset of a reintroduction. For example, population genetic effects which arise as a consequence of conservation management may not be realistically detectable until many years after the event (e.g. Raisin et al., 2012). Similarly, the emergence or introduction of novel parasites and pathogens within reintroduced populations can be almost impossible to predict despite rigorous monitoring protocols (Ewen et al., 2007). The existence of long-term datasets of

restored and reintroduced populations are therefore a valuable resource which offer an alternative and better approach to examine in detail temporal changes in genetic diversity, population structure and levels of inbreeding, variables which are assumed to be important in reintroduction success and population recovery (Keller et al., 2012, Groombridge et al., 2012).

The primary aim of most species recovery programmes is to increase population sizes from very low numbers, but recovering these bottlenecked species can result in populations with reduced levels of genetic diversity due to inbreeding (Groombridge et al., 2000, Keller et al., 2001, Jamieson, 2011). More complex difficulties can arise in the form of sequential bottlenecks created during the course of conservation management, such as the founding of multiple reintroduced populations (Taylor and Jamieson, 2008, Keller et al., 2012). Population bottlenecks are often the result of fundamental shifts in ecological processes, for example, habitat loss or degradation, resulting in the need for prolonged human intervention and conservation management in order to successfully propagate such species. Genetic problems such as inbreeding which can arise in small populations are well known to conservation managers tasked with recovering endangered species, but are perhaps some of the most overlooked components of long-term monitoring programmes of reintroductions. Indeed, Frankham (2010) recognised a general lack of consideration of genetic issues in the management of wild populations and emphasised the need for increased awareness among wildlife managers of the genetic implications associated with managing threatened populations. More broadly, loss of genetic diversity in naturally outbreeding populations reduces the evolutionary potential of species to adapt to environmental fluctuations (Keller et al., 2012) and has been associated with reduced reproductive success (Reed and Frankham, 2003) and increased extinction risk (O'Grady et al., 2006). In addition, threatened populations often exist in fragmented landscapes consisting of small, isolated pockets of individuals with limited dispersal, thereby further increasing spatial associations between

relatives and ultimately resulting in fine-scale genetic structure among population fragments (Slatkin, 1987, Beck et al., 2008, Randall et al., 2010).

As a consequence of these insidious and often overlooked genetic considerations, monitoring genetic structure within species across various spatial and temporal scales is now a fundamental division of conservation biology and is recognised as a vital tool in conservation management (Schwartz et al., 2007, Robert, 2009, Groombridge et al., 2012, Jamieson and Lacy, 2012). By applying a fine-scale approach to monitor levels of genetic diversity, the effects of different management techniques (Nussey et al., 2005) or stochastic events such as disease outbreaks (Randall et al., 2010) can be assessed relative to the viability and reproductive success of the threatened population, thereby providing managers with an adaptive framework with which to manage threatened species and improve the chances of success for future reintroduction programmes. Unfortunately, long term datasets detailing and monitoring the recovery of endangered species are rare, however those which do exist could prove invaluable as more and more species require some form of management.

The consideration of population genetic viability within threatened species has become possible due to an increased recognition of its relevance and the wide scale availability of relatively inexpensive molecular techniques available for the characterisation of individual level genetic variation such as microsatellite markers. In avian systems, such techniques have largely been used to identify dispersal patterns and to estimate effective population size (Temple et al., 2006, Beck et al., 2008, Lee et al., 2009). Thanks to the foresight of a number of conservation managers working with species over the course of several decades, a limited number of long term monitoring studies of restored populations of threatened species are available. One such example is the successful recovery of the Mauritius (or echo) parakeet (*Psittacula echo*), an endangered species which has, as a consequence of a sustained field monitoring effort spanning three decades, been documented in unprecedented detail resulting in the collection of over 900 individual blood samples across this time period and

the existence of an extensive social pedigree incorporating life history data on every known individual since the early 1990s. This long term conservation programme has therefore provided an ideal opportunity to investigate the temporal and spatial genetic effects of managing the recovery of a threatened species and using reintroduction techniques to re-establish populations in a fragmented landscape.

3.2 Study system

In 2010/11, the population of Mauritius parakeets consisted of ~80 known breeding pairs and an estimated total population size of ~500 individuals, the population having been recovered from a bottleneck of just ~20 known individuals in the 1980s (Jones, 1987, MWF, 1996-2012, Jones and Merton, 2012). By employing techniques such as captive breeding and reintroduction, as well as manipulations of nesting pairs and supplemental feeding, an intensive conservation management programme spanning more than three decades prevented the extinction of this island endemic species (Jones, 2004). As a result of this management and ongoing monitoring, detailed pedigree records of each known individual have been collected, alongside blood samples from the vast majority of post-bottleneck individuals.

Previous work has shown that prior to conservation intervention this species existed as at least two genetically fragmented subpopulations (Raisin et al., 2012); a northern subpopulation (shown in Figure 1 as BF and GG) and a southern subpopulation (BO in Figure 1), separated by a plateau and habitat degradation in the Black River Gorge National Park, Mauritius (20°23'S 57°23'E). Raisin et al. (2012) showed that as a result of conservation management (nest rescues, translocations and releases) genetic variation was homogenised across the geographically separated subpopulations. An outbreak of psittacine beak and feather disease (PBFD) in the cohort of captive-bred birds released in 2004/5 subsequently spread throughout all subpopulations (Kundu et al., 2012) and threatened the recovery of this species; as a consequence of this disease outbreak intensive management ceased in an attempt to limit the spread of infection. Therefore, captive breeding, nest

manipulations, translocations and population supplementation were all halted in an effort to reduce the risk of disease spread but routine colour-banding and blood sampling of nestlings continued, together with provision of artificial nestboxes and supplemental food. Consequently, the current population consists of three putative subpopulations (Figure 1); Bel Ombre (BO), Brise Fer (BF) and Grande Gorge (GG).

The provision of artificial nestboxes and supplementary food has been instrumental in the recovery of this species. During the breeding season 2010/11, 47 of 61 pairs (73%) which successfully fledged chicks used artificial nestboxes, and of these 33 (70%) occurred in the BF subpopulation (see Figure 1) (MWF 1996-2011). Supplemental food has been provided at two locations (BO and BF) previously used as release sites when the populations were augmented by captive reared individuals trained to use specially designed feeding hoppers. The regular provision of supplemental food for the recovering population of Mauritius parakeets has resulted in an increase in productivity since 2001 as new generations of individuals have learnt to utilise this resource (Figure 2 illustrates the growth in number of fledglings produced per breeding pair). Approximately a third of breeding pairs, however, do not take supplemental food. Most of these individuals constitute the Grande Gorge subpopulation (with the remainder occurring in remote areas of BO); they are the remnants of the original relict populations, mostly nesting in natural tree cavities and occurring in the remotest and most inaccessible parts of the National Park. Observational data suggest that despite an overall increase in the species' population size, natural dispersal events (leading to gene-flow) between the three subpopulations are rare (MWF, 1996-2012).

Mauritius parakeets reach sexual maturity at 2-3 years old, form monogamous pair bonds which usually persist for several breeding seasons (Jones and Duffy, 1993) and wild individuals have been known to live for over 17 years (MWF, 1996-2012). Juvenile survival to one year has decreased since 2005 and is thought to be associated with mortality due to PBFD and associated secondary infections (Jones and Merton, 2012). Figure 3 illustrates the turnover in the breeding population in terms of recruitment and retirement of breeding

individuals. Despite the increased productivity of breeding pairs, annual recruitment of new individuals into the breeding population has remained relatively constant since population augmentation ceased in 2005.

In this study, we use fine-scale, spatio-temporal genetic analyses as a tool to monitor population fragments of a recovering, bottlenecked parakeet species under varying degrees of management. We assess genetic structure of the breeding population across the entire 16-year recovery period and relate this to various aspects of population management in order to assess long term viability and to evaluate the sustainability of current management processes. We use a temporal analysis of allelic richness and expected heterozygosity in order to detect any evidence of reduction in genetic diversity as a result of fragmentation effects amongst small subpopulations and the effects of inbreeding. Genetic differences among subpopulations are evaluated with respect to changes in management procedures in response to the documented disease outbreak. We predict that the cessation of translocations and releases has resulted in enhanced genetic structure among subpopulations owing to a lack of gene flow via migration. Furthermore, we predict that, in accordance with theoretical expectation, levels of genetic variation will reflect subpopulation size with the larger breeding subpopulations displaying greater diversity (Frankham, 1996). Finally, we assess temporal patterns of relatedness among breeding individuals at each subpopulation with respect to sex and we examine whether or not levels of allelic richness and heterozygosity predict fledgling recruitment.

3.3 Materials and methods

3.3.1 Sample collection

Blood samples were collected between 1993 and 2011 in conjunction with the conservation management programme directed by the Mauritian Wildlife Foundation. As part of disease monitoring efforts, nestlings were sampled via jugular or brachial venipuncture when the eldest chick was around 45 days old, approximately 10-15 days before fledging. All known

nestlings were also banded with a unique colour combination of rings to aid identification in the field. Owing to the comprehensive nature of this conservation programme, breeding pairs have been consistently very closely monitored and therefore almost all nesting attempts have been recorded, many in readily accessible nest boxes. This sustained field monitoring effort has resulted in an extensive set of blood samples from over 95% of all fledglings produced since 2004 and detailed observational data concerning those individuals which have been recruited into the breeding population each year. Nestling sex was derived using the sexually dimorphic primer set Z002B (Dawson, 2007) and confirmed using blood samples from individuals of known sex from sexually dimorphic adult individuals.

3.3.2 Estimation of inbreeding and loss of genetic diversity

Measurement of inbreeding (the probability that any alleles at any given locus are identical by descent) and inbreeding depression (the reduction in fitness as a consequence of inbreeding) are most accurately characterised by calculating the individual inbreeding coefficient *(f)* from genealogical relationships in pedigreed populations (Pemberton, 2008, Taylor et al., 2010). However, few wild populations exhibit the level of pedigree required for accurate estimation of *f* due to the presence of individuals with unknown ancestry, the occurrence of extra-pair paternity (Griffith et al., 2002) and assumptions associated with relationships among founding individuals (founding individuals are traditionally assumed to be unrelated). Whilst the collection of data for the Mauritius parakeet population has been intensive across an extended time period, some of these difficulties have limited the pedigree reconstruction for this species. For example, despite the existence of a comprehensive pedigree for the Mauritius parakeet, the depth of this pedigree is relatively shallow (maximum average generation value for an individual since founding is 4.75) owing to individual longevity. Furthermore, the cryptic nature of the parakeet's natural breeding behaviour has meant that many individuals with unknown ancestry have been subsequently detected in the population. For example, approximately 30% of all individuals fledged in 2010 were associated with an unknown parent or grandparent in the observational pedigree

(MWF, 1996-2011). As a consequence of these limitations, we estimated level of inbreeding, loss of genetic diversity and relatedness from molecular data generated by genotyping all sampled individuals using a suite of microsatellite markers developed for the Mauritius parakeet (Raisin et al., 2009).

Approximately 50 microlitres of blood was taken from each individual, mixed with 1.5 millilitre of absolute ethanol and stored in a screw-topped rubber-sealed microfuge tube at room temperature prior to DNA extraction. We used an ammonium acetate precipitation protocol (Nicholls et al., 2000) to extract genomic DNA from all blood samples collected between 2008 and 2011. All individuals were genotyped at 20 species-specific polymorphic microsatellite loci developed by Raisin et al., (2009) and the resulting genotypes were added to an existing genotype dataset for this species (Raisin et al., 2012). All individuals were sex-typed using the Z002B primer set (Dawson, 2007) in order to check that all the loci used were autosomal and to help identify any contamination, sample mix-up or labelling errors. Null allele frequencies were estimated using *Cervus* (Marshall et al., 1998). We checked that the alleles amplified were of the size expected (based on the sequenced clone), and also rechecked the scoring for all alleles of the same locus that differed by 1bp and of any allele that was 20bp or more different in size to the other alleles observed. Each locus was tested for deviation from Hardy-Weinberg equilibrium using the exact probability test in *Genepop* (Raymond and Rousset, 1995). Pairs of loci were checked for linkage disequilibrium using *Genepop* (Raymond and Rousset, 1995) and a sequential Bonferroni correction for multiple tests was applied (Rice, 1989). Genotyping error rates, allelic dropout and miss-scoring were estimated by repeating the DNA extraction, amplification and genotyping process for a randomly selected 10% of individuals.

3.3.3 Statistical analyses

The Mauritius parakeet has a single breeding season each year; eggs are laid between September and November with the main fledging period occurring during December and January. Individuals were therefore arranged into the different annual cohorts corresponding to the year within which they hatched or occurred as a breeding adult. Consequently, analysis was conducted separately on breeding adult individuals and fledglings given that only a fraction of those fledged individuals are recruited into the breeding population (see Figures 1 and 2). Breeding adults were identified as those individuals who were known to have produced eggs as a pair during a breeding season and therefore many adult individuals were present in consecutive annual cohorts owing to multiple reproductive seasons. Thus, the difference in genetic variation between any two consecutive cohorts is a result of the turnover in the breeding population due to the recruitment and retirement of individuals into and out of the breeding population. Similarly, fledged individuals were assigned to annual cohorts defined by the year in which they were produced as eggs, facilitating genetic analyses associated with recruitment probability. All individuals were assigned a code relating to the geographical subpopulation in which they were produced (fledglings) or were found to be nesting (adults).

We used a number of different approaches for quantifying genetic differentiation. Allele frequencies in any sexually reproducing population are determined by the random sampling of genes from the previous generation (genetic drift) and in small populations these frequencies will fluctuate with greater amplitude compared to large populations, increasing the chance that rare alleles are lost. A recent debate has called into question the use of Wright's (Wright, 1965) *F*-statistics on data obtained from neutral highly polymorphic markers to measure genetic differentiation and several authors note that its correct usage lies with measuring the fixation of alleles and not population differentiation (Hedrick, 2005, Jost, 2008, Meirmans and Hedrick, 2011, Edelaar and Björklund, 2011). Therefore, changes in the structure of genetic variance were assessed using Wright's (Fst) F-statistics (1965) and

the harmonic mean of Jost's D (Jost, 2008) at different spatial and temporal levels. To identify any temporal changes in gene frequencies at the global scale across the entire study period we calculated temporal Fst and D_{est} among breeding cohorts using FStat (Goudet, 1995) and SMOGD (Crawford, 2010) irrespective of any spatial affiliation. Similarly, spatial differentiation was assessed at the global scale by grouping individuals into their respective subpopulation irrespective of time. Temporal changes in spatial structure were then assessed by analysing each cohort separately with respect to subpopulation. Temporal variation at the local scale was then assessed separately for each subpopulation to examine finer scale differences. Results obtained using the two different methods showed very similar patterns and therefore only those derived using Fst are presented here; results obtained using Jost's D are available as supplementary material (Table S1).

An estimate of Fst significantly greater than zero was taken to indicate a greater level of genetic differentiation among groups than within groups (Holsinger and Weir, 2009). Pairwise Fst values were generated between each group pair and taken to represent genetic differentiation between each pairwise combination. Linear regression analysis was used to identify any significant trends over time by using 'Year' (i.e. annual breeding season) as an explanatory variable and the appropriate estimate of differentiation as the response.

Standard estimates of genetic diversity were also calculated for each sample group. Number of alleles, allelic richness (Ar), gene diversity (unbiased heterozygosity: He) and Weir and Cockerham's (Weir and Cockerham, 1984) estimate of fixation index (Fis) were calculated using FStat (Goudet, 1995). Allelic richness was rarefied to the smallest observed sample size common to each locus; Ar and He were averaged over all loci to produce mean group values. Deviation from Hardy-Weinberg equilibrium (HWE) was investigated in each cohort or group of samples and values of Fis were tested for significance using permutation tests in FStat (Goudet, 1995). Variation in Ar and He among cohorts was analysed on a temporal scale by using a Kruskal-Wallis ANOVA for non-parametric data and variation between subpopulations was assessed using Wilcoxon signed-rank tests for pairwise comparisons of

non-normal data. Linear regression analysis was used to identify any significant trends in diversity estimates over time. Estimates of parental relatedness were investigated within and among subpopulations using Internal Relatedness (IR) measures (Amos et al., 2001). All statistical analyses, unless otherwise stated, were carried out using R (R Development Core Team, 2012).

3.4 Results

3.4.1 Genotyping

A total of 897 individuals were genotyped (399 females, 408 males and 90 unknown sex), spanning a maximum of 5 generations from 1993-2010. Estimated frequencies of null alleles and deviations from Hardy-Weinberg equilibrium were calculated using 25 unrelated individuals from a single population. Genotyping error rates were less than 5% and the estimated frequencies of null alleles per locus were less than 0.1% for all loci and. Two loci (*Peq16* and *Peq21*) were found to be sex-linked (Z-linked) based on the absence of heterozygotes in females and were therefore omitted from future genetic analyses. One pair of loci was found to be in linkage disequilibrium (*Peq09* and *Peq12*) and therefore one of these (*Peq09*) was excluded from the analyses. All loci were found to be in Hardy-Weinberg equilibrium (including *Peq16* and *Peq21* when assessed in male individuals only). These findings are congruent with results obtained by previous genetic analyses of this species (Raisin et al., 2009, Raisin et al., 2012). Amplification was poor for individuals at *Peq19* and consequently this locus was also dropped. The final dataset comprised 897 individuals genotyped at 16 loci.

A total of 109 alleles were identified across the entire dataset; average number of alleles per locus ranged from 4 to 10.8 (mean: 6.8) and average expected heterozygosity ranged from 0.27 to 0.81 (mean 0.64).

3.4.2 Global analyses

Temporal variation

Breeding adults from all putative subpopulations were pooled into appropriate annual cohorts to identify any global variation in genetic diversity through time. Each cohort therefore represented the available genotypes of individuals which were known to be breeding in any given year. Temporal global Fst values indicated no significant variation among breeding cohorts (Fst = -0.004; CI: -0.005 to -0.001). None of the pairwise Fst values was significantly different from zero after Bonferroni corrections for multiple comparisons ($P > 0.00033$).

Values were averaged over all loci to derive annual diversity indices (Table 1). No single annual cohort deviated significantly from HWE. Unbiased, expected heterozygosity ranged from 0.64 to 0.680 but showed no significant temporal variation (χ^2 (d.f. = 17) = 5.24, P = 0.99). Allelic richness ranged from 3.317 to 3.662 and also showed no significant temporal variation at the global scale (χ^2 (d.f. = 17) = 4.40, P = 0.99). Mean annual allelic richness correlated strongly with average expected heterozygosity ($r^2 = 0.86$, $P < 0.001$), demonstrating the suitability of either variable to describe genetic variability. Linear regression analysis of mean annual Ar and He detected significant temporal trends indicating an overall decrease (see Figure 4) in both of these variables over the 16 year study period (Ar: $r^2 = -0.48$, $P < 0.001$; He: $r^2 = -0.72$, $P < 0.001$).

Spatial variation

All breeding adults genotyped across the study period were divided among their putative geographic subpopulations (BF: $n = 94$, BO: $n = 39$, GG: $n = 53$) to investigate genetic differentiation among them irrespective of time. Global Fst values indicated significant spatial structure among the three subpopulations (Fst = 0.04; (95% CI: 0.027 to 0.056)). All

pairwise Fst values were significantly different from zero ($P < 0.017$) after applying a Bonferroni correction for multiple comparisons (Table 2).

A total of 100 alleles were identified within breeding individuals and estimates of genetic diversity varied among subpopulations, with the BO subpopulation displaying the lowest mean values and BF subpopulation the highest (Table 3). Alleles common to all three subpopulations amounted to 75% of the total number, whilst private alleles were detected in all three subpopulations (BF: = 6, BO: = 2, GG: = 5).

Heterozygosity and allelic richness (Ar) were highest in BF and lowest in the BO subpopulation, however Wilcoxon rank sum tests revealed no significant differences in Ar or He between any of the subpopulation pairs when averaged over all loci and across the entire study period (BF-BO: Ar, $P = 0.97$, He, $P = 0.59$; BF-GG: Ar, $P = 0.87$, He, $P = 0.68$; BO-GG: Ar, $P = 0.89$, He, $P = 1$).

Temporal variation of spatial structure

Individual genotypes were then assigned to their appropriate annual breeding cohorts with respect to population subdivision. In doing so, individuals were therefore potentially represented in more than one annual cohort group owing to multiple reproductive seasons. Fst values were calculated annually to investigate the temporal variation in genetic structure among subpopulations resulting from the cumulative effects of recruitment and retirement (due to death or disappearance) of adults into - and out of - the breeding population. Fst values were significantly greater than zero in all years (1994, 1995, 1997 and 1998 breeding seasons were omitted due to low sample size) and ranged from 0.134 (95% CI: 0.97 to 0.171) in 2003 to 0.032 (95% CI: 0.019 to 0.038) in 2010 (Figure 5).

Pairwise comparisons of Fst among subpopulations highlighted the significant genetic variation between the subpopulations in each year (Table 4). No significant temporal trends in annual Fst estimates over the entire study period were observed. However, between 2003

(the last year of successful releases) and 2010 Fst over time showed a significant negative correlation with time (r^2 = -0.84, P < 0.01).

Pairwise Fst values between BO and GG subpopulations were consistently greater than zero between each breeding cohort with the exception of 1996. Variation between BF and GG subpopulations was significant in three consecutive years during the period 2007 to 2009. Significant genetic differentiation was identified between BF and BO subpopulations in the years 2002, 2003, 2006, 2007 and 2009. Significant variation among all three subpopulations was revealed for 2007 and 2009. Figure 6 illustrates the variation in pairwise Fst differentiation among the subpopulations over the entire study period.

3.4.3 Subpopulation level analyses

Temporal analyses within subpopulations

Temporal genetic variation was investigated for each subpopulation separately by grouping genotypes into their respective subpopulations. Temporal Fst values indicated no differentiation among years in any of the three subpopulations; BF = -0.007 (95% CI: -0.003 to -0.001); BO = -0.0015 (95% CI: -0.007 to -0.0024) and GG = -0.010 (95% CI: -0.006 to -0.015). No significant pairwise Fst values were found among years in any subpopulation after Bonferroni corrections for multiple comparisons (P > 0.000476).

Indices of genetic diversity were calculated for each subpopulation per breeding cohort (Table 5). No significant variation was found for either of the diversity indices in any of the subpopulations (Table 6). However, mean allelic richness decreased over time in both BF (9.5% 1995-2010) and GG (7.5% 1997-2010) subpopulations, and increased in the BO subpopulation (23% 2003-2010). Linear regression analyses revealed a significant temporal trend in diversity estimates across the study period within BF (He: r^2 = -.0.36, P < 0.05, Ar: r^2 = -0.58, P < 0.001) and GG (He: r^2 = -0.11, P = 0.1, Ar: r^2 = -0.23, P < 0.05). Similar patterns were not reflected in the BO subpopulation over the entire study period (He: r^2 =

0.12, P = 0.11, Ar: r^2 = 0.11, P = 0.12), however both estimates of diversity were strongly positively correlated with time in this subpopulation between 2002 and 2010 (He: r^2 = 0.92, P < 0.001, Ar: r^2 = 0.88, P < 0.001).

Variation among subpopulations within cohorts

Figure 7 and Table 6 illustrate the variation in average diversity estimates among the three subpopulations. The BF subpopulation displayed higher mean values than the other subpopulations in most years with BO subpopulation displaying the lowest values. Wilcoxon rank sum tests to investigate variation in diversity estimates among subpopulations within each cohort revealed significant differences between BO and BF in; 1999 (Ar: P = 0.016, He: P < 0.01), 2000 (Ar: P = 0.011, He: P = 0.019), 2001 (He: P = 0.02), 2002 (Ar: P = 0.012, He: P < 0.001) and 2003 (P = 0.049), and between BO and GG in 2003 (P = 0.02). No significant differences in Ar between BF and GG were found in any cohort (all P values > 0.1908). Between 1999 and 2003 there were consistent differences between BO and BF in one or both measures of diversity.

3.4.4 Effects of population size

The total free-living population on Mauritius has increased in size over the study period; the number of females known to lay eggs has increased from three in 1993 to approximately 80 in 2011 representing a significant positive trend in growth over time (r^2 = 0.95, P < 0.001). We therefore examined whether the increase in population size was a contributing factor in the observed temporal patterns of genetic diversity. Local subpopulation size was significantly and negatively correlated with mean annual Ar and He in both BF (Ar: r^2 = -0.55, P < 0.001, He: r^2 = -0.62, P < 0.001) and GG (Ar: r^2 = -0.36, P < 0.05, He: r^2 = -0.21, P < 0.05) subpopulations and positively correlated within the BO subpopulation (Ar: r^2 = 0.21, P < 0.05, He: r^2 = 0.22, P < 0.05).

3.4.5 Parental relatedness

Figure 8 illustrates subpopulation level internal relatedness (IR). Average annual IR was highest in BO (mean = 0.11) and was significantly greater than BF (mean = 0.07, $P < 0.01$) and GG (mean = 0.02, $P < 0.001$) subpopulations. The GG subpopulation showed the lowest level of IR, significantly lower than in BF ($P < 0.001$). At the global scale IR did not show any significant temporal trend ($r^2 = 0.003$, $P > 0.06$). However, significant temporal trends were apparent in all three subpopulations; IR decreased over time in both BF ($r^2 = -0.58$, $P < 0.01$) and BO ($r^2 = -0.31$, $P < 0.05$) subpopulations, and increased in the GG subpopulation ($r^2 = 0.65$, $P < 0.001$). Significant temporal trends were also found when males and females were analysed separately (see Figure 9). A strong positive correlation was found between female IR and year ($r^2 = 0.82$, $P < 0.0001$). Among males, the trend was weak but significantly negative ($r^2 = 0.01$, $P = 0.01$).

3.4.6 Fledgling recruitment

Measures of genetic diversity were investigated as predictors of recruitment probability for fledglings produced between 1999 and 2007 (those produced after that time were considered to have not yet been detected as attempting to breed). Average annual diversity estimates were not significantly different between recruited and non-recruited individuals at the global scale (Wilcoxon rank sum; Ar: $P = 0.7$, He: $P = 0.83$). Recruitment was also investigated within separate cohorts and subpopulations of fledglings. No significant differences in annual mean diversity measures were found between recruited and non-recruited individuals from any year (Wilcoxon rank sum; all P values > 0.52) or within any subpopulation (all P values > 0.38).

3.5 Discussion

3.5.1 Global patterns of spatio-temporal variation

As expected, no significant variation in temporal Fst estimates was found among breeding cohorts at the global scale. A small founder population size as a consequence of the species'

severe bottleneck, individual longevity, multiple reproductive seasons and a relatively short study period are all likely to have contributed to the observed lack of temporal genetic structure. Allelic richness and expected heterozygosity were averaged over loci and did not significantly vary over time according to Kruskal-Wallis ANOVA; however linear regression of both indices of genetic diversity over time indicated a significant declining temporal trend during the study period. This disparity is a possible consequence of using diversity estimates averaged across variable loci resulting in large deviation about the mean. Averaging diversity indices over loci is not ideal (Devillard et al., 2011) and small sample sizes will reduce statistical power, however this is a restriction common to studies involving threatened populations consisting of few individuals. The estimated decline in global annual allelic richness and expected heterozygosity (averaged over all loci and breeding individuals) since 1995 was 9.5% and 6.5% respectively. Many studies report far greater loss of genetic diversity in avian species, for example; expected heterozygosity fell by 39% over 100 years in Galapagos finches (Hoeck et al., 2010) and 22% over 50 years in Greater Prairie Chickens (Bellinger et al., 2003). The reduction found within this study is comparatively small, but nonetheless remains a concern in view of the relatively short study period and is similar to that reported from a translocated population of Takahe (7.5% in 15 years), a species with a comparable generation time and over a similar sampling duration (Grueber and Jamieson, 2008).

The significant decrease in Fst over time since 2003 is most likely attributable to two cohorts of individuals sourced from captive and wild stock and released at the BO subpopulation between 2002 and 2003; out of 53 individuals released 17 were recruited. Not all of these individuals were recruited in the same year however, which may explain the observed gradual decline in Fst. Despite this homogenisation, significant genetic differentiation was still detected among all three subpopulations as recently as 2009, indicating that genetic differentiation has not been erased entirely by the homogenising effects of conservation management. Not surprisingly, the most isolated subpopulation (BO) shows the greatest

extent of differentiation, supporting observational evidence that little or no dispersal has occurred into this area. This subpopulation is also the smallest and as predicted displays the lowest diversity of the three subpopulations compared to the largest subpopulation (BF) which contains the highest level of diversity. Pairwise comparisons indicate that the two most differentiated subpopulations are BO and GG, further evidence suggesting that little or no migration has occurred between them.

3.5.2 Local patterns of temporal variation and effects of population size

Despite an increase in population size in all three subpopulations, subpopulation level analyses reveal an overall decreasing trend over time in genetic diversity estimates within the two largest subpopulations (BF and GG) versus an upward trend within BO (especially since 2002). The reduction in diversity estimates in the two larger subpopulations is indicative of the effects of genetic drift. The considerable increase in diversity estimates observed in BO is most likely to be a combined result of (i) relatively recent releases (49 individuals released between 2002 and 2003) bringing novel alleles into the population, and (ii) the sampling of fledglings hatched to a small number of adult males who have never been caught or recorded as successfully breeding. This latter contributory factor is supported by the genetic data; alleles previously unseen in any of the subpopulations were detected for the first time in 2010/11 season in two broods within the BO subpopulation.

A consistent and statistically significant difference in estimates of genetic diversity between the two subpopulations BO and BF was found during the period 1999 to 2003. This period coincides with the most intensive period of management; the effects of management appear to have reduced this difference after 2003 as juveniles which were redistributed among the populations as part of the captive breeding and release programme were recruited into the breeding population. It is likely that this difference was also prevalent before 1999, however small sample sizes limited the power to test for its presence.

3.5.3 Parental relatedness

Average parental relatedness was significantly higher in BO and BF than in the GG subpopulation. This result is also reflected in the considerable differences among subpopulation level inbreeding as indicated by the Fis values (Table 5). Taken together, one possible explanation for these findings is the increased density of pairs over time as a result of provision of artificial nest boxes that have been used as nest sites by breeding pairs in BF and BO subpopulations in close proximity to supplemental feeding stations (feeding stations are not present in the GG subpopulation). The increase in relatedness among GG individuals since 2003 is likely due to a combination of a lack of appropriate cavities in which to nest and a one-way direction of gene flow involving the translocation and release of individuals during intensive management. Managed releases were carried out at only two of the three main subpopulations, BO and BF; comprising individuals that were often rescued as malnourished chicks from wild nests. This management appears to have resulted in a net effect of removing genetic material from GG, a subpopulation which although not geographically isolated appears to be genetically fragmented.

The differences in temporal trends between the sexes suggest that females are the more philopatric sex. Choice of appropriate nest sites is limited due to a lack of mature, cavity forming trees, a limitation which is believed to have been one of the major factors that affected population growth before pairs started to accept artificial nest boxes (Jones and Merton, 2012).

3.5.4 Fledgling recruitment

We found no evidence to suggest that recruited fledglings have on average higher genetic diversity indices than non-recruits, a result that appears to indicate that post-fledging survival is not predicted by genetic diversity. If our set of neutral genetic markers is a satisfactory predictor of genome-wide heterozygosity, then there appears to be no correlation between multi-locus heterozygosity and recruitment as has been found in other studies (e.g.

Hansson et al., 2001). However, individuals which died in the nest before fledging (or eggs which failed to hatch) were not sampled, which may present a bias in these results.

3.5.5 Recommendations for future management

Our study has prompted several key recommendations concerning the long term genetic management of the Mauritius parakeet that are likely to promote the evolutionary viability of this species. Many of the management recommendations we advocate here may apply equally to other similarly managed species.

(a) Dispersal of individuals among population fragments is to be encouraged, in order to prevent further loss of genetic diversity. This benefit can be achieved via the management technique of translocation of individuals between sites, which also has the effect of homogenising genetic differences between subpopulations and reducing population genetic structure. However, encouraging natural dispersal should be of prime concern and is fundamental if this species is to sustain viability into the future.

(b) For a species such as the Mauritius parakeet, where natural nest sites are a limiting factor to population growth (Jones, 1987), the provision of artificial alternatives gives conservation managers a valuable and dual purpose tool. With a meticulous approach to nest site selection managers have the ability to redress or prevent the negative genetic effects of inbreeding and population fragmentation by encouraging dispersal.

(c) The provision of artificial nest sites and supplemental food are vital to the success of many avian reintroduction programmes and can facilitate the collection of detailed and valuable post-release monitoring data such as that obtained for this study. However, where provision of artificial nests and supplemental food is used simultaneously, consideration must be given to the effects at those locations of an increased density of breeding pairs. Reducing spatial associations among related individuals should be encouraged wherever possible, particularly for long-lived and monogamous species.

3.6 Conclusions

Rapidly declining species often require intervention that demands an immediate focus on boosting numbers of individuals where considerations of genetic factors are of secondary importance to the more immediate threat of extinction caused by habitat destruction or introduced predators. Post-recovery genetic analysis of such species recoveries is often concerned with quantifying the loss of genetic diversity of species at the global level as a result of population bottlenecks, which risks neglecting the presence of fine-scale genetic structure within recovered populations. The long term viability of such populations depends upon the presence of evolutionary mechanisms to maintain and generate genetic diversity highlighting the importance of long term strategies to minimize inbreeding (Jamieson, 2011). By using a fine-scale approach to examine post-recovery spatio-temporal trends in genetic diversity, we have identified the existence of within-population genetic structure indicating a lack of gene flow among isolated subpopulations risking further loss of diversity and therefore, evolutionary potential. Conservation managers are becoming increasingly aware of the need for standardised long term monitoring strategies if conservation reintroductions are to be successful (Sutherland et al., 2010). Our study represents a valuable step forward in this respect and provides evidence to support the assertion that dispersal in restored and fragmented populations has a major influence over levels of inbreeding in wild populations and should be encouraged (Szulkin and Sheldon, 2008).

3.7 Figures and tables

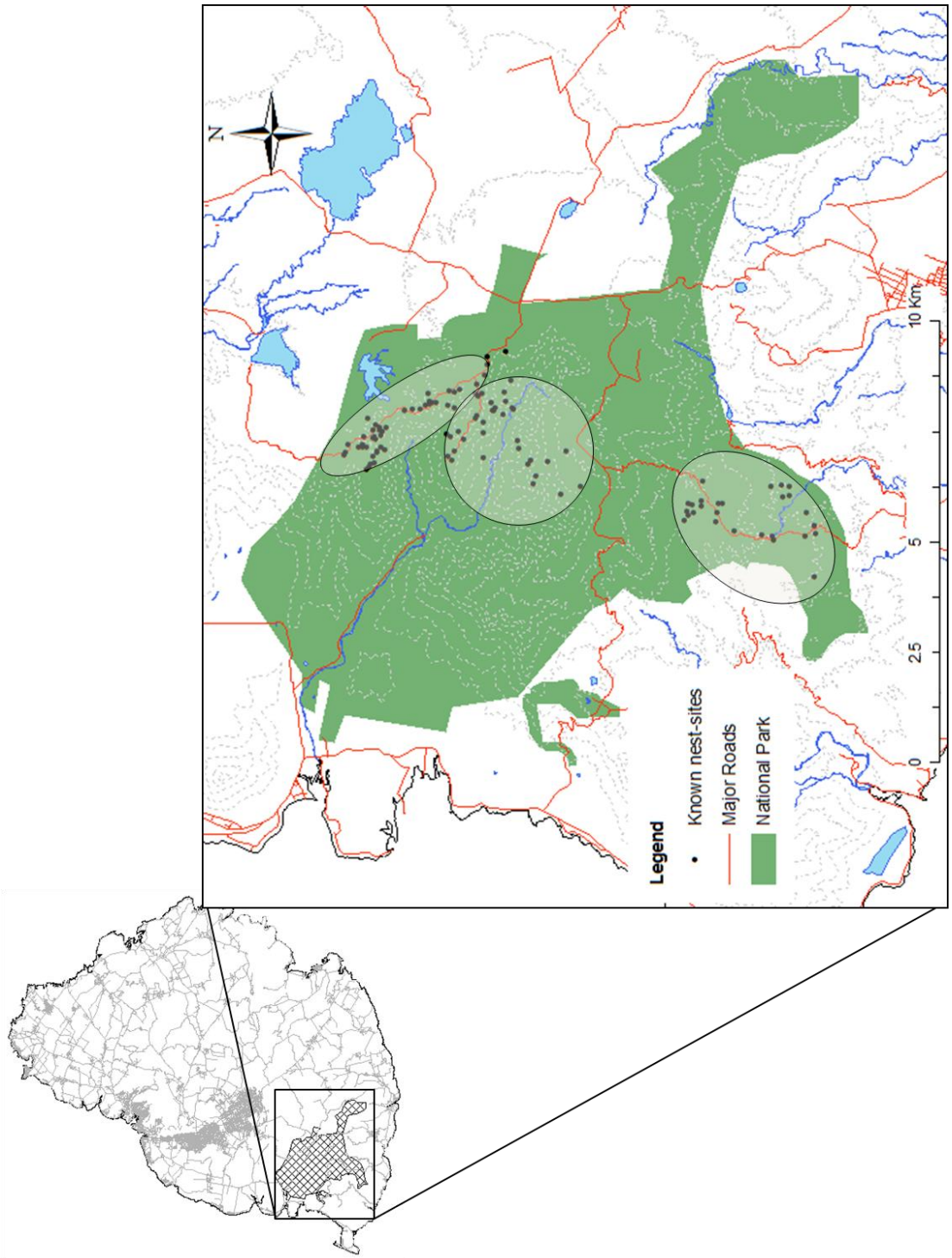

Figure 1: The Black River Gorge National Park and the three putative subpopulations of Mauritius parakeet (from north to south; Brise Fer [BF], Grande Gorge [GG] and Bel Ombre [BO]). Inset: location of the national park in the south-west of Mauritius.

Figure 2: Growth of the Mauritius parakeet population during the course of its managed recovery. N, number of known/monitored breeding pairs (filled circles), number of fledglings produced (open circles) and number of individuals released as part of the conservation programme (shaded bars).

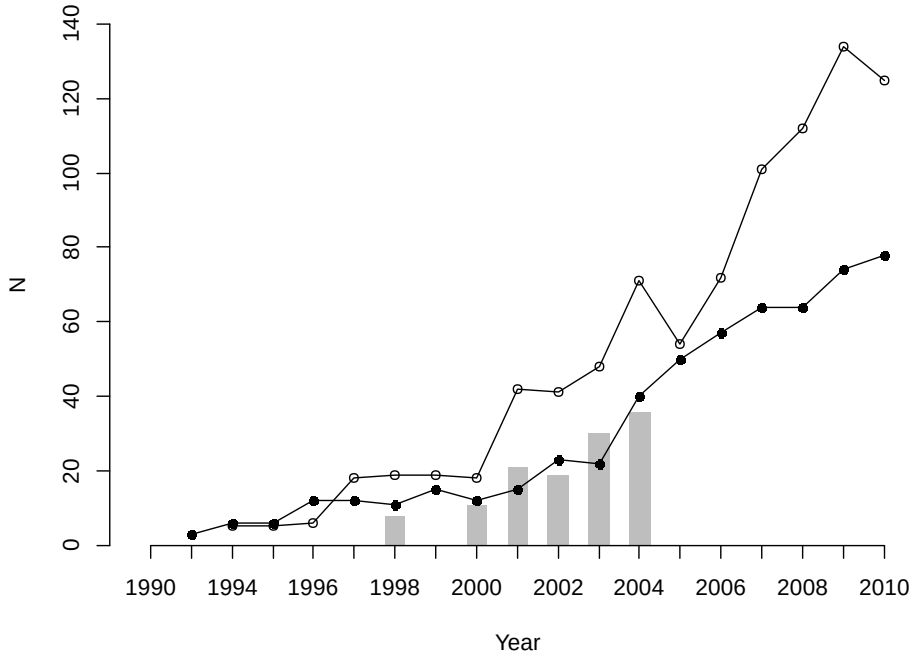

Figure 3: Turnover in breeding population of the Mauritius parakeet based upon number of available genotypes per year. Grey bars indicate total number of breeding individuals per year. Black bars indicate number of newly recruited individuals. The number of individuals retiring from the breeding population in each year is shown at the top of the bars.

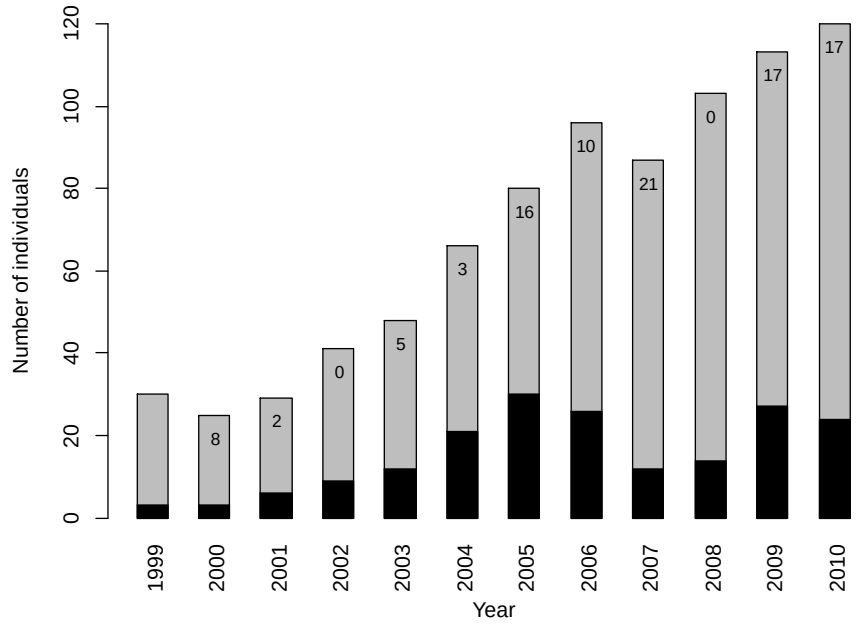

Figure 4: Annual mean expected heterozygosity (open circles) and allelic richness (solid circles) of breeding adults within the Mauritius parakeet population and temporal trends over the study period (dashed, expected heterozygosity; solid, allelic richness).

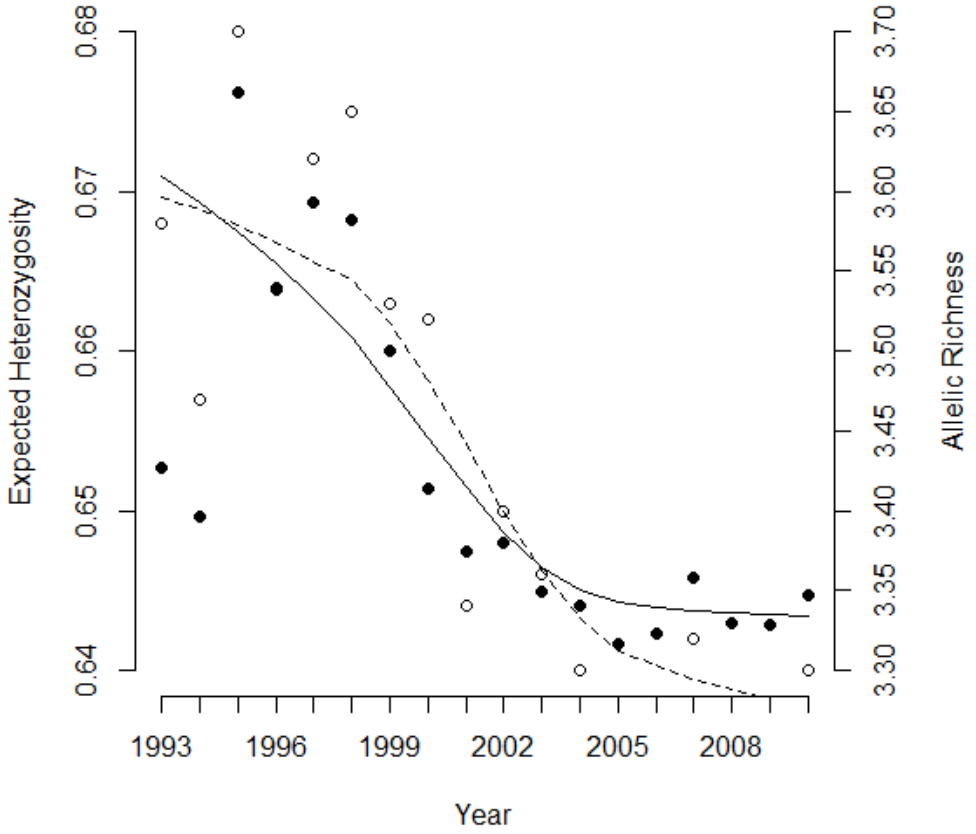

Figure 5: Annual global Fst estimates for the Mauritius parakeet population from 1996-2010. Estimates for 1997 and 1998 were not calculated due to low sample sizes.

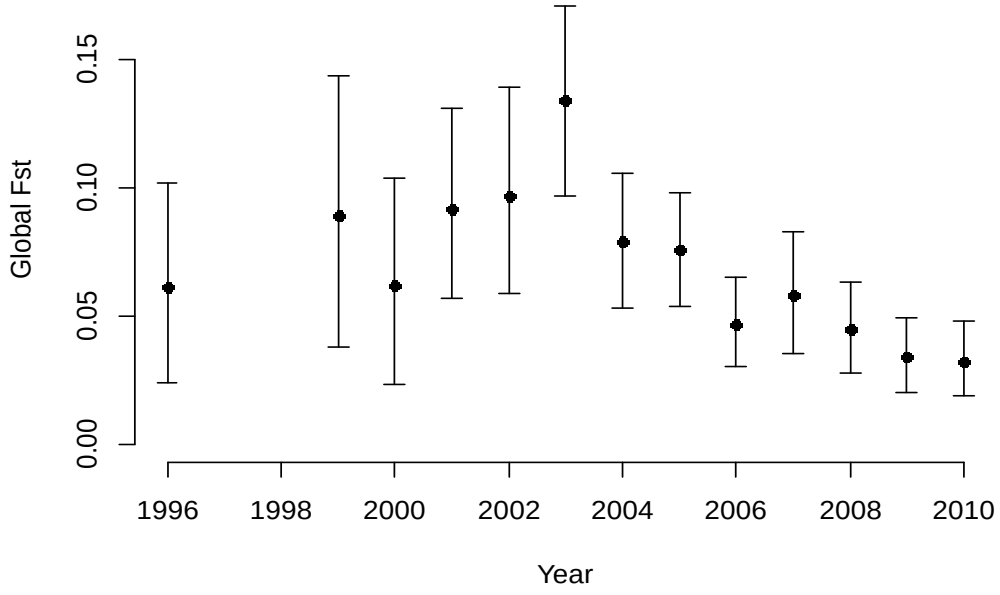

Figure 6: Pairwise Fst estimates illustrating changes in genetic differentiation between pairs of subpopulations over time. (BF:Brise Fer, BO: Bel Ombre, GG: Grande Gorge).

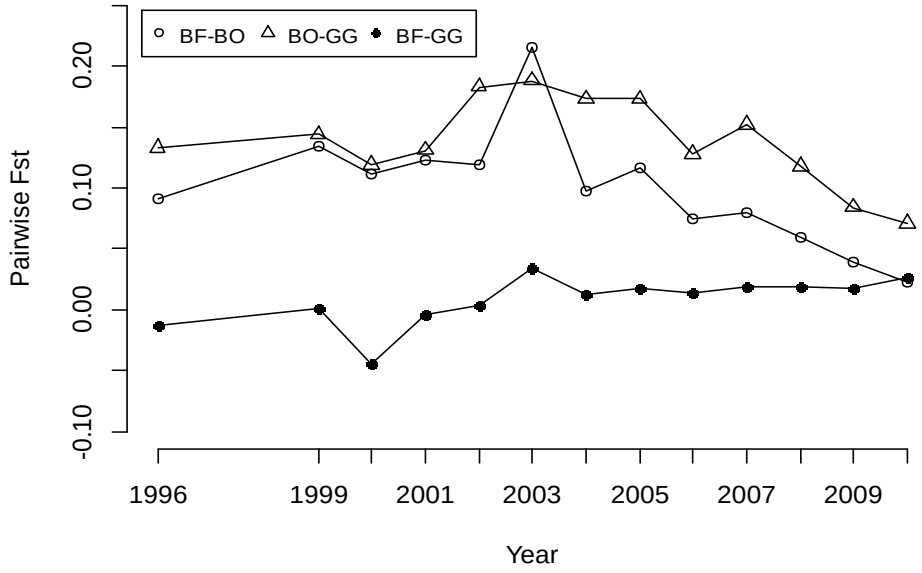

Figure 7: Temporal trends in annual average allelic richness and expected heterozygosity (He) for each of the three subpopulations, Brise Fer (BF), Bel Ombre (BO) and Grande Gorge (GG).

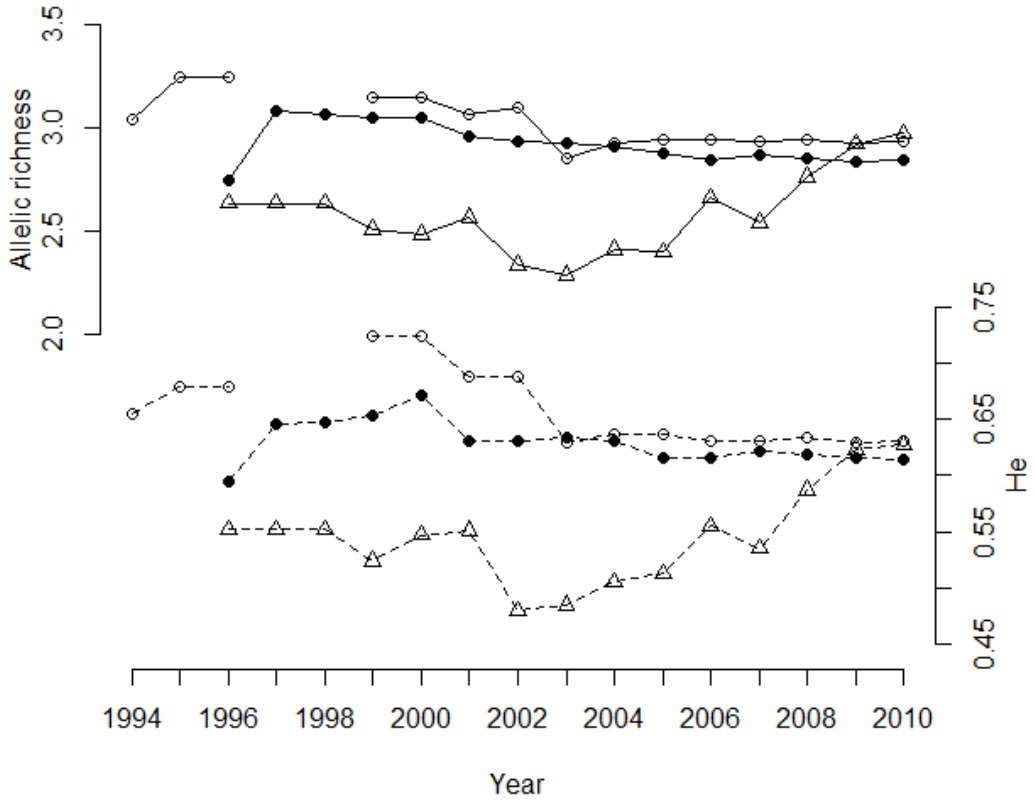

Figure 8: Temporal trend in internal relatedness (IR) for the three subpopulations, Brise Fer (BO), Bel Ombre (BO), and Grande Gorge (GG).

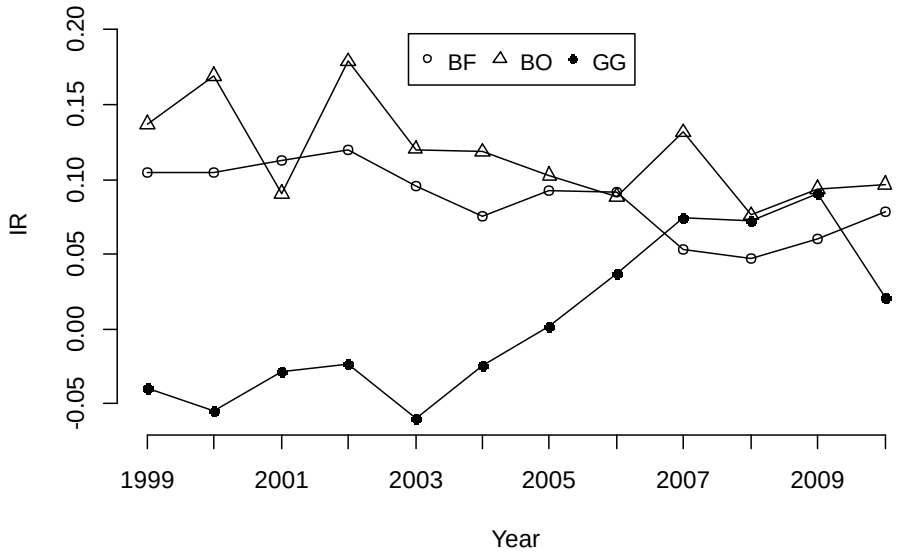

Figure 9: Annual mean internal relatedness (IR) for males (filled circles) and females (open circles) of breeding adults within the recovered Mauritius parakeet population. Bars represent standard error.

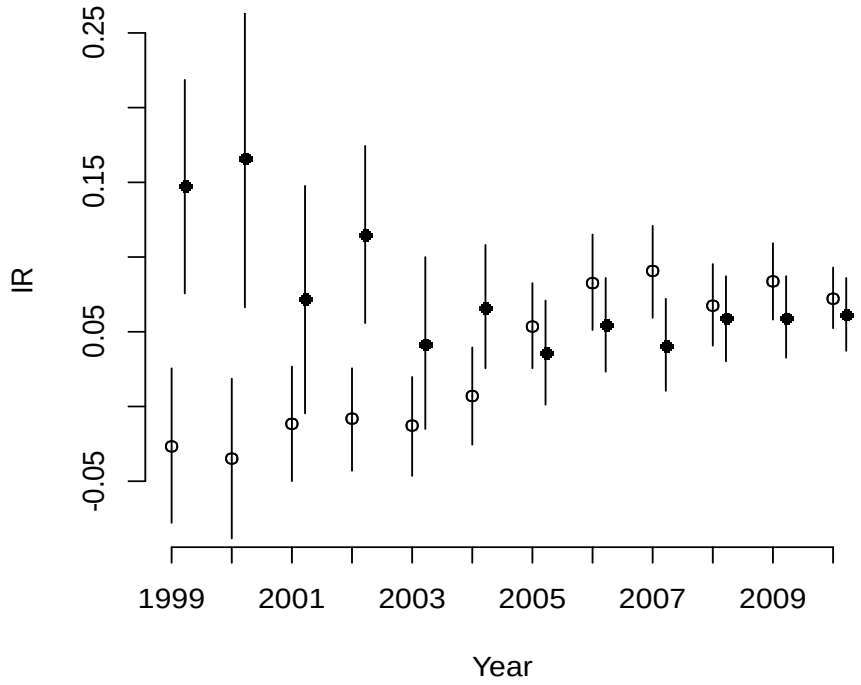

Table 1 Indices of genetic diversity and sample size for the global breeding population per cohort for the entire study period including number of alleles (Na), number of individuals sampled (N), expected heterozygosity (He), allelic richness (Ar) and estimate of fixation index (Fis).

Year	Na	N	He	Ar	Fis
1993	62	5	0.668	3.427	0.141
1994	67	9	0.657	3.396	0.044
1995	77	9	0.680	3.662	0.088
1996	79	16	0.664	3.538	0.040
1997	81	15	0.672	3.593	0.030
1998	81	16	0.675	3.582	0.069
1999	81	19	0.663	3.500	0.052
2000	75	16	0.662	3.414	0.052
2001	75	25	0.644	3.374	0.021
2002	77	32	0.650	3.380	0.042
2003	77	37	0.646	3.349	0.012
2004	81	51	0.640	3.340	0.029
2005	82	68	0.636	3.317	0.051
2006	88	80	0.636	3.323	0.067
2007	90	76	0.642	3.358	0.073
2008	87	91	0.638	3.330	0.059
2009	87	102	0.638	3.329	0.067
2010	90	108	0.640	3.347	0.065

Table 2 Spatial variation among subpopulations; Brise Fer (BF) Bel Ombre (BO) and Grande Gorge (GG). Fst values are below diagonal. Above diagonal are significant P values.

	BF	BO	GG
BF		**0.0167**	**0.0167**
BO	0.046		**0.0167**
GG	0.015	0.092	

Table 3 Summary of subpopulation diversity indices; number of alleles (Na), number of individuals (N), expected heterozygosity (He) and allelic richness (Ar). Subpopulations; Brise Fer (BF), Bel Ombre (BO) and Grande Gorge (GG).

	Na	N	He	Ar
BF	91	94	0.648	4.829
BO	83	38	0.617	4.727
GG	87	53	0.626	4.788

Table 4 Fst estimates between subpopulation pairs per cohort; Brise Fer (BF), Bel Ombre (BO) and Grande Gorge (GG). Pairwise values indicating significant genetic variation ($P < 0.01667$) are in bold.

Year	BF-BO	BF-GG	BO-GG
1996	0.091	-0.013	0.133
1999	0.134	0.001	**0.144**
2000	0.111	-0.044	**0.119**
2001	0.123	-0.004	**0.131**
2002	**0.119**	0.004	**0.183**
2003	**0.215**	0.034	**0.188**
2004	0.098	0.013	**0.173**
2005	0.116	0.017	**0.173**
2006	**0.075**	0.014	**0.128**
2007	**0.080**	**0.019**	**0.152**
2008	0.059	**0.019**	**0.118**
2009	**0.039**	**0.018**	**0.084**
2010	0.023	0.027	**0.071**

Table 5 Summary of number of individuals sampled (N), expected heterozygosity (He), allelic richness (Ar) and estimate of fixation index (Fis), for each subpopulation Brise Fer (BF), Bel Ombre (BO) and Grande Gorge (GG) within breeding cohorts.

Subpopulation	BF				BO				GG			
Year	N	He	Ar	Fis	N	He	Ar	Fis	N	He	Ar	Fis
1994	5	0.66	3.04	-0.05	0	NA	NA	NA	4	NA	NA	NA
1995	5	0.68	3.25	0.01	2	NA	NA	NA	2	NA	NA	NA
1996	5	0.68	3.25	0.01	4	0.55	2.64	-0.04	7	0.59	2.75	0.05
1997	0	NA	NA	NA	4	0.55	2.64	-0.04	11	0.65	3.08	-0.05
1998	1	NA	NA	NA	4	0.55	2.64	-0.04	11	0.65	3.06	-0.02
1999	4	0.72	3.15	0.14	5	0.52	2.51	-0.07	10	0.65	3.05	-0.03
2000	4	0.72	3.15	0.14	5	0.55	2.49	0.02	7	0.67	3.05	-0.05
2001	4	0.69	3.07	0.11	8	0.55	2.57	-0.07	13	0.63	2.96	-0.06
2002	7	0.69	3.10	0.09	6	0.48	2.34	-0.09	19	0.63	2.93	-0.05
2003	7	0.63	2.86	0.05	9	0.48	2.29	-0.16	21	0.63	2.93	-0.08
2004	18	0.64	2.93	0.07	11	0.51	2.41	-0.12	22	0.63	2.91	-0.05
2005	31	0.64	2.94	0.09	13	0.51	2.40	-0.12	24	0.62	2.88	-0.03
2006	42	0.63	2.94	0.08	11	0.56	2.66	-0.05	27	0.62	2.85	0.00
2007	39	0.63	2.93	0.05	12	0.53	2.54	-0.04	25	0.62	2.87	0.05
2008	51	0.63	2.95	0.03	13	0.59	2.77	-0.03	27	0.62	2.86	0.04
2009	60	0.63	2.93	0.04	15	0.62	2.92	0.05	27	0.62	2.84	0.04
2010	63	0.63	2.93	0.07	21	0.63	2.97	0.07	24	0.61	2.85	-0.02

Table 6 Summary of temporal variation of genetic diversity indices within subpopulations over the study period. Brise Fer (BF), Bel Ombre (BO) and Grande Gorge (GG).

	Ar		He	
Subpopulation	Range	(χ^2, d.f. = 14) P	Range	(χ^2, d.f. = 14) P
BF	2.857 - 3.246	(5.56) 0.97	0.629 - 0.688	(13.31) 0.50
BO	2.289 - 2.974	(16.65) 0.27	0.480 - 0.627	(13.18) 0.51
GG	2.750 - 3.083	(3.47) 0.99	0.594 - 0.671	(3.16) 0.99

Table S1: Estimates of genetic differentiation between subpopulations using two different methods; Jost's D, and Fstat. Subpopulations are identified as Brise Fer (BF), Bel Ombre (BO), and Grande Gorge GG).

Year	Jost's D	Fst	BF-BO Jost	BF-BO Fst	BF-GG Jost	BF-GG Fst	BO-GG Jost	BO-GG Fst
1996	0.07	0.06	0.06	0.09	0.00	-0.01	0.12	0.13
1999	0.08	0.09	0.13	0.13	0.00	0.00	0.11	0.14
2000	0.05	0.06	0.11	0.11	-0.03	-0.04	0.10	0.12
2001	0.09	0.09	0.12	0.12	0.00	0.00	0.14	0.13
2002	0.12	0.10	0.12	0.12	0.00	0.00	0.21	0.18
2003	0.17	0.13	0.20	0.22	0.02	0.03	0.24	0.19
2004	0.12	0.08	0.10	0.10	0.00	0.01	0.22	0.17
2005	0.14	0.08	0.14	0.12	0.01	0.02	0.21	0.17
2006	0.09	0.05	0.09	0.07	0.01	0.01	0.16	0.13
2007	0.10	0.06	0.08	0.08	0.01	0.02	0.17	0.15
2008	0.09	0.05	0.07	0.06	0.01	0.02	0.16	0.12
2009	0.06	0.03	0.04	0.04	0.01	0.02	0.10	0.08
2010	0.05	0.03	0.02	0.02	0.02	0.03	0.08	0.07

Chapter 4. Effects of contrasting evolutionary histories on genetic variation and immune function in birds: a case-study using endemic and invasive parakeets on Mauritius

Abstract

Island populations face depauperate parasite communities in comparison to continental systems and host immune function variables are predicted to reflect this contrasting pathogen pressure. Inbreeding and reduced genetic diversity are common characteristics of endemic island species and can result in an increased susceptibility to novel parasites and pathogens. Two parakeet species occur on Mauritius; an endangered island endemic (*Psittacula echo*) and an introduced, invasive and continentally evolved congeneric species (*P. krameri*). I use this system to investigate the relationships between individual-level genetic diversity and variables associated with immune function between nestlings of two species reflecting contrasting evolutionary histories but similar recent population bottlenecks. Individual-level multilocus heterozygosity was similar for both species but *P. krameri* individuals exhibited increased cellular and humoral immunocompetence. Estimates of genetic diversity were not associated with individual-level variables of immune function among *P. echo* but multilocus heterozygosity was positively associated with the magnitude of PHA response among *P. krameri*. Nestlings of the endemic and endangered island species reveal an attenuated immune system when compared to a sympatrically occurring congeneric species but genetic diversity measured by neutral markers is comparable.

4.1 Introduction

Maintaining an appropriate and effective immune system is vital to maximise an individual's fitness but the elimination, avoidance or regulation of parasites and pathogens is associated with life history trade-offs and costs (Sheldon and Verhulst, 1996, Owens and Wilson, 1999, Lochmiller and Deerenberg, 2000, Norris and Evans, 2000, Lee, 2006). Investigations of vertebrate immune function among free-living individuals are critical to understanding the

extent to which genetic, environmental and life history factors influence immune systems and can play a vital role in the conservation of endangered species and populations.

As a consequence of a vast range of biotic and abiotic factors, host defences are driven by selection and determined by the contemporary and evolutionary interactions between exposure to diverse parasite and pathogen communities and a variety of other ecological, genetic and environmental factors associated with species' life history traits (Schulenburg et al., 2009, Horrocks et al., 2011) leading to substantial variation in immune function systems both within and among species (Lee, 2006, Matson et al., 2006). This variation is becoming increasingly apparent as the effects of emerging infectious diseases (EIDs) on human health and biodiversity continue to cause global concern (Daszak et al., 2000, Jones et al., 2008). The diversity of infectious diseases, parasites and pathogens is subject to the generally observed patterns of species richness attributed to area and latitude. In other words, island host species can expect to face a depauperate parasite (used here in a broad sense to include infectious agents, viruses and bacteria) community in comparison to those on continental land masses and diversity should decrease with increasing latitude (Piersma, 1997, Møller, 1998, Ricklefs and Wikelski, 2002, Matson and Beadell, 2010).

Despite the expectation of relatively reduced parasite pressure on islands, endemic island species generally display increased extinction risk compared to continental species (Pimm et al., 1995, Purvis et al., 2000) due to a suite of factors associated with evolutionary mechanisms in these arguably simplified ecosystems. Reduced species richness and higher levels of endemism on islands compared to continental land masses (MacArthur and Wilson, 1967) means that island endemics are more likely to have evolved in the absence of predators and competitors, making them ill-equipped to tolerate introduced species (Blackburn et al., 2004). Additionally, relatively stable environmental conditions have resulted in specialised species which have evolved to exist in very narrow ecological niches and are therefore sensitive to habitat transformation (Simberloff, 1995) and other human-mediated changes (Fordham and Brook, 2010). Furthermore, island species are prone to the

negative stochastic effects associated with small population size (Lande, 1988), they display lower genetic diversity than phylogenetically similar mainland counterparts (Frankham, 1998, Spielman et al., 2004b), leading to an increased risk of inbreeding (Frankham, 1997).

Together, inbreeding and loss of genetic diversity is predicted to increase disease susceptibility in natural populations (Acevedo-Whitehouse et al., 2003, Spielman et al., 2004a, Hawley et al., 2005, Hale and Briskie, 2007b). Therefore the introduction of novel parasites to an island system which, by definition, is likely to already be sensitive to sudden changes has been demonstrated to negatively impact upon endemic host species which have evolved in isolation of such parasites and are immunologically naive as a result (Wikelski et al., 2004). Avian systems have been studied extensively in this context and examples of this phenomenon in birds endemic to island systems include the archipelagos of Hawaii (Warner, 1968, Atkinson et al., 1995) and the Mascarene Islands in the Indian Ocean (Swinnerton et al., 2005a, Bunbury et al., 2008, Jones and Merton, 2012). Contrastingly, continentally-evolved species which constitute much larger population sizes are exposed to a greater variety of infectious diseases and are more likely to occupy a diverse array of habitats covering a broader latitudinal range.

Alongside these different evolutionary genetic influences on host susceptibility across continental-island and latitudinal gradients, a species' pace of life is thought to predict, along with a variety of other life history variables, investment in immune function (Ricklefs and Wikelski, 2002). Avian species' pace of life can be roughly predicted by latitudinal occurrence; those which occur close to the equator generally display a slower pace of life (Wikelski et al., 2003, Wiersma et al., 2007) characterised by smaller clutch sizes (Lack, 1947, Dunn et al., 2000), fewer clutches per year, slower growth rates (Ricklefs, 1968, McCarty, 2001), longer lifespan and greater parental investment in rearing young (Martin, 1996, McNamara et al., 2008). Theory predicts that species which lead a slow pace of life may display a 'reorganised' immune system characterised by greater investment in specific and humoral-mediated immune defences as opposed to non-specific and cellular responses

(Tieleman et al., 2005, Lee, 2006, Hasselquist, 2007) owing to the probability of repeated and frequent exposure to parasites during a long lifetime with many breeding attempts. Conversely, temperately occurring species characterised by a shorter lifespan and fewer highly productive breeding attempts should invest more in less costly innate, non-specific and inflammatory immune strategies in order to maximise lifetime reproductive effort (Lee, 2006, Hasselquist, 2007). The implication that tropical species lead a more "leisurely lifestyle" (Hamilton, 1966, Steiger et al., 2009) is reflected by the contrasting patterns in environmental fluctuation, seasonal variation and temperature extremes between tropical and temperate regions. Tropical species encounter very little variation in temperature and slight seasonal fluctuations when compared to temperate species which must adapt to distinct seasonal cycles or invest vast amounts of energy into migration (Wikelski et al., 2003).

These predicted patterns of genetic diversity, parasite community diversity and immune function evolution result in a conundrum when trying to position within this framework avian species endemic to tropical oceanic islands. Are they naive hosts in a pathogen poor community as a result of isolation and size, or have they evolved within a pathogen diverse habitat as predicted by latitude? Furthermore, given their insular history of evolution, how well equipped are they immunologically and how do they compare to a mainland congeneric? Lastly, what role does genetic diversity and inbreeding play in the immune function of individuals within an endemic island species and how does this compare to continental species? Many of these questions are emerging as increasingly important within conservation biology and in particular for the restoration of endangered populations of island endemic species, many of which are facing the challenges of emerging infectious disease.

Island environments are amongst the most exposed systems to invasive species (Mooney and Cleland, 2001), the majority of which, often dispersed as a result of recent human intervention, have evolved on continental systems. Consequently, an island endemic species and a recently introduced phylogenetically similar and continentally-evolved invasive species can sometimes be present on the same island, providing a valuable comparative

opportunity to explore differences in genetic and immunological relationships. Successful establishment of introduced invasive avian species from continents is often attributed to 'enemy release'; whereby a species finds itself free from the suite of parasites and predators it has evolved to co-exist with (Keane and Crawley, 2002, Torchin et al., 2003). The Mascarene archipelago is a particularly clear example of how vulnerable island ecosystems are to introduced species (Cheke and Diamond, 1987, Simberloff, 1992, Jones et al., 1996, Cheke and Hume, 2009) with no fewer than 10 avian extinctions since human colonisation alongside the arrival of 19 naturalised exotic bird species on Mauritius alone (Cheke and Hume, 2009), most of which represent direct competition with the remaining endemic avifauna and are considered as reservoirs of a variety of parasites and pathogens. Psittacine beak and feather disease (PBFD) (Greenwood, 1996), trichomoniasis (Swinnerton et al., 2005a), avian pox (Swinnerton et al., 2005b) and leukocytozoon (Bunbury et al., 2007) have all been considered to be closely associated with or transmitted by invasive bird species on Mauritius. These potentially novel parasites continue to limit population growth and long term viability of several reintroduced populations of endemic Mauritius birds.

One of the most recent avian introductions to Mauritius has been the Indian ringneck (or rose-ringed) parakeet; *Psittacula krameri.* Two sub-species of *P. krameri* (*P. k. borealis* and *P. k. manillensis*) are native to the Indian sub-continent and at least one of these sub-species was introduced to Mauritius in the 1880s via the pet trade (Cheke and Hume, 2009). The current population is estimated to be in excess of 10,000 individuals and is thought to constitute both Indian sub-species (C. Jones, pers. comm.). This continentally-evolved invasive species exists sympatrically with its congeneric relative, the endemic Mauritius (or echo) parakeet *P. echo,* the last remaining native Psittaciforme in the Mascarenes. This endangered species is found only in the few remaining pockets of native vegetation with a current population of around 500 individuals (MWF, 1996-2012) following a successful recovery from fewer than 20 in the 1980s when the species was considered to be the world's rarest parrot (Jones, 1987, Jones and Duffy, 1993). Direct competition exists between the

two species for suitable nest sites (Jones, 1987) and it has been suggested that *P. krameri* may be responsible for the introduction of PBFD (Kundu et al., 2012), a major factor contributing to the observed high juvenile mortality among *P. echo* (Jones and Merton, 2012). Today the endemic species is the subject of an intensive conservation programme directed by the Mauritian Wildlife Foundation (MWF) and supported by the Government of Mauritius.

These two parakeet species, which diverged during the Pliocene period around 2-6 million years ago (Groombridge et al., 2004a, Kundu et al., 2011) have evolved to be morphologically very similar and are virtually inseparable to an inexperienced ornithologist. However, since the evolutionary split of the island form from the continental form they have adapted to very different environments; *P. echo* to a small sub-tropical oceanic island in relative environmental stability compared to *P. krameri*, accustomed to a vast subcontinent, a range of different habitat types spanning a much larger latitudinal breadth and therefore adapted to a more diverse parasite community leading to interspecies differences in optimal immune strategies between the two potential hosts (Lindström et al., 2004).

The existence of these two parakeet species on Mauritius provides the opportunity to investigate the interactions between innate immune function and genetic diversity within and between two phylogenetically matched and sympatrically occurring species. Accordingly, I test the following hypotheses:

(i) I predict that genetic variation measured by allelic richness and heterozygosity will be lower in *P. echo* than *P. krameri* at both the population and individual level owing to the effects of the documented population bottleneck and levels of inbreeding associated with the subsequent recovery and reintroduction of an endangered species (Keller et al., 2012).

(ii) If considerable variation in immune function exists and is under selection, then indices of immune function will be predicted by genetic diversity at the individual level for both species.

(iii) I predict that *P. krameri* will, as a result of adaptation to a continental (and therefore a more diverse) parasite community, demonstrate a more effective constitutive innate immune system than *P. echo* when challenged with a novel antigen.

(iv) Alternatively, if immune systems are indeed 'reorganised' among island hosts as suggested by Matson (2006) and Whiteman et al., (2006) then we might expect to see *P. echo* display a more effective cellular or humoral immune function component when compared to *P. krameri*. Matson's (2006) evidence points to an up-regulation in innate and inducible over acquired immunity among island bird species which may be a result of a loss of genetic variability and subsequent shift in the balance of what is recognised as an extraordinarily complex system and therefore trade-offs may exist for similar reasons between cellular (T-cell mediated) and humoral (B-cell mediated) components of immunity. Accordingly, Whiteman et al., (2006) found that small island populations with reduced genetic variation showed increased levels of (humorally mediated) natural antibodies (NAbs) when compared to larger and more genetically diverse island populations.

4.2 Materials and methods

4.2.1 Immune function assessment

(i) Phytohaemagglutinin (PHA) challenge

The exact physiological processes underpinning an inflammatory response to PHA injection are unclear and have been the subject of numerous experimental studies (Smits et al., 1999, Kennedy and Nager, 2006, Martin et al., 2006a, Tella et al., 2008, Vinkler et al., 2010) since the procedure was first described in aves by Goto et al., (1978). However, the response is known to reflect an individual's innate T-cell mediated immunocompetence (Goto et al., 1978, Tella et al., 2008) and has also been shown to elicit an acquired cellular response (Martin et al., 2006a). Whilst it is apparent that the response to PHA cannot be attributed to T-lymphocyte function alone and given the ongoing uncertainty over its exact immunological effects in a highly complex system, it is perhaps best referred to as a non-

specific, 'measure of inflammatory potential' (Vinkler et al., 2010) which involves both innate and cellular components of the immune system (Martin et al., 2006a, Tella et al., 2008).

The PHA test has been widely used in studies of avian ecoimmunology to characterise immune function potential or to demonstrate the life history trade-offs associated with the costs of immunity. Most studies agree that magnitude of response is positively correlated with the propensity of an individual to mount an immune response, or indeed a measure of its current immunological status (Saks et al., 2006, Vinkler et al., 2011). For example, the magnitude of PHA induced wing-web swelling has been positively associated with nestling survival (Christe et al., 1998, Saino et al., 2003, Moreno et al., 2005), increased nutritional status (Hasselquist and Nilsson, 2012, Saino et al., 1997), plumage colouration (Saino and Møller, 1996, Saks et al., 2003), genetic diversity (Hale and Briskie, 2007b, Fossøy et al., 2009), parasite infection status and general body condition (Navarro et al., 2003, Moreno et al., 2005). However, it is widely recognised that an optimal strategy for any individual presented with an immune challenge depends upon the costs associated with mounting a response such that a maximum immune response may not necessarily be optimal (Viney et al., 2005, Graham et al., 2011).

(ii) Cellular and humoral assessment

The evaluation of haematological characteristics offer the avian eco-immunologist a relatively simple, non-destructive method of assessing the general health of an individual at a single moment in time with only a small blood sample and relatively little specialist equipment, making this approach ideally-suited to field-based studies.. Haematocrit or packed cell volume (PCV) is a measure of the proportion of whole blood composed of erythrocytes and reflects oxygen uptake and metabolic activity (Feldman et al., 2000); extreme values can be suggestive of anaemia, dehydration, nutritional deficiencies

(Campbell and Ellis, 2006) and have been shown to reflect levels of parasitism (Brommer et al., 2011) and genetic variation among nestlings (Potti et al., 1999).

Examination of the leukocyte profile from blood slides is also widely used in field studies that investigate avian immune defence (reviewed in Davis et al., 2008). Generally used to infer levels of stress (Gross and Siegel, 1983, Ilmonen et al., 2003, Groombridge et al., 2004b, Garamszegi et al., 2006) concentrations of different leukocytes reflect the stimulation of both innate and acquired immune defences (Feldman et al., 2000) and the assessment of the heterophil to lymphocyte ratio (H:L) in particular has been associated with, for example, body condition (Palacios et al., 2009), food limitation (Gross and Siegel, 1983, Banbura et al., 2011), parasite infection (Gangoso et al., 2009), experimental immunological stimulation (Sarv and Hõrak, 2009), genetic diversity (Hale and Briskie, 2007b) and nesting environment factors such as brood size and hatch date (Cláudia Norte et al., 2009).

Assessment of the B-lymphocyte governed humoral immunity is facilitated by the examination of serum proteins including antibodies and complement. Natural antibodies are immunoglobulin molecules and as such are a component of the innate humoral immune system which recognises antigens and neutralises them or marks them for elimination by complement mediated lysis (Janeway et al., 1999). The haemolysis-haemagglutination assay (HL-HA) (Matson et al., 2005) is a technique designed to characterise innate humoral immunity by assessing the ability of NAbs to recognise a foreign antigen (rabbit red blood cells) and the subsequent lysis of that antigen by complement. In avian ecoimmunology studies the HL-HA has been used to illustrate, for example, a decrease in NAb levels and variability associated with inbreeding in small island populations (Whiteman et al., 2006), how species exhibiting a slow pace of life increase investment in humoral and acquired immune development (Martin et al., 2006b, Lee et al., 2008) and that differences in innate humoral immunity exist between island endemic and mainland species (Beadell et al., 2007).

4.2.2 Sample collection and PHA challenge

Nests of Indian ringneck and Mauritius parakeets on Mauritius were monitored regularly in order to determine the date of first egg-laying and approximate hatch date of chicks. This enabled each nest to be accessed shortly after or during hatching to determine hatch order based upon size and development of each chick which were then marked on the toes with permanent ink to aid future identification. Nests were accessed when the eldest nestling was estimated to be 45 days, approximately 10 days before fledging. I followed the protocol of Smits et al., (1999) by administering 20µl of a 5:1 PHA (Sigma-Aldrich L8754) in phosphate buffer (PBS; Oxoid, Dulbecco A – BR0014G) solution to the right wing-web of each nestling.

At least three measurements of patagium thickness at the injection site were taken to the nearest 0.01mm using a pressure-sensitive micrometer (Silverline 282378) prior to PHA exposure and the average of these measurements was taken as the pre-injection thickness. The injection site was marked with a permanent marker pen and individual body mass was then recorded prior to returning the chicks to the nest. All *P. echo* nestlings at this point were individually identifiable owing to metal rings fitted at approximately 10 days old as part of the ongoing conservation and population monitoring programme. After 24hrs (±2 hrs) the nest sites were accessed for a second time to record the PHA response measurement and to take blood samples for genetic and additional immunological tests. Post-injection swelling was recorded in an identical manner to the pre-injection measurement and PHA response was then recorded as the average post-injection measurement minus the average pre-injection measurement. Measurement repeatability was carefully assessed and was confirmed to be high for all three readings both before and after PHA injection ($r > 0.90$). Body mass for each individual was recorded for a second time in addition to wing length (carpal joint to longest primary feather) and tail length (longest tail feather).

Blood samples were then taken via jugular venipuncture. Approximately 0.8ml of blood was drawn using a 25 gauge needle attached to a 1ml non-anticoagulant treated syringe. At least two blood smears were made per individual using the wedge method (Clark et al., 2009) using a drop of blood directly from the syringe before the remainder of the blood was divided into collection tubes for subsequent analysis. Two to three drops of blood were stored in absolute ethanol for DNA extraction for genetic analysis, 0.5ml blood was then transferred into heparin treated collection tubes (Teklab H1230) for subsequent antibody analysis using the general haemolysis haemagglutination assay (Matson et al., 2005) described below. Blood slides were air dried and all samples were transported back to the field laboratory for further processing where the slides were fixed in 100% methanol before being stained with Leishman's stain solution (Fisher PB05) following the manufacturers guidelines. Packed cell volume was recorded for each individual using a small amount of heparinised blood and a haematocrit centrifuge. Plasma was extracted from each heparinised blood sample by centrifugation and stored at -80ºC until it could be transported back to the UK.

4.2.3 Laboratory methods

Blood slides were initially examined so that only the best quality slide for each individual (with respect to smudge cells and monolayer consistency) was selected for leukocyte profiling. Slides were then examined blindly with respect to species and date of collection at x1000 magnification and the first 100 leukocytes were identified and counted following Campbell and Ellis (2006), Clark et al., (2009) and Ritchie et al., (1993). For each individual, numbers of heterophils, lymphocytes, eosinophils, basophils and monocytes were recorded as the total number of cells out of 100 and subsequently a ratio of heterophils to lymphocytes (H:L) was calculated. All blood slides were examined by the same person (S.J.T) and only attributed an individual identification after examination.

General haemolysis-haemagglutination Assay

Plasma was transported back to the UK on dry ice for assessment using the general haemolysis - haemagglutination assay (Matson et al., 2005). I followed the protocol of Matson et al., (2005) with the following modifications. Twenty-five microlitres of 1.0 M PBS (Oxoid, Dulbecco A – BR0014G) were added to all wells in columns 2-12. Twelve and a half microlitres of eight individual plasma samples were then added to columns 1 and 2. A multi-channel pipette was then used to serially dilute the plasma solution between columns 2 and 11 resulting in dilutions ranging from 1:3 in column 2 to 1:59049 in column 11. Column 1 was used as a positive control as it only contained plasma, and column 12 was used as a negative control given that it contained only PBS and no plasma. Chicken plasma was used as a control in every third microplate to ensure assay consistency. To each well of the microplate, 12.5µl of a 1% rabbit red blood cell suspension (Harlan Laboratories, UK. S.B-0009) was added and the plates incubated at 37ºC for 90 minutes then scanned and agglutination by NAbs recorded and scored according to Matson et al., (2005). Complement mediated lysis was then recorded in a similar manner after the plates were stored at ambient temperature for a further 60 minutes. Consistency of lysis and agglutination scores for chicken plasma was high (n = 10, lysis score = 4 in all plates, agglutination score = 9 in all but one replicate where agglutination was scored at 8).

4.2.4 Microsatellite analyses

All individuals were genotyped using a suite of 21 fluorescently labelled autosomal microsatellite markers developed specifically for *P. echo* (Raisin et al., 2009) and sexed using the sexually dimorphic primer set Z-002B (Dawson, 2007). DNA was extracted using an ammonium acetate precipitation method (Nicholls et al., 2000) and samples from each species were amplified separately in slightly different multiplex arrangements prior to analysis on an ABI 3730 DNA analyzer (Applied Biosystems). Genotypes were scored using GeneMapper (v. 4.0) software. The PCR amplification procedure for *P. echo* individuals

consisted of five multiplex (MP) sets modified from those developed by Raisin et al (2009, 2012); (MP1: *Peq04, Peq05, Peq09, Peq11, Peq13, Peq18, Peq19*; MP2: *Peq02, Peq03, Peq07, Peq12*; MP3: *Peq06, Peq10, Peq14, Peq21*; MP4: *Peq16, Peq17, Peq20*; MP5: *Peq01, Peq 15,* Z-002B). *P. krameri* individuals were amplified in four multiplex sets; (MP1: *Peq01, Peq04, Peq05, Peq11, Peq13, Peq14*; MP2: *Peq09, Peq12, Peq16, Peq18, Peq19*; MP3: *Peq03, Peq07, Peq10, Peq17, Peq20, Peq21*; MP4: *Peq02, Peq15, Z-002B*). All loci were tested for linkage disequilibrium and deviation from Hardy-Weinberg equilibrium using *Genepop* (Raymond and Rousset, 1995) with exact probability tests corrected for multiple comparisons using a sequential Bonferroni correction (Rice, 1989). Null allele frequencies were assessed using *Cervus* (Marshall et al., 1998). Gene diversity (GD) and allelic richness (AR) per locus were calculated for each species using *Fstat* (Goudet, 1995) and values were averaged over all loci and for each population to assess interspecies differences using Wilcoxon-rank sum tests.*Multilocus heterozygosity measures*

I used the R (R Development Core Team, 2012) package 'Rhh' (Alho et al., 2010) to calculate individual level multilocus heterozygosity. Standardised heterozygosity (SH; Coltman et al., 1999), was preferred over other similar measures such as homozygosity by loci (HL; Aparicio et al., 2006), internal relatedness (IR; Amos et al., 2001) or mean d^2 (Coulson et al., 1998) because it is recognised as the simplest measure of individual variation in studies concerning HFCs (Chapman et al., 2009) and comes with few interpretational difficulties associated with other metrics (Hansson, 2010). In addition I found all three measurements (SH,IR and HL) to be very highly correlated in both species (see Results; Table 1) indicating the suitability of any of these metrics to represent individual variation.

4.2.5 Statistical analysis

I summarised levels of MLH for each species using all three Rhh measures of variability and used Pearson's product-moment correlations to determine the similarity of these measures. Heterozygosity-heterozygosity correlations for each species were calculated to assess any signal of inbreeding within the microsatellite genotype dataset and to evaluate the suitability of this genotype sets to represent genome-wide heterozygosity (Balloux et al., 2004). Simple T-tests, adjusted for different sample sizes, were then used to look at interspecific differences in measures of diversity under the assumption that *P. echo* would show reduced MLH and increased HL/IR due to its documented bottleneck (Jones, 1987) compared to *P. krameri*. Indices of immune function were then summarised for each species and interspecies differences were investigated using T-tests and Wilcoxon rank sum tests for non-parametric data.

I used generalized linear mixed models (GLMMs) to examine the extent to which variation in immune function variables were explained by individual SH, morphology, gender and variables associated with nesting environment. GLMMs incorporate the benefits of fitting linear mixed models with random effects and generalised linear models which can incorporate non-normal errors (Bolker et al., 2009). To account for any statistical non-independence of siblings from the same nest I included the random effects of 'nest ID' in all models. Where the response variables followed a normal distribution I fitted GLMMs using the restricted maximum likelihood (REML) parameter estimation procedure and the lmer function as part of the 'lme4' package in R (Bates et al., 2010) with a Gaussian error structure and identity link. The models which included monocyte counts as the response variable were fitted in a similar manner but with a poisson error structure. Counts of basophils and eosinophils are notoriously difficult to assess statistically given their unusual, zero-inflated count distribution; such data cannot be made normal by transformation (Bolker et al., 2009) and therefore zero-inflated GLMMs were employed using the 'glmmADMB' package in R (Skaug et al., 2008) with a poisson error function. Lysis titres were

transformed for both species into a binary 'low or high' variable (using a lysis score of zero as 'low' for *P. echo* and zero or one for *P. krameri*) and analysed by fitting a GLMM with a binomial error structure. Models were checked visually for heterogeneity of variance, residual normality and influential data points.

These models were fitted in three stages;

(i) Initially, I created simple bivariate models which included the index of immunity as the response variable and SH as a single explanatory covariate to examine relationships between genetic variability and immunity for each species. I then used likelihood ratio tests to identify the significance of each explanatory variable, comparing the fit of each model to that of the same model after removal of the single explanatory variable leaving just an intercept and random term.

(ii) To examine the variation in immune function variables explained by non-genetic factors I included in the models a carefully selected set of covariates. I limited the parameters to those variables known from the literature to affect immune function and those identified as important and biologically plausible from initial, exploratory analyses. Explanatory variables were investigated for collinearity using correlation matrices and variance inflation factors (VIFs) (Zuur et al., 2010) and relationships between them were examined graphically using pair plots. Welch's T-tests and Wilcoxon signed-rank tests for non-parametric data were used to explore the relationships between each immune function variable and the fixed factor variables of gender and year for both species. Body mass and wing length were included as covariates given that size, growth rate and condition of nestlings have previously been shown to influence PHA response and other parameters of immunity (Hõrak et al., 1999, Alonso-Alvarez and Tella, 2001, Moreno et al., 2005, Martin et al., 2006a). Hatch date (Sorci et al., 1997, Moreno et al., 2005), and time of day (Clark et al., 2009, Forsman et al., 2010) were included in addition to year and gender (derived from molecular analysis) as fixed factors as well as the interaction between gender and SH given that some studies have

reported gender specific genetic effects on immune response variables (Martin et al., 2006a, Drobniak et al., 2010). To avoid over-parameterisation (Burnham and Anderson, 2002) I only included hatch order and clutch size variables where initial exploratory analysis and visual examination suggested that these may be potential predictors of the immune function variable of interest.

(iii) Finally I examined the relationships between magnitude of PHA response and haematological parameters to identify interspecific differences. Increase in wing-web swelling was used as the response variable and all other haematological values were included as explanatory covariates in order to identify the species-specific causes of a response to PHA mitogen stimulation. Collinearity of explanatory variables was investigated via correlation matrices and VIFs. For these analyses GLMMs with a Gaussian error structure were fitted for each species separately and all PHA-challenged individuals across both years were included.

An information theoretic (IT) and model averaging approach was used to assess model suitability based on the application of Akaike's information criteria (Burnham and Anderson, 2002, Burnham et al., 2011, Garamszegi, 2011). This approach to model selection as an alternative to null-hypothesis testing and stepwise methods has gained widespread support recently given its suitability to estimate effect sizes of multiple predictor variables which may display large standard errors or be too weak to be included in traditional methods relying upon arbitrarily set significance thresholds (Johnson and Omland, 2004, Whittingham et al., 2006, Grueber et al., 2011a). Input variables were centralised following Gelman (2008) by standardising to a mean of zero and a standard deviation of 0.5 in order that the parameter estimates of variables measured on different scales can be relatively interpreted after model averaging (Gelman, 2008, Grueber et al., 2011a). Model averaging was implemented in the multi-model inference package MuMIn for R (Bartoń, 2009). The threshold at which models are selected for averaging is somewhat subjective and appropriate levels have been suggested between $\Delta AIC < 2$ and $\Delta AIC < 10$ (Burnham and Anderson,

2002, Bolker et al., 2009). I averaged each set of candidate models using a threshold of ΔAIC < 4 or used summed weights to derive the 95% confidence set when ΔAIC < 4 produced extremes of model numbers.

4.3 Results

A total of 38 *P. krameri* and 152 *P. echo* nesting attempts were monitored. The final dataset consisted of indices of immune function and multilocus heterozygosity estimates for 259 *P. echo* nestlings from 122 clutches (of 79 different females) spanning two consecutive breeding seasons during 2009/10 ($n_{clutches}$ = 61) and 2010/11 ($n_{clutches}$ = 61) and a total of 61 *P krameri* nestlings were successfully sampled from 27 clutches during the same period ($n_{clutches}$ = 10 during 2009/10 and $n_{clutches}$ = 17 during 2010/11). Each of these clutches were reared by a different female. Average clutch size among *P. krameri* (3.34 ±0.90) was significantly greater (t = 3.58, d.f. = 41.4, P < 0.001) than *P. echo* (2.8 ±0.46) and average number of fledglings per nest was also significantly greater among *P. krameri* (*P. krameri*: mean = 2.2 ±1.32; *P. echo* mean: = 1.7 ±0.61; t = 2.28, d.f. = 40.5, P = 0.03).

4.3.1 Genotyping and individual multilocus heterozygosity

Analysis of genotype data revealed two sex-linked microsatellite loci *(Peq 21, Peq 16*) and two loci displaying linkage disequilibrium in both species (*Peq 09 and Peq 12*). Therefore *Peq 09, Peq 16* and *Peq 21* were dropped from any further analysis in both species. Amplification at *Peq 04, Peq 06* and *Peq 18* was poor among *P. krameri* individuals and therefore these loci were also dropped from further analysis in this species. The final genetic dataset therefore consisted of *P. echo* individuals genotyped at 17 microsatellite loci and *P. krameri* genotyped at 14 of these. Estimated frequencies of null alleles per locus were less than 0.1% for all loci in both species and genotyping success was in excess of 95%. Although mean allelic richness was higher in *P. krameri* (6.64 ±2.19) than in *P. echo* (5.41 ±1.73) this did not represent a significant difference (W = 81.5 P = 0.14). Similarly, values

of mean gene diversity were not significantly different between species (*P. krameri*: 0.68 ±0.13, *P. echo*: 0.63 ±0.18; W = 111.5 P = 0.78).

Pearson's product-moment correlations revealed strong associations between all molecular measures of diversity (Table 1) for both species indicating the suitability of any of these measures to describe individual-level multi-locus variability. Standardised multilocus heterozygosity among *P. echo* ranged from 0.57 to 1.52 (mean = 1.02 ±0.19) and from 0.57 to 1.34 (mean = 0.99 ±0.20) among *P. krameri*. There was no significant difference between the two species for any measure of MLH (Table 2). Positive and significant heterozygosity-heterozygosity correlations were observed in both species; *P. echo*, r = 0.19, 95% CI, 0.09 – 0.29; *P. krameri* r = 0.14, 95% CI, 0.01 – 0.32.

4.3.2 Immune function variables

Values of all immune function variables measured are summarised in Table 3. To control for possible effects of PHA on haematological values results are presented for only those individuals subjected to the PHA challenge. Sample sizes vary among the recorded variables because it was not possible to record all parameters for all individuals. Simple T-test comparisons reveal that wing-web swelling as a result of PHA injection was significantly larger on average (t = 4.58, P < 0.001) in *P. krameri* (1.14mm ±0.62) than in *P. echo* (0.73mm ±0.32) (Tables 3 and 4, Figure 1). *P krameri* individuals also displayed higher average PCV values, monocyte counts and lysis titres whilst average individual basophil counts were higher in *P. echo* (see Table 3 for details). Mean body mass for *P. echo* was 157.50g (±10.58) pre-PHA injection and 156.34g (±11.16) post-injection indicating no significant difference (t = 1.98, P = 0.06). Mean body mass for *P. krameri* was however, significantly lower post-PHA injection (mean pre = 146.20g ±14.10, mean post = 141.51g ±14.86, t = 2.00, P < 0.001). At this stage in nestling development a loss of body mass is expected given that mass in *P. echo* is known to reach an asymptote at around day 35 (MWF, 1996-2012).

The haemolysis haemagglutination assay revealed that opsonisation of rabbit red blood cells was significantly more likely among *P. krameri* individuals than *P. echo* (Table 3). Out of 54 *P. krameri* individuals, 46 (> 85%) showed some level of complement mediated lysis (Figure 2). Contrastingly, out of 108 *P. echo* individuals only 60 (56%) showed opsonisation (χ^2 = 11.8, d.f. = 1, P < 0.001). Additionally, among *P. echo* individuals, a significant annual effect was observed; during 2009/10 22 out of 54 individuals (41%) showed some level of lysis whilst in 2010/11 38 out of 54 nestlings (70%) demonstrated some level of successful complement mediated lysis (χ^2 = 8.44, d.f. = 1, P < 0.01).

4.3.3 Relationships between immune function variables and MLH

Simple bivariate GLMMs revealed that SH is not a good predictor of immune function variables in either species (Table 5). However, I did observe a significant effect of removing SH from one model indicating the existence of a positive relationship between PHA induced wing-web swelling and MLH in *P. krameri* (β = 0.40, SE = 0.19, χ^2 = 4.0, d.f. = 1, P < 0.05; Table 5 and Figure 3).

4.3.4 Predictors of immune function variables

Individual sample size for *P. krameri* individuals in 2009/10 was low (n = 17) and initial exploratory analyses (Welch's T-tests and Wilcoxon signed-rank tests) revealed significant inter-annual effects in many of the measured immune function parameters for both species (*P. echo*: PHA; t = 5.12, P < 0.001, basophils; W = 2570, P < 0.001 , monocytes; W = 5.80, P < 0.001, Lysis; W = 934, P < 0.001, agglutination; t = 3.34, P < 0.01, *P. krameri*: PHA; t = 9.6, P < 0.001, basophils; W = 468, P < 0.01, agglutination, t = 4.07, P < 0.001). I therefore restricted the dataset for this analysis to 2010/11 for both species to eliminate the effects of small sample size and to control for inter-annual variation which might be influenced by temporal changes in environmental factors such as temperature and rainfall. Assessment of collinearity among explanatory variables for *P. echo* revealed that the highest correlation was between wing length and hatch order (r = -0.45) indicating that older nestlings were

generally larger. All VIFs were below 1.6 and therefore this set of explanatory variables was not considered to demonstrate high collinearity (Zuur et al., 2009). Similarly, among *P. krameri* individuals, the highest correlation was between wing length and hatch order (r = -0.46) and all VIFs were below 1.70.

Response to phytohaemagglutinin (PHA)

The candidate model set for *P. krameri* when ΔAIC < 4 consisted of 9 models. Model averaging revealed that gender and SH were significant predictors of PHA increase in *P. krameri* (Table 6). Female *P. krameri* nestlings responded more strongly to PHA stimulation (Figure 4) and the magnitude of wing-web increase was positively related to standardised heterozygosity (Table 6 and Figure 3). An identical approach to model averaging for the candidate set for *P. echo* revealed that only the null model remained when ΔAIC < 4 suggesting that there were no significant predictors of PHA response among *P. echo* individuals. Increasing ΔAIC to < 6 revealed 4 candidate models for *P. echo* besides the null model. Confidence intervals of parameter estimates for all of the predictors included zero indicating that none of the predictor variables affect PHA increase in *P. echo* (Table 6).

Heterophil/ lymphocyte ratio (H:L)

Only one significant predictor of H:L ratio was observed for *P. krameri* showing a negative relationship with wing length (Table 7 and Figure 5), indicating that smaller chicks generally displayed a lower H:L ratio. No significant predictor variables were seen for *P. echo* (Table 7). However, the null model appears as the one with the lowest AIC score in both species and the confidence interval for the significant effect in *P. krameri* is very close to zero (-0.52, -0.01).

Packed cell volume (PCV)

The final model set for *P. krameri* consisted of only two models at ΔAIC < 4 and so ΔAIC was increased to < 6 (Table 8). This made little difference to the final model selection and only included one additional model. Wing length was the only significant predictor and revealed a weak positive relationship indicating that larger chicks generally display increased PCV (Figure 6). The model selection table for *P. echo* consisted of only one model at ΔAIC < 4. Two significant predictors of PCV among *P. echo* were observed; gender and wing length. Males tend to have lower PCV and larger individuals tend to show increased PCV (Table 8 and Figure 6). This model was repeated to include the interaction effect of gender and wing length but this made no difference to the model outcome and the interaction did not appear in any models at ΔAIC < 4.

Eosinophils

A significantly positive relationship was observed between body mass and eosinophil count among *P. krameri* (Table 9). Hatch day was the only significant predictor among *P. echo*, suggesting that individuals hatching later in the season produce fewer eosinophils (Table 9). The relative importance score of body mass among *P. echo* individuals was low (0.15) whereas time of day (importance score of 0.32) and SH (importance score, 0.4) were more important predictors.

Basophils

The time of day predictor for analysis of basophil counts in each species was transformed to a continuous ordinal variable (1 = early morning – 4 = early evening) to overcome problems associated with heteroscedasticity when this variable was treated as categorical. A significant positive relationship was found between number of circulating basophils and time of day among *P. krameri* (Table 10). No significant predictors for basophil count were

revealed among *P. echo*, however the strongest predictor was hatch day indicating a weakly positive relationship with a relative importance factor of 0.52 (Table10).

Monocyytes

No single predictor had a significant effect on the number of circulating monocytes among *P. krameri*. However a significant interaction effect was detected between gender and SH (Table 11) and was included in seven out of the nine models where $\Delta AIC < 4$. Male individuals revealed a negative relationship between SH and monocytes; individuals with a higher SH tended to display fewer monocytes whilst the opposite pattern was detected among females (Figure 7). No such observations were apparent for *P. echo* individuals; the only predictor variable with confidence intervals which did not span zero was hatch day (Table 11) indicating a negative relationship between number of monocytes and date of hatch.

Among *P. echo*, counts of all rare white blood cells (eosinophils, basophils and monocytes) were most strongly predicted by hatch day. Individual eosinophil and monocyte counts were observed to decrease significantly over the hatching period (Table 11). Figure 8 illustrates these relationships by showing the raw counts of rare white blood cells per individual against the corresponding hatch day for each species. This figure also serves to illustrate the extended breeding period of *P. krameri* when compared to *P. echo*.

Lysis

No apparent effect of any of the predictor variables on lysis was observed in *P. krameri* (Table 12). A significant effect of time of day on lysis in *P. echo* is the result of no single individual sampled in the afternoon (n = 15) presenting a high lysis score.

Agglutination

A significant negative relationship was observed between wing length and agglutination in *P. krameri*, indicating that smaller individuals show increased agglutination (Table 13.), however graphical inspection of this model revealed that a single data point was influencing this result and its subsequent removal resulted in the null model outperforming all others. Model averaging after removal of this point suggested that no predictors significantly affected agglutination. No significant predictors were observed for *P. echo* and the best performing model was the null model. Relative importance factors indicated that wing length and clutch size were the most influential (albeit non-significant) predictors of agglutination for both species.

4.3.5 Haematological predictors of PHA increase

Investigations into collinearity of the haematological predictors revealed a lack of strong correlations especially among *P. echo* individuals (Table 14) suggesting a relatively homeostatic nature when compared to the correlations and VIFs among *P. krameri*. The highest correlations among *P. krameri* were observed between PHA increase and agglutination by NAbs ($r = 0.54$) and between H:L ratio and complement mediated lysis ($r = -0.47$). Only one correlation was observed where $r > 0.30$ among *P. echo* individuals (compared to six among *P. krameri*); agglutination and lysis titres ($r = 0.44$). Variance inflation factors did not exceed 1.80 for any variable in either species. Results of analyses from GLMMs using PHA induced wing-web swelling as the response variable are shown in Table 15. Among *P. krameri* nestlings, an increased PHA response was strongly associated with a higher agglutination titre (Table 15 and Figure 9). Weaker significant relationships were observed between PHA response and two cellular predictors; a negative relationship with basophil concentration and a positive relationship with monocyte concentration (Figure 9). Among *P. echo* nestlings only one of the haematological variables was observed as a significant predictor of PHA response (Table 14 and Figure 10); individuals demonstrating

an increased PHA response had a greater probability of a reduced eosinophil, however the proximity to zero of the upper confidence interval and the low relative importance factor indicates that this relationship is not strong (Table 15).

4.4 Discussion

It has been suggested that as a result of contrasting evolutionary mechanisms concerning parasite communities on islands and continents, island-dwelling hosts should encounter a depauperate pathogen community when compared to their mainland counterparts (Wikelski et al., 2004), thereby relaxing any selective maintenance of immune function-related genetic diversity, leading to the eventual loss of such diversity concordant with the effects of drift and mutation in small populations (Frankham, 1997). Many pathogens are highly host-specific (Janeway et al., 1999) and if island endemic species are exposed to a relatively reduced pathogen community then this coevolution may be enhanced (e.g. Cornuault et al., 2012) leaving endemic island fauna naive to a wide range of novel, continentally evolved parasites and infectious diseases but well equipped to tolerate a small number of specific types. In contrast, continentally evolved species may have evolved less immunological specificity, leading to an increased ability to tolerate a wider range of parasites. Alternatively, island species may experience a reorganisation of immune function as a result of evolutionary interactions between the innate and acquired components of defence (Matson, 2006). Matson (2006) suggests that a reduction in genetic diversity which impacts upon one arm of the immune function could result in an increase in the efficacy of the other, and provides evidence to suggest that insular hosts may have developed more effective innate, non-specific immune defences at the cost of reduced humoral adaptive systems.

4.4.1 Genetic diversity and population history

Despite documented evidence that *P. echo* endured a considerable bottleneck which at its most severe saw the population comprised of less than 20 individuals in the 1980s (Jones and Duffy, 1993), I found no evidence to suggest that measures of population level diversity

or individual MLH were different between the endemic and introduced *Psittacula* species which occur sympatrically on Mauritius. Likewise, heterozygosities among loci were positively correlated in both species indicating similarly low levels of identity by descent (Balloux et al., 2004). This lack of interspecies difference in genetic measures is perhaps explained by the nature of colonisation of Mauritius by *P. krameri* from a small number of escaped or released cage-birds in the 1880s (Cheke and Hume, 2009), indicating that the current population may have been established from a small number of individuals, broadly comparable to the bottleneck experienced by the endemic species. It is therefore likely that the contemporary populations of both species originated from a similar number of founders. The fact that *P. krameri* currently outnumbers *P. echo* by at least an order of magnitude suggests that the dramatic growth trajectory of this invasive species on Mauritius cannot be attributed to greater genetic diversity alone even though it is considered to have originated from a continental population and to therefore have had the intrinsic benefits of a greater initial pool of immunological, genetic and phenotypic variation. Given this apparent lack of difference in genetic variation between two species following bottleneck events of similar proportions over a broadly comparable time-scale, it is unlikely that differences in immune function can be attributed simply to contemporary genetic factors and are therefore more likely associated with contrasts in evolutionary life history determined by continental vs. island adaptation or by differences in pace of life.

4.4.2 Effects of evolutionary origin on immune function

Out of the eight variables of immune function used in this study, five of them differed significantly between the two closely related Psittacula species. Response to PHA mitogenic stimulation was greater in *P. krameri* and I interpret this as indicating an increased capacity by *P. krameri* to mount an innate and non-specific immune response to a novel antigen in comparison to the abilities of the insular endemic *P. echo*. Furthermore, *P. krameri* exhibited an elevated complement-mediated humoral response as measured by the lysis of a foreign antigen by natural antibodies via the general haemolysis-haemagglutination assay relative to

the island form. The magnitude of swelling as a result of PHA stimulation was significantly positively predicted by both cellular (monocytes) and humoral (agglutination) components of immune function among *P. krameri* but no positive relationship was observed among *P. echo*. These contrasts suggest a fundamental species-related divergence in immune function efficacy and provide some evidence to support the hypothesis that continentally evolved species, adapted to coexist with a more diverse community of pathogens are more capable of producing an innate immune response to a novel challenge than an island evolved host. Furthermore, these two species differ in their pace of life, with theory predicting a more effective innate immunity for faster living species. Juvenile *P. krameri* develop faster and clutch sizes are larger compared to *P. echo*. Furthermore, the native distribution of *P. krameri* spans the entire Indian sub-continent and is therefore adapted to a vast range of environmental conditions and habitats, its profile therefore matching that of a faster living species than *P. echo*.

4.4.3 Determinants of individual level variation

Gender-based differences were observed in both species but for different immune function indices. Male *P. krameri* nestlings produced a smaller PHA response than females, whilst male *P. echo* nestlings displayed lower PCV. There is no difference in size or mass between the sexes of either of these species at this age and sexual differences in nestling immune variables have previously been attributed to variation in hormone production, specifically testosterone which may incur an increased cost in males (Folstad and Karter, 1992, Muehlenbein and Bribiescas, 2005, Fargallo et al., 2007).

There was no significant difference in average PCV values between adults of these species (*P. echo*: n = 48, mean PCV = 48.27 ±4.33; *P. krameri*: n = 40, mean PCV = 48.7 ±4.52, t = 1.98, P = 0.65) and therefore the difference observed between nestlings of the same age (which is known to affect PCV; Clark et al., 2009) is unlikely to be due to purely taxonomic differences and may be explained by enhanced metabolic rate of *P. krameri* when compared

to *P. echo*. Little information exists regarding the exact fledging period for *P. krameri* in the wild and estimates of between 42 and 60 days have been made (Butler, 2003, Shwartz et al., 2009), however it is apparent that on Mauritius at least, the fledging period for *P. krameri* is shorter than that for *P. echo* by approximately one week (*P. krameri*: 46-61 days, *P. echo*: 50-69 days (MWF, 1996-2012)) and therefore nestlings from different species are likely to display differing developmental progress at the same age.

Hatch day was a significant predictor for two of the four cellular indices among *P. echo* with both eosinophil and monocyte counts negatively associated with date of hatch. Each of the four cellular variables among *P. krameri* corresponded to different predictors; body mass, wing length, time of day and an interaction effect of sex and SH perhaps indicative of a more labile cellular immunity among *P. krameri*.

Increased production of basophils has been associated with exposure to PHA in previous studies of avian ecoimmunology (Martin et al., 2006a, Vinkler et al., 2011), chlamydial infection in other Psittaciforme species (Fudge, 1997, Campbell and Ellis, 2006) and allergic hypersensitivity reactions in other vertebrates (Dvorak et al., 1970, Sullivan and Locksley, 2009). Basophils and eosinophils were generally more commonly found in *P. echo* than in *P. krameri*. This difference was significant in the case of basophils and approached statistical significance for eosinophils. The number of circulating basophils was not significantly related to the size of PHA swelling in *P. echo*. However, basophil counts were significantly negatively associated with magnitude of PHA response in *P. krameri* thus representing an interpretational challenge in this study.

4.5 Conclusions

Enemy release?

The successful colonisation of *P. krameri* in Mauritius can perhaps be attributed to factors associated with the enemy release hypothesis (Torchin et al., 2003, Colautti et al., 2004) previously associated with the spread of this species in Europe (Shwartz et al., 2009). Unlike in its native range, *P. krameri* faces no native mammalian or avian predators in Mauritius and according to theory (MacArthur and Wilson, 1967, Wikelski et al., 2004) is exposed to a reduced parasite diversity. Those novel parasites that it does encounter probably lack the evolutionary structure to produce disease in a new host species that it has not coevolved with (Lee and Klasing, 2004), resulting in a net effect of reducing the cost of maintaining a continental immune defence. Conversely it is highly probable that with the introduction of *P. krameri* to Mauritius were parasites novel to *P. echo*; infectious agents much better adapted to cause disease and incur costs related to an increase in immune activity in a naive host. For example, an outbreak of PBFD in the population of *P. echo* in 2004 (Kundu et al., 2012) illustrated the rapid evolution of a virus in this species given favourable environmental conditions. Whilst it is not known for certain that this disease was introduced to Mauritius with *P. krameri*, the rapid adaptation documented in the viral genome (Kundu et al., 2012) and initially high levels of host mortality post-outbreak (MWF, 1996-2012) would suggest a lack of historical host-parasite coevolution in *P. echo*, at least with the particular genetic isolate of the virus which caused the initial high mortality. Although unable to provide conclusive proof, the evidence from this study reflects a fundamental difference in innate immune function between two very closely related species which cannot be attributed to species-level differences in genetic diversity. Given the different evolutionary origins of these species the evidence presented here suggests support for the enemy release hypothesis.

Evolutionary origin or contemporary genetic factors?

Ultimately there appear to be few significant predictors of immune variation among *P. echo* when compared to *P. krameri* and those that exist tend to be associated with environmental (e.g. hatch day or time of day) rather than physiological (e.g. wing length or body mass) parameters. Innate immune function among *P. echo* measured by the parameters used here is less variable and less responsive compared to *P. krameri* and the exact reasons for this present an interpretational challenge. Evidence from the suite of microsatellite markers used here suggests that neutral diversity alone does not explain the documented variation in immune function The fact that both the endemic and invasive species also have broadly similar bottleneck profiles further supports the idea that the observed species-level differences in immune function are associated with evolutionary affinity. However, one possible explanation is that a loss of immunogenetic diversity resulting from a similar population bottleneck differs between the species but is not detected by using a small number of neutral microsatellite markers.

Excluding the potential effects of reduced genetic diversity to explain the apparent difference in immune function between these two species can therefore, to some extent be justified due to the similarity in measures of MLH and shared bottleneck profiles. A more reasonable explanation could just be a pure selection effect of island life and a depauperate parasite community which therefore confers selection to 'relax' the immunogenetic diversity among *P. echo* over evolutionary time; fewer parasites result in a reduced requirement for a wide variety and level of immune responses.

I found no evidence of a reorganisation of immune system between cellular and humoral components between these two species. Among cellular components, *P. krameri* produced more monocytes (which also predicted PHA response) but *P. echo* produced more basophils, a result which was not significantly associated with any other variable. Both humoral and cellular components were involved in the response to a novel immune challenge among *P.*

krameri. In contrast, no humoral element was observed to be involved in the response to PHA among *P. echo* and the only cellular association with magnitude of wing-web swelling was a decline in eosinophil count. A reorganisation of immune systems is therefore unlikely given the lack of any positive relationships between PHA response and immune function variables in *P. echo*.

The results of this study suggest that both the cellular and humoral components of innate immune function are less effective among nestlings of an island endemic species compared to a congeneric non-native, invasive continental species, despite both populations experiencing effects of the same recent environment, parasite community and proportionally similar founding events.

4.6 Figures and tables

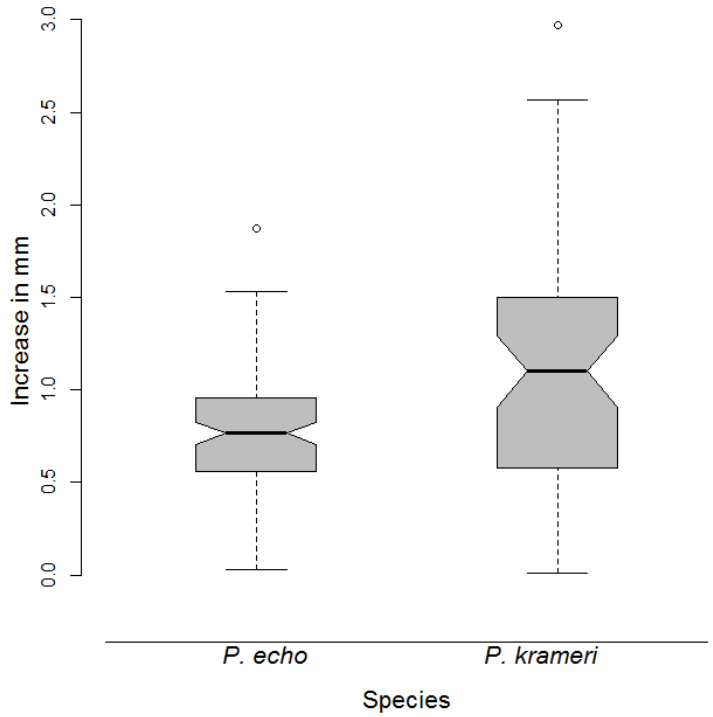

Figure 1 Boxplots showing increase in wing web thickness in response to phytohaemagglutinin challenge for both species.

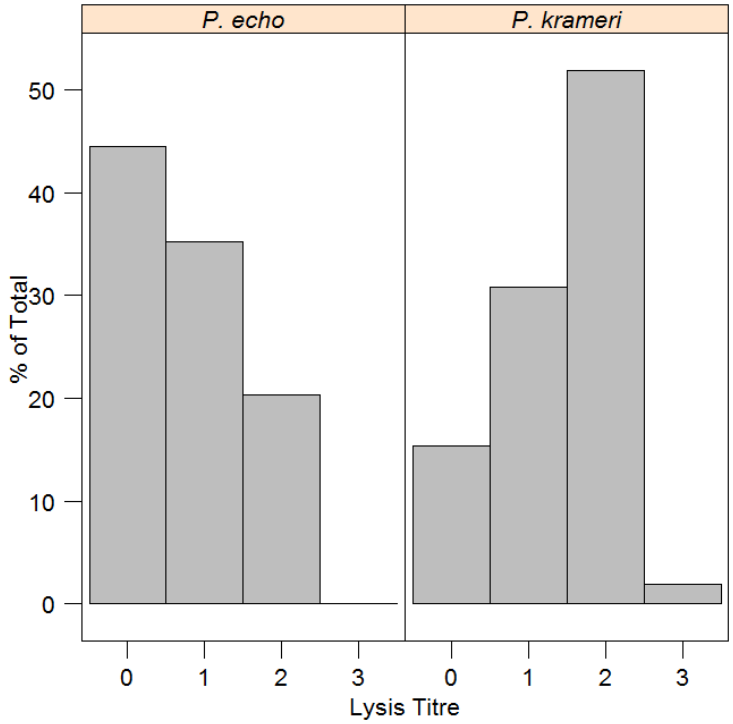

Figure 2 Lysis titre scores for both species derived from the haemolysis-haemagglutination assay featuring all individuals challenged with phytohaemagglutinin.

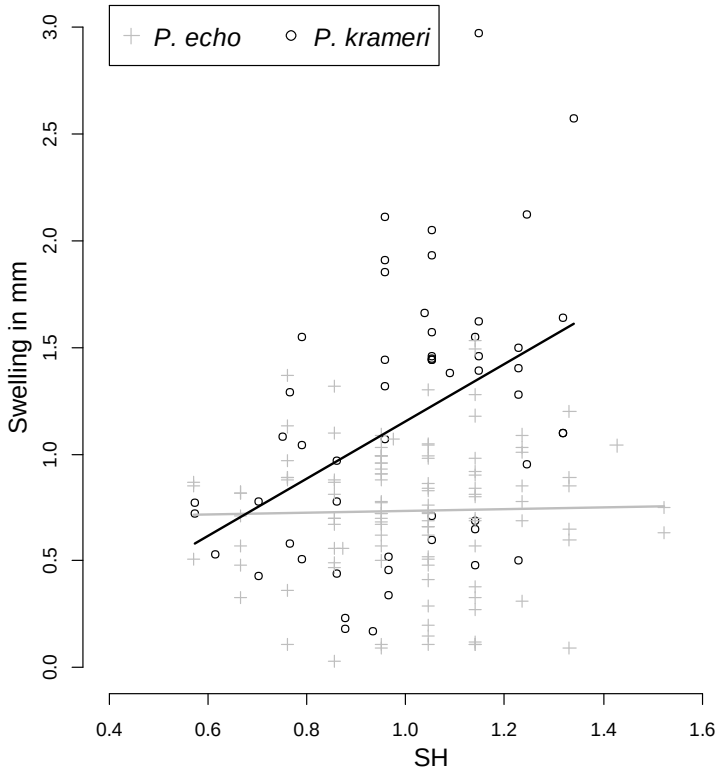

Figure 3 Relationship between standard heterozygosity (SH) and phytohaemagglutinin induced win-web swelling for both species. The regression line shows predicted values from generalised linear models with Gaussian error structure and identity link.

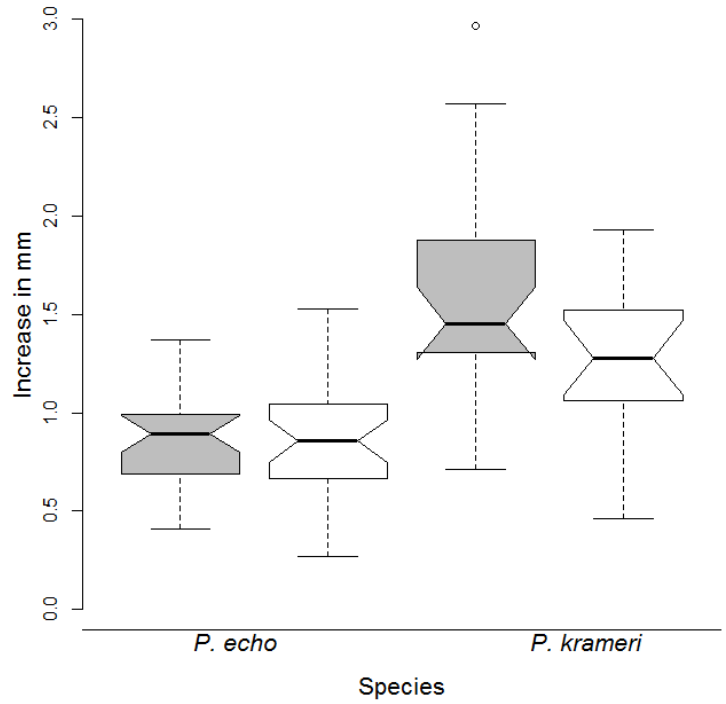

Figure 4 Phytohaemagglutinin induced swelling for both species in 2010/11 illustrating the difference between females (filled boxes) and males (open boxes) among *P. krameri*.

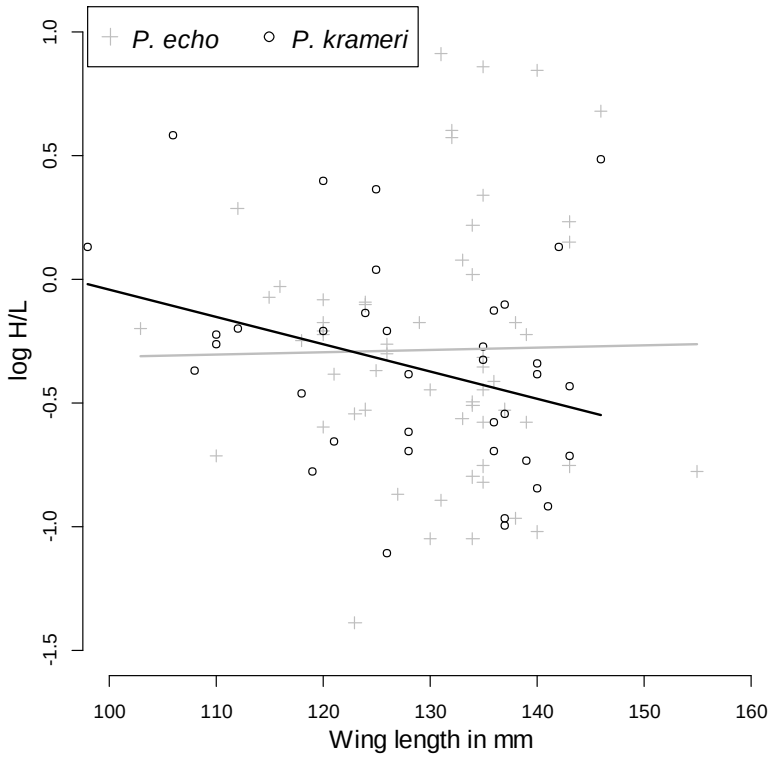

Figure 5 Relationship between wing length and log heterophil/ lymphocyte ratio (H:L) for both species. The regression line shows predicted values from generalised linear models with Gaussian error structure and identity link.

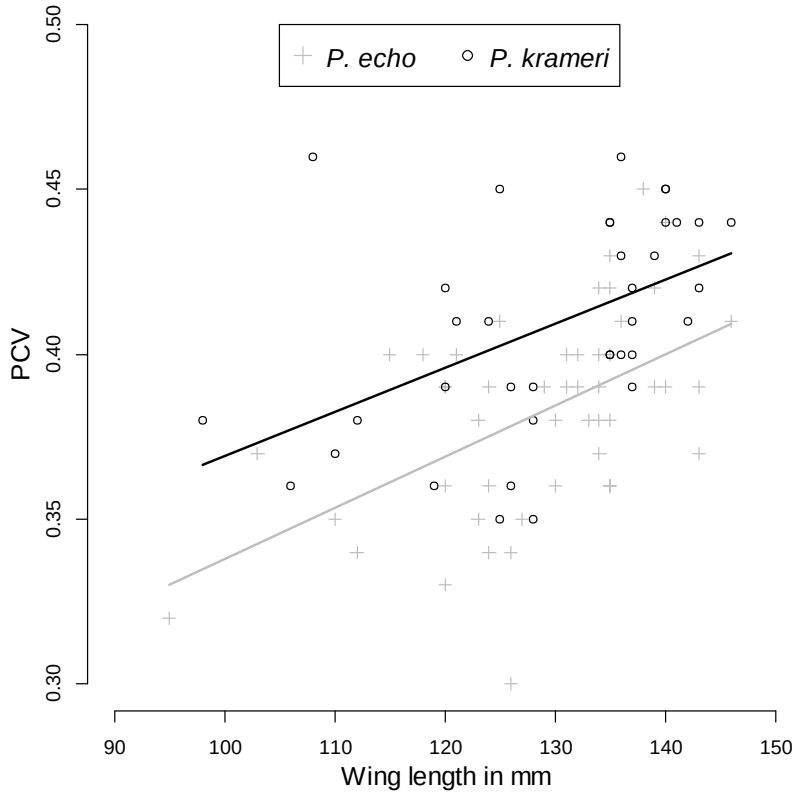

Figure 6 Relationship between packed cell volume (PCV) and wing length for each species. The regression line shows predicted values from generalised linear models with Gaussian error structure and identity link. The distance between the regression lines reflects the significant difference in PCV between the two species from Table 3.

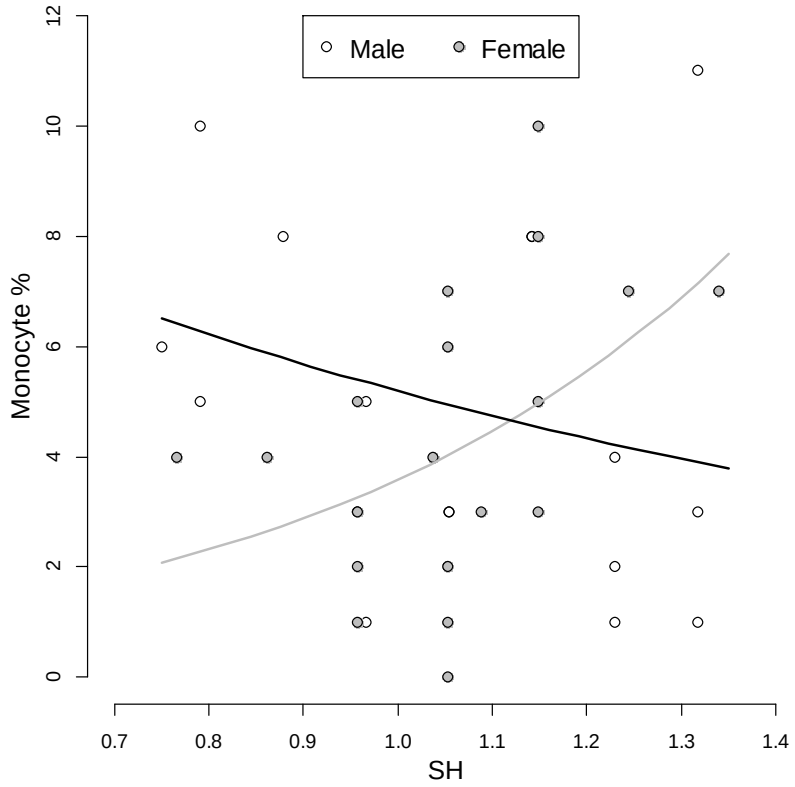

Figure 7 Relationship between monocyte count and standardised heterozygosity for *P. krameri*. Predicted values from generalised linear models with poisson error and log link are shown as the regression lines to illustrate the significant interaction from Table 11.

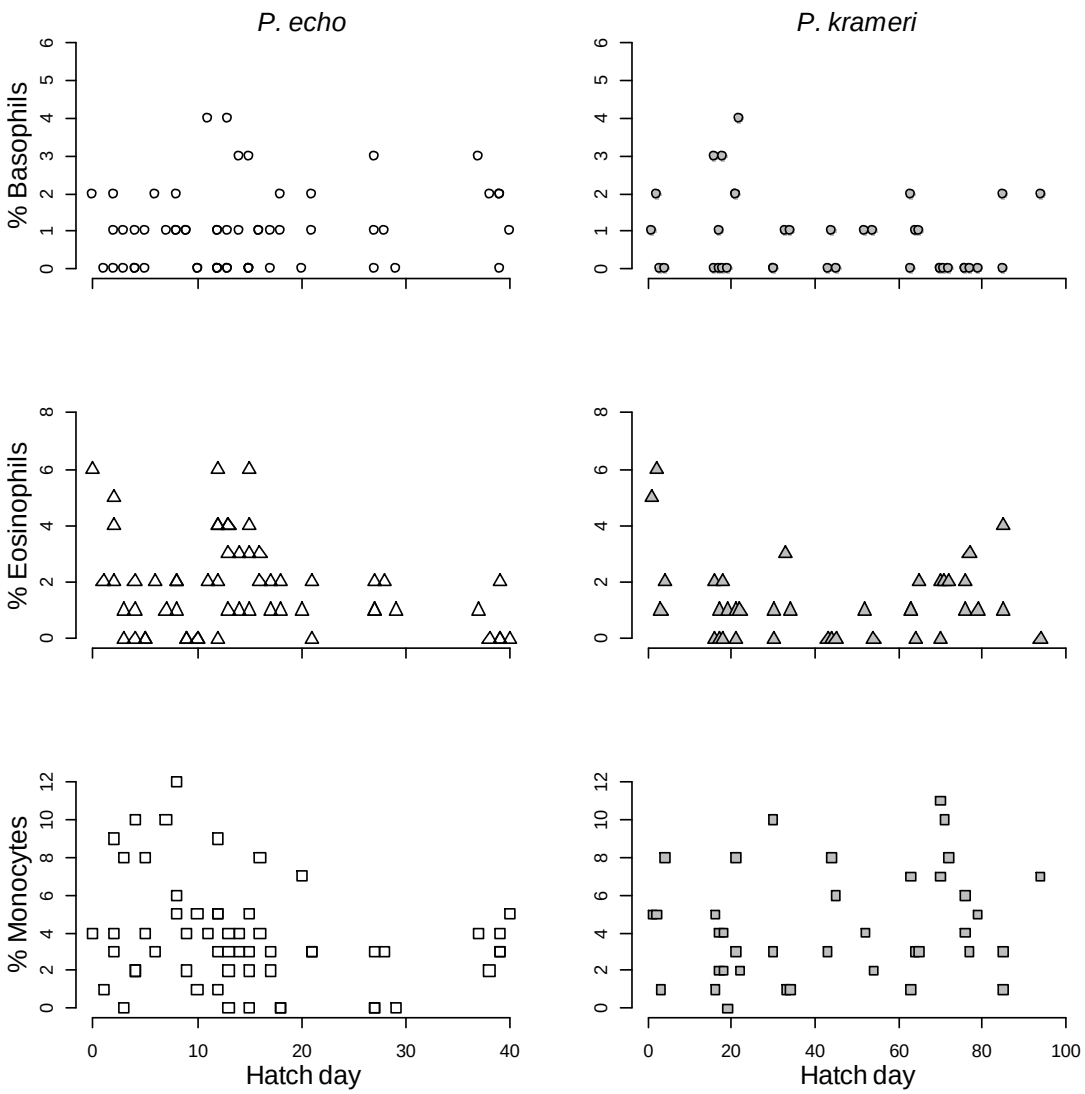

Figure 8 Relationship between hatch day and proportions of rare leukocytes. Hatch day was found to be the most important predictor of all counts of rare leukocytes among *P. echo*. Note the difference in length of breeding 'window' between the species; all *P. echo* individuals hatched within a 40 day period in contrast to more than 100 days for *P. krameri*.

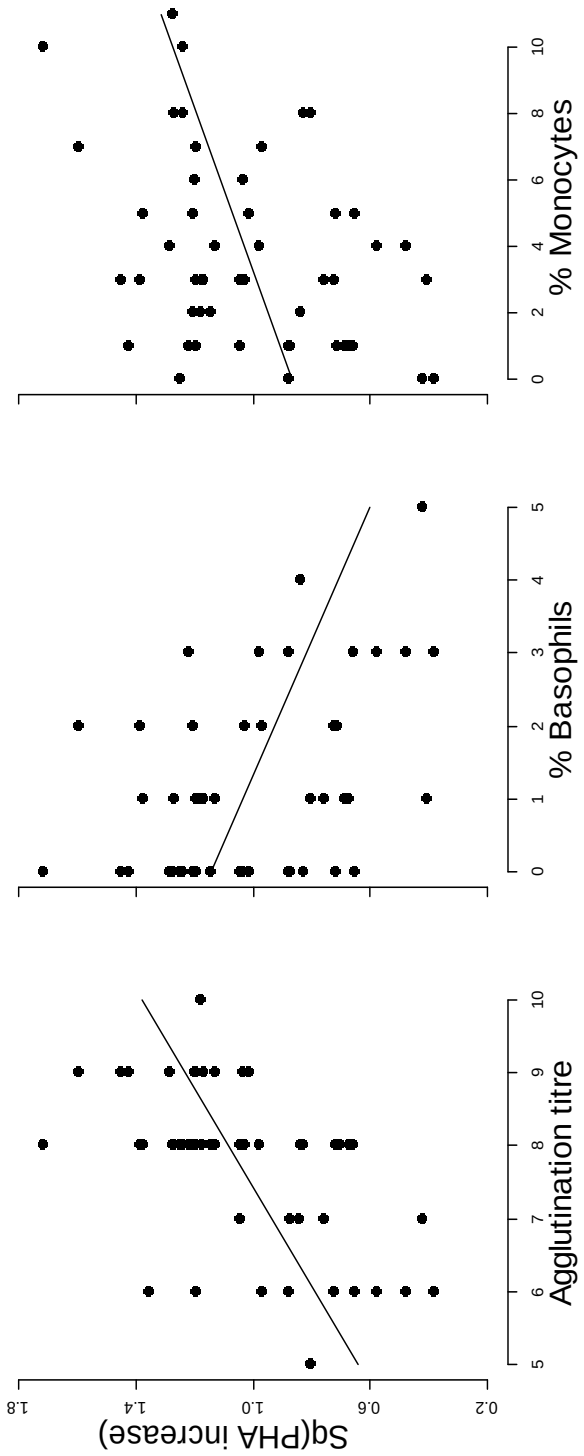

Figure 9 Significant relationships between PHA induced wing-web swelling and haematological indices for *P. krameri*. Regression lines using predicted values from generalised linear models from Table 14 are shown to illustrate the direction of significant relationships.

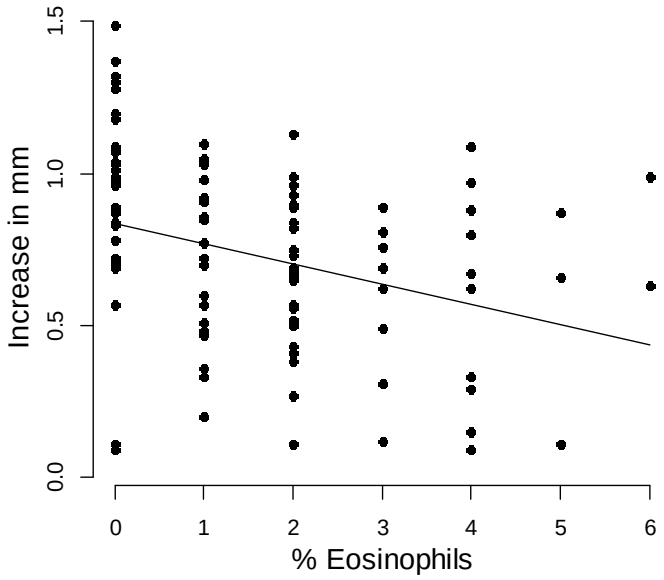

Figure 10 Relationship between eosinophil concentration and wing-web swelling among *P. echo*. Regression line drawn using predicted values from generalised linear model to illustrate significant relationship detailed in Table 14.

Table 1 Matrix of correlation coefficients of molecular measures for both species. Below diagonal represents P. krameri, above diagonal represents P. echo. All P values <0.001.

	SH	HL	IR
SH		-0.97527	-0.97949
HL	-0.97906		0.967607
IR	-0.97201	0.967049	

SH Standardised heterozygosity
HL Homozygosity by loci
IR Internal relatedness

Table 2 Multilocus heterozygosity (MLH) measures for both species and results of T-test comparisons.

	P. echo		*P. krameri*				
MLH	**mean**	**sd**	**mean**	**sd**	**t**	**d.f**	**P**
SH	1.02	0.19	0.99	0.20	0.29	115.69	0.77
HL	0.33	0.12	0.29	0.14	1.19	109.76	0.06
IR	0.02	0.17	0.0008	0.18	0.75	115.31	0.46

For P. echo on only 14 loci; mean SH = 1.00, HL = 0.33, IR = 0.01.
SH Standardised heterozygosity
HL Homozygosity by loci
IR Internal relatedness

Table 3 Values of immune function variables for each species. Bold text indicates significantly higher mean values from T-tests.

Immune response variable	*P. echo*			*P. krameri*					
	n	**mean**	**SD**	**n**	**mean**	**SD**	**t (W)**	**d.f.**	**P**
PHA	113	0.73	0.32	56	**1.14**	0.63	-4.58	69.56	<0.001
PCV	102	0.38	0.03	56	**0.41**	0.03	-4.21	93.33	<0.001
H:L	113	0.84	0.51	54	0.83	0.54	0.03	99.23	0.98
Eosinophil†	113	1.63	1.55	54	1.20	1.35	1.87 (3599)	117.62	0.07 (0.09)
Basophil†	113	**1.80**	1.59	54	1.18	1.27	2.70 (3819)	126.84	<0.01 (0.01)
Monocyte†	113	2.49	2.52	54	**3.89**	2.86	-3.08 (2150)	92.90	<0.01 (<0.01)
Lysis†	108	0.76	0.77	54	**1.41**	0.77	-5.07 (1677)	106.91	<0.001 (<0.001)
Agglutination	108	7.80	1.15	54	7.78	1.09	0.20	110.62	0.84

† Results are presented for both Welch's T-test and Wilcoxon rank sum test for non-parametric data (in parentheses).
PHA Phytohaemagglutinin
PCV Packed cell volume
H:L Heterophil/ lymphocyte ratio

Table 4 Wing-web measurements in mm.

	Pre (SD)	Post (SD)	Increase (SD)
P. echo	0.53 (±0.09)	1.23 (±0.35)	0.73 ±(0.32)
P. krameri	0.52 (±0.12)	1.66 (±0.60)	1.14 (±0.62)

Table 5 Relationships between standardised heterozygosity (SH) and immune variables for both species including slope (β), and standard error (SE) derived from generalised linear mixed models. Chi squared statistic (χ^2) and P values represent significance of removing the explanatory term in each model as tested by likelihood ratio tests.

Immune response variable	Explanatory variable	*P. echo*				*P. krameri*			
With nest ID as random effect		**β**	**SE**	**χ^2**	**P**	**β**	**SE**	**χ^2**	**P**
PHA†	SH	0.05	0.15	0.08	0.78	0.40	0.19	4.00	**0.04**
PCV†	SH	0.3^{-03}	0.01	$0.\ 3^{-03}$	0.97	-0.02	0.02	0.49	0.48
H:L†	SH	0.24	0.28	0.75	0.39	-0.35	0.33	1.03	0.31
Eosinophils‡	SH	0.50	0.52	0.95	0.33	0.47	0.85	0.30	0.58
Basophils‡	SH	-0.57	0.50	1.3	0.25	0.10	0.90	0.01	0.91
Monocytes‡	SH	0.43	0.51	0.73	0.40	0.28	0.5	0.30	0.59
Lysis §	SH	0.61	0.88	0.53	0.47	5.56	6.13	0.45	0.50
Agglutination †	SH	0.15	0.58	0.07	0.80	0.89	0.77	1.12	0.29

† GLMMs with Gaussian error structure.
‡ Zero inflated GLMMs with poisson error structure
§ GLMMs with binomial error structure.

Table 6 Effects of each predictor variable on individual level response to PHA using generalised linear mixed models and model averaging. Bold text indicates a significant effect where lower and upper confidence levels (CIL, CIU) around the slope do not include zero.

Response: PHA increase	Predictor	β	SE	CIL	CIU	Relative importance
P. krameri	Intercept	1.41	0.10	1.21	1.62	
	Sex (Male)	-0.52	0.14	**-0.79**	**-0.26**	0.96
	SH	0.34	0.16	**0.02**	**0.65**	0.43
	Hatch day	0.39	0.20	-0.01	0.79	0.42
	Clutch size	-0.36	0.20	-0.75	0.04	0.31
	Sex (Male):SH	-0.29	0.30	-0.88	0.30	0.07
	Body mass	0.21	0.16	-0.11	0.53	0.09
P. echo	Intercept	0.87	0.04	0.80	0.94	
	Body mass	0.09	0.07	-0.05	0.23	0.09
	SH	-0.08	0.07	-0.22	0.06	0.08
	Hatch day	-0.01	0.07	-0.16	0.13	0.04
	Sex	-0.01	0.07	-0.15	0.13	0.04
	Wing	0.00	0.07	-0.14	0.14	0.04
	Clutch size	0.00	0.07	-0.14	0.14	0.04

Table 7 Effects of each predictor variable on individual level heterophil to lymphocyte ratio (H:L) using generalised linear mixed models and model averaging.

Response: H:L	Predictor	β	SE	CIL	CIU	Relative importance
P. krameri	Intercept	-0.36	0.07	-0.49	-0.22	
	Wing	-0.26	0.13	**-0.52**	**-0.01**	0.26
	Body mass	0.23	0.15	-0.07	0.53	0.13
	Hatch day	0.17	0.14	-0.12	0.45	0.08
	Sex (M)	0.12	0.14	-0.16	0.4	0.06
	SH	0.11	0.15	-0.19	0.41	0.06
P. echo	Intercept	-0.26	0.13	-0.51	0.00	
	Sex (Male)	-0.19	0.11	-0.41	0.02	0.19
	Clutch size	0.16	0.16	-0.16	0.47	0.10
	TimeS2	-0.30	0.23	-0.76	0.15	0.09
	TimeS3	-0.43	0.26	-0.93	0.07	0.09
	TimeS4	0.51	0.49	-0.45	1.47	0.09
	Body mass	-0.13	0.14	-0.41	0.15	0.08
	SH	0.12	0.13	-0.14	0.37	0.07

Table 8 Effects of each predictor variable remaining after model averaging on individual level packed cell volume (PCV) using generalised linear mixed models.

Response: PCV	Predictor	β	SE	CIL	CIU	Relative importance
P. krameri	Intercept	0.41	0.01	0.39	0.42	
	Wing	0.03	0.01	**0.01**	**0.05**	0.49
	Hatch day	-0.03	0.01	-0.06	0.00	0.03
P. echo	Intercept	0.38	0.00	0.38	0.39	
	Sex (Male)	-0.03	0.00	**-0.04**	**-0.02**	1.00
	Wing	0.03	0.00	**0.02**	**0.04**	1.00

Table 9 Effects of important predictor variables on individual eosinophil count using generalised linear mixed models and model averaging.

Response: Eosinophils	Predictor	β	SE	CIL	CIU	Relative importance
P. krameri	Intercept	0.23	0.17	-0.11	0.57	
	Body mass	0.73	0.33	**0.07**	**1.39**	0.83
	Hatch day	-0.33	0.30	-0.94	0.29	0.3
	Wing	0.22	0.31	-0.41	0.84	0.18
	SH	0.29	0.35	-0.42	1.00	0.24
	Sex (Male)	-0.04	0.29	-0.63	0.55	0.11
P. echo	Intercept	0.68	0.19	0.29	1.08	
	Hatch day	-0.69	0.27	**-1.22**	**-0.16**	0.97
	SH	0.28	0.19	-0.11	0.67	0.4
	Time2	-0.25	0.32	-0.89	0.39	0.32
	Time3	0.12	0.33	-0.55	0.79	0.32
	Time4	0.95	0.51	-0.07	1.98	0.32
	Wing	0.06	0.30	-0.53	0.66	0.15
	Sex (Male)	-0.01	0.22	-0.45	0.44	0.15
	Body mass	0.07	0.25	-0.43	0.57	0.15

Table 10 Model averaging results of generalised linear mixed models of basophil count.

Response: Basophils	Predictor	Slope	SE	CIL	CIU	Relative importance
P. krameri	Intercept	-0.56	0.27	-1.11	-0.02	
	Time	1.94	0.57	**0.78**	**3.09**	1.00
	Sex (Male)	-0.29	0.38	-1.07	0.49	0.15
	SH	0.21	0.39	-0.58	0.99	0.13
	Hatch day	0.13	0.42	-0.72	0.97	0.12
	Body mass	0.02	0.40	-0.80	0.84	0.11
	Wing	-0.01	0.35	-0.71	0.70	0.11
P. echo	Intercept	0.10	0.21	-0.32	0.52	
	Hatch day	0.41	0.25	-0.09	0.90	0.52
	Wing	0.34	0.28	-0.22	0.89	0.37
	Time	0.38	0.37	-0.36	1.12	0.35
	Sex (Male)	-0.16	0.27	-0.70	0.38	0.20
	Body mass	0.01	0.29	-0.57	0.59	0.14
	SH	0.00	0.28	-0.57	0.56	0.14

Table 11 Model averaging results of generalised linear mixed models of monocyte count.

Response: Monocytes	Predictor	Slope	SE	CIL	CIU	Relative importance
P. krameri	Intercept	1.38	0.14	1.11	1.66	
	Sex	0.26	0.17	-0.07	0.59	0.89
	SH	0.26	0.23	-0.20	0.72	0.89
	Body mass	0.37	0.19	-0.01	0.75	0.45
	Sex (Male:SH)	-1.33	0.44	**-2.20**	**-0.46**	1.00
	Wing	0.17	0.20	-0.21	0.56	0.21
	Hatch day	0.12	0.24	-0.35	0.59	0.14
	TimeS2	-0.35	0.32	-0.98	0.29	0.04
	TimeS3	-0.29	0.28	-0.83	0.26	0.04
	TimeS4	-0.62	0.25	-1.10	-0.13	0.04
P. echo	Intercept	1.10	0.14	0.83	1.37	
	Hatch day	-0.52	0.26	**-1.03**	**-0.01**	0.75
	SH	0.32	0.18	-0.03	0.67	0.63
	Wing	0.29	0.19	-0.09	0.67	0.47
	Body mass	-0.24	0.21	-0.66	0.19	0.30
	Sex (Male)	0.08	0.16	-0.24	0.40	0.20
	TimeS2	0.33	0.33	-0.31	0.98	0.02
	TimeS3	-0.17	0.37	-0.89	0.55	0.02
	TimeS4	-0.02	0.69	-1.38	1.33	0.02
	Sex (Male):SH	0.23	0.28	-0.32	0.78	0.02

Table 12 Predictors of complement mediated lysis titre from generalised linear mixed models after model averaging.

Response: Lysis	Predictor	β	SE	CIL	CIU	Relative importance
P. krameri	Intercept	0.47	0.43	-0.37	1.30	
	Wing	0.65	0.74	-0.81	2.11	0.19
	Body mass	0.58	0.81	-1.00	2.17	0.22
	SH	-0.50	0.83	-2.11	1.12	0.16
	Sex (Male)	0.39	0.73	-1.04	1.82	0.11
	Hatch day	0.21	0.87	-1.51	1.92	0.10
P. echo	Intercept	-1.04	0.36	-1.75	-0.33	
	Time	-2.21	0.86	**-3.89**	**-0.53**	1.00
	Wing	-1.03	0.73	-2.45	0.40	0.44
	Sex (Male)	0.85	0.69	-0.50	2.21	0.36
	Hatch day	-0.72	0.70	-2.09	0.64	0.29
	Clutch size	-0.36	0.81	-1.95	1.23	0.16
	SH	0.02	0.66	-1.27	1.30	0.13
	Body mass	-0.19	0.70	-1.55	1.17	0.15

Table 13 Predictors of agglutination by natural antibodies from generalised linear mixed models after model averaging.

Response: Agglutination	Predictor	β	SE	CIL	CIU	Relative importance
P. krameri	Intercept	8.12	0.14	7.84	8.39	
	Wing	-0.57	0.26	**-1.09**	**-0.06**	0.63
	Clutch size	-0.21	0.27	-0.75	0.32	0.12
	SH	-0.18	0.30	-0.77	0.42	0.11
	Hatch day	0.16	0.30	-0.43	0.75	0.12
	Sex (Male)	-0.09	0.27	-0.61	0.43	0.06
	Body mass	0.10	0.28	-0.45	0.66	0.10
P. echo	Intercept	8.17	0.14	7.90	8.44	
	Clutch size	-0.51	0.31	-1.12	0.10	0.47
	Wing	0.44	0.28	-0.10	0.99	0.42
	SH	-0.23	0.28	-0.78	0.32	0.14
	Hatch day	0.17	0.29	-0.40	0.75	0.13
	Sex (Male)	0.16	0.28	-0.39	0.71	0.12
	Body mass	-0.08	0.30	-0.66	0.50	0.11

Table 14 Correlation matrix and variance inflation factors (VIF) of immune function variables for both species. Above diagonal is *P. krameri*, below diagonal is *P. echo*. Abbreviations: phytohaemagglutinin response (PHA), heterophil to lymphocyte ratio (H:L), packed cell volume (PCV), eosinophil concentration (Eosin), basophil concentration (Baso), monocyte concentration (Monoc), complement mediated lysis (Lys), agglutination by natural antibodies (Agg).

	PHA	H:L	PCV	Eosin	Baso	Mono	Lys	Agg	VIF
PHA		0.16	-0.35	0.06	-0.38	0.32	0.00	0.54	1.80
H:L	0.02		-0.20	0.15	0.20	0.11	-0.47	0.01	1.63
PCV	-0.13	0.29		-0.05	0.37	-0.26	0.12	-0.24	1.35
Eosin	-0.15	0.16	0.12		0.11	0.05	0.06	-0.08	1.08
Baso	-0.21	-0.04	-0.01	0.03		-0.34	0.04	-0.26	1.60
Mono	0.07	0.05	0.20	0.16	-0.27		-0.16	0.05	1.26
Lys	0.19	0.03	-0.04	0.12	-0.08	0.27		0.19	1.47
Agg	0.05	0.06	-0.01	0.11	-0.15	0.02	0.44		1.58
VIF	1.13	1.12	1.17	1.11	1.17	1.27	1.43	1.31	

Table 15 Haematological predictors of PHA increase for individuals across both years. Results of model averaging of generalised linear mixed models.

Response: PHA increase	Explanatory variable	Slope	SE	CIL	CIU	Relative importance
P. krameri	Intercept	1.04	0.03	0.98	1.11	
	Agglutination	0.30	0.07	**0.16**	**0.45**	1.00
	Basophil	-0.19	0.07	**-0.33**	**-0.04**	0.59
	Monocyte	0.17	0.07	**0.03**	**0.31**	0.54
	PCV	-0.08	0.07	-0.22	0.05	0.08
P. echo	Intercept	0.73	0.04	0.65	0.80	
	Eosinophil	-0.14	0.06	**-0.26**	**-0.02**	0.47
	Lysis	0.10	0.06	-0.02	0.22	0.16
	Basophil	-0.08	0.06	-0.20	0.04	0.06

Chapter 5. Conservation reintroductions: Can genotype and immunological phenotype be used to select individuals for reintroduction?

Abstract

Conservation reintroductions often lack sufficient post-release monitoring, consistently applied methodologies and *a priori* defined targets which allow objective assessment. As such many attempts fail after considerable input of human and financial resources. The ability to 'cherry-pick' juvenile individuals for release which display traits associated with increased establishment success and survival probability would benefit reintroduction biology. This study combines data concerning individual-level immunocompetence, multilocus heterozygosity, survival and BFDV (beak and feather disease virus) infection status from a managed and monitored population of endangered parakeets to identify such individuals. Future reintroductions of this species are planned and this research identifies several key considerations for selection of individuals for a release cohort. Results suggest that recruitment probability is biased towards male fledglings. The recruitment probability of fledglings of non-supplementary fed adults was negatively associated with hatch day whilst that of supplementary fed broods was positively associated with hatch day. Infection status of BFDV was not predicted by individual heterozygosity, and was not associated with any of the variables associated with immune function. Based on these results recommendations are given for selecting individuals for a release cohort which would maximise establishment success and population persistence.

5.1 Introduction

Interventional and intensive management of populations is becoming increasingly necessary as a last resort to prevent the extinction of species in human-altered landscapes. Conservation reintroductions - the release of an organism into a part of its native range from which it has become extirpated (IUCN, 1987) - have become a vital tool in efforts to halt the

declining trend in global biodiversity (Butchart et al., 2010). However, the *a priori* identification of individuals for release that could maximise the chances of a successful reintroduction is rarely carried out (Seddon, 1999, Fischer and Lindenmayer, 2000, Armstrong and Seddon, 2008, Soorae, 2010, Sutherland et al., 2010). Nevertheless, methodologies for achieving such bespoke selection of individuals based upon objective scientific data may prove valuable for enhancing the successful establishment, overall survival and recruitment of individuals upon their reintroduction.

Armstrong and Seddon (2008) propose 10 key questions for consideration to encourage reintroduction biologists to adopt a more integrated approach to reintroduction planning, the first four of which are concerned with the establishment probability and persistence of the released population:

1. How is establishment probability affected by size and composition of the group?

2. How are post-release survival and dispersal affected by pre- and post-release management?

3. What habitat conditions are needed for persistence of the reintroduced population?

4. How will genetic makeup affect persistence of the reintroduced population?

The probability of successful initial establishment and long term population persistence are underpinned by, amongst various factors, elements of genetic diversity and parasite management. The term 'parasites' is used here to broadly describe infectious agents including viruses, protozoa, ectoparasites and helminths (Cunningham, 1996, Sainsbury and Vaughan-Higgins, 2012). The potential impacts of infectious diseases on both the founder population and species present in the receiving habitat are increasingly being considered (Ballou, 1993, Beck et al., 1993, Cunningham, 1996, Ewen et al., 2012, Sainsbury et al., 2012, Sainsbury and Vaughan-Higgins, 2012), whilst the importance of maintaining genetic diversity has been widely recognised by conservation biologists for some time (Allendorf,

1986, Haig et al., 1990, Leberg, 1993, Frankham, 1995, Jamieson et al., 2006). However, small populations (a trait of most reintroduced populations) are characterised by reduced genetic diversity (Lande, 1988, Frankham, 1997) and the negative effects of this can be intensified among endangered populations due to inbreeding. Consequently any further bottlenecks as a result of reintroduction potentially increases the risk of inbreeding depression (Groombridge et al., 2000, Keller et al., 2001, Taylor and Jamieson, 2008, Keller et al., 2012). Maximising the genetic representation of a species within a release group is therefore a key consideration in order to minimise the negative effects of reduced genetic diversity (Reed and Frankham, 2003), reduced evolutionary adaptive potential (Franklin and Frankham, 1998) and ultimately, increased extinction risk (O'Grady et al., 2006).

Inbreeding and loss of genetic diversity are also associated with increased disease susceptibility (Acevedo-Whitehouse et al., 2003, Spielman et al., 2004a, Ilmonen et al., 2008) and the success of many high profile reintroduction programmes has been challenged by the threat of novel or endemic infectious diseases including that of the black-footed ferret (*Mustela nigripes*) (Thorne and Williams, 1988), the pink pigeon (*Nesoenas mayeri*) (Swinnerton et al., 2005a), the grey wolf (*Canis lupus*) (Almberg et al., 2009) and the Canadian lynx *(Lynx canadensis*) (Wild et al., 2006). However, identifying and quantifying the potential threat of infectious diseases is a challenge in reintroduction biology; often these threats are not realised until post-reintroduction (Ewen et al., 2007, Raisin et al., 2012). Additionally, determining prevalence is a very poor indicator of the impact of disease on a population (McCallum and Dobson, 1995), which highlights the uncertainty of such initiatives and the importance of systematic post-reintroduction monitoring (Armstrong and Seddon, 2008, Sutherland et al., 2010). More subtly, the maintenance of delicate host-parasite relationships often depends upon host population density (Lyles and Dobson, 1993) and therefore when host populations become fragmented and reduced to levels below that required for the maintenance of such relationships, parasite populations may be extinguished (Cunningham, 1996).

This result of reduced population size among endangered species can have the effect of making populations immunologically naive and therefore more prone to the adverse effects of subsequent epidemics (Cunningham, 1996) which may not be apparent until, through conservation intervention for example, the population recovers, allowing previously lost host-parasite relationships to return. Added to this, the threat of extinction as a result of disease among re-established populations increases if a reservoir of disease-tolerant hosts is available, thereby interrupting previous host-parasite dynamics and providing the necessary foundation for epidemics to occur (Hudson et al., 2006, Smith et al., 2009). Therefore, conducting wildlife reintroductions of genetically-impoverished endangered species into disease landscapes is not straightforward but establishment success may be increased by selecting individuals based upon some measure of immunity (Møller and Cassey, 2004).

Incorporating elements of ecological immunology or 'ecoimmunology' into the field of reintroduction biology offers the potential to tackle some of these issues. Ecological immunologists attempt to characterise an individual's susceptibility or tolerance to parasites and pathogens in free-living populations whilst considering the life history trade-offs and costs associated with mounting an immune response (Norris and Evans, 2000, Sheldon and Verhulst, 1996, Pedersen and Babayan, 2011, Martin et al., 2011). Measuring immune function within natural populations in the face of environmental and ecological stressors can form a vital contribution to understanding how individuals allocate finite resources in order to maximise their fitness, and is central to explaining host life history strategies and their evolution. Consequently, a portfolio of techniques designed to quantify an individual's immune function in natural populations is becoming increasingly accessible (reviewed by Boughton et al., 2011) and employing these techniques when designing reintroductions potentially allows the identification of those individuals which, when faced with an immune challenge, will respond in the most robust way and thereby maximise fitness and overall survival of the release cohort.

Of course, establishing self-sustaining populations through reintroductions can only be achieved when the principal threat of extinction has been removed (Griffith et al., 1989, Seddon, 1999) which implies that, when the principal threat is defined by habitat destruction or degradation for example, some form of long term management is required to maintain population viability. The success of such a reintroduction programme must therefore be measured by reproductive output and population growth relative to the level of management required and the sustainability of that management. Examples of avian reintroduction programmes which incorporate some level of ongoing management are numerous and include monitoring and managing lead poisoning among Californian condors (*Gymnogyps californianus*) (Walters et al., 2010), nest box provisioning for Mauritius kestrels (*Falco punctatus*) (Nicoll et al., 2003) and several cases of supplemental feeding (Elliott et al., 2001, Jones, 2004, Edmunds et al., 2008, Chauvenet et al., 2012). These reintroduction successes (and the lessons learned from documented failures) can be used as templates in the development of methods to establish new populations and can provide valuable insight regarding the selection of founding individuals owing to the existence of marked individuals, a feature of many conservation reintroduction programmes. Such marked populations are often closely monitored providing detailed individual level life history and pedigree data. Combining this knowledge with indices of individual level immune function and genetic diversity can allow practitioners to 'cherry-pick' individuals in order to maximise the chance of successful establishment and long term persistence.

The endangered Mauritius (or echo) parakeet (*Psittacula echo*) has, through an intensive conservation programme, been recovered from the immediate risk of extinction and represents an example of a monitored and managed, reintroduced population. This species exists as a small fragmented population that persists despite the outbreak of a highly infectious disease in 2004/5 which continues to affect mainly sub-adult individuals (Kundu et al., 2012). The establishment of new populations on Mauritius has long been considered in an effort to further increase numbers and areas of potentially suitable habitat exist which

would not be colonised through natural dispersal (Jones and Duffy, 1993). Moreover, it has been suggested that this species could be sufficiently closely related to the extinct Reunion parakeet *P. eques eques* on the neighbouring island of La Réunion to represent a taxon substitute; ideally placed to fill (via introduction from Mauritius) the empty ecological niche left by that extinct parakeet species (Temple, 1981, Cheke, 2008, Hansen, 2010).

Mauritius parakeets have been known to live for >17 years in the wild, they generally reach adult maturity at three years old and form monogamous pair bonds which may persist for several breeding seasons (MWF, 1996-2012). Before intensive management began a male biased sex skew was apparent and it was not uncommon for a female and dominant male to allow 'helpers' at the nest site, often male individuals who would contribute to feeding offspring (Jones, 1987). More recently this is less common and the adult sex ratio has reached approximate parity (MWF, 1996-2012). Extra pair paternity is thought to be low and was recently genetically verified at ~ 11% (Raisin, 2010). Current (2012) population numbers are estimated at over 500 individuals with ~80 known breeding pairs; deforestation, habitat degradation, and the introduction of non-native predators and competitors had reduced the population no more than 20 individuals in the 1980s (Jones, 1987, Jones and Duffy, 1993, Safford and Jones, 1998, MWF, 1996-2012). Population numbers were initially bolstered in the 1990s by the development of captive breeding and hand-rearing techniques and the manipulations of wild broods which included rescuing underdeveloped chicks from failing nests. The subsequent introduction of both artificial nestboxes and supplemental feeding further promoted population growth and continues to do so (Jones and Merton, 2012, MWF, 1996-2012). Between 1997 and 2005 a total of 139 individuals were released to augment the wild population before an outbreak of psittacine beak and feather disease (PBFD) in 2004/5 prompted the end of the intensive rescue, rear and release period, a decision that was made to minimise management-mediated disease spread. The disease outbreak is assumed to have originated in the last release cohort of 36 hand-reared individuals in 2004/5, of which only 4 (11%) are known to have survived to two years

(MWF, 1996-2012). Of the 103 individuals which were released prior to this cohort, 50 (49%) subsequently survived to enter the breeding population (MWF, 1996-2012).

An important factor in facilitating post-release monitoring in this species was the advent of techniques designed to train young, hand-reared individuals to take supplemental food from feeding stations which were specifically designed to exclude other bird species. Once fledged and released, these reintroduced birds were easily observed during their regular attendance at these feeding stations, and this enabled the conservation team to take a daily 'register' of the released individuals and to monitor those which returned to the feeding stations. This ongoing practice facilitated the collection of daily records of attendance (and health monitoring) for all supplementary fed individuals. Since 2005 the population has been managed on a less intensive scale, but nevertheless continues to grow aided by the provisioning of artificial nestboxes and supplemental food. Around 80% of all individuals known to be alive in 2011 use the supplementary food to varying degrees, some are ever-present regulars whereas others are opportunistic or sporadic visitors and may only be recorded once in a month or less (MWF, 1996-2012). The vast majority of nesting attempts occur in artificial nestboxes (73% in 2011) and accessible natural tree cavities and therefore nearly all attempts are regularly monitored and the outcome recorded, resulting in an extensive dataset concerning individual level reproductive success of this once critically endangered species.

Almost all (~95%) breeding adults and their fledglings are individually identifiable owing to unique colour combinations of metal rings fitted to each nestling as part of this monitoring process. Furthermore, blood samples exist for the majority of the contemporary population and since 2005 blood samples have been collected from every known fledgling with very few exceptions resulting in the existence of genotype information for approximately 900 individuals spanning more than twenty years (Raisin et al., 2012, Chapter 3, this thesis) and PBFD infection status for over 400 since 2005 (Kundu et al., 2012).

The possibility of forthcoming releases of this species to establish new populations represents a unique opportunity to maximise the probability of establishing reintroduced populations by selecting individuals most likely to persist in a disease environment. Additionally, this study aims to provide empirical results that highlight the value of post-release monitoring and benefit the discussion concerning the issue of how to optimise levels of reintroduction success. To this end I address four key questions intended to identify suitable sub-adult individuals with which to use as founders for the forthcoming reintroduction of Mauritius parakeets:

1. How is recruitment affected by environmental, management and genetic factors?
2. Is PBFD status predicted by environmental, management and/or genetic factors?
3. How are indices of individual immune function affected by environmental, management and genetic factors?
4. Ultimately, can I identify which individuals would be more suitable for reintroduction based upon a combination of genetic, immunological and life history factors?

5.2 Materials and methods

5.2.1 General methods

The study was conducted using data collected over a period of 7 years, from 2005 to 2011 in the Black River Gorges National Park, Mauritius (20º23'S, 57º23'E). For the purposes of this study a nesting attempt was defined as the occupation of a nest site by an adult female individual that resulted in at least one egg being laid. Secondary clutches, laid as a result of initial clutch or brood failure were included and recorded as separate nesting attempts. The regular monitoring and accessing of nest sites meant that clutch size, brood size at hatching and number of fledglings was recorded for nearly all known nesting attempts with a few exceptions where nesting attempts were discovered late or cavity access was impossible, therefore in these instances clutch size and number hatched were not known; these cases

were not included in the analyses. The number of fledglings from each nesting attempt was recorded as the number of individuals alive in the nest at the last monitoring check (~10 days before fledging) minus any dead individuals discovered at a post-fledging monitoring check (~10 days after fledging) and therefore represents the brood size at fledging. Given that the breeding season for this species spans the end of the calendar year; eggs are usually laid between September and November with individuals fledging between December and February, I refer to year hatched or fledged as the year in which an individual was produced as an egg.

Provisioning of supplemental food occurred at two sites throughout this study period; these two sites which were used as release locations for population augmentation were monitored regularly in order that adult individuals which take supplementary food could be identified. I used this data to assign a binary category of 'supplemental fed' or 'non-supplemental fed' to each nesting attempt. If at least one of the adult pair were known to take supplemental food then the nesting attempt was considered supplementary fed, therefore nesting attempts categorised as non-supplementary fed featured a breeding pair which relied exclusively on naturally available food resources. Each individual nestling was also categorised in this manner. Whilst observational data of sightings at feeding stations suggest that some individuals use this resource more than others, the different levels of consumption are difficult to quantify and are not considered in this study. However, I did calculate the Euclidean distance of each nest site to the nearest feeding station and each nest site was identified as a natural cavity or an artificial box.

Hatch order was determined by checking size, weight and other developmental signs for each nestling within a brood during monitoring checks timed to coincide within a day or two of hatching and the date of hatch for each individual was estimated to ±2 days using these cues. I used this data to assign each individual a hatch day relative to the first individual which hatched in any given year. Therefore, the first individual known to hatch in each year was assigned a hatch day of zero and the hatch day of each subsequent individual was the

number of days after the first. Age of female parent was derived from the detailed field-studbook kept for this species but I did not consider age of male parent owing to the numerous cases where males were unidentified or where an identifiable male's age was unknown owing to it being first recorded as an adult.

I used previously described molecular methods (Chapter 3) to determine genotype and gender of each individual where blood samples were available using 21 microsatellites developed for this species by Raisin et al., (2009) and the sexually dimorphic primer set Z002B (Dawson, 2007). Multi-locus heterozygosity (standardized heterozygosity; SH) was calculated following Coltman et al., (1999) from these genotypes using the package Rhh (Alho et al., 2010) in the R environment (R Development Core Team, 2012) and individual inbreeding coefficients (*f*) were derived from detailed pedigree records using the R package Pedigree (R Development Core Team, 2012). Owing to the existence of many founding, unknown or unidentified individuals in the pedigree for which *f* could not therefore be determined, inbreeding coefficients were only assigned to individuals where the depth of pedigree allowed us to identify all four grandparents resulting in minimum estimates of inbreeding coefficients (Keller, 1998, Jamieson et al., 2003). All abbreviations and acronyms used in the text, tables and figures are summarised in Box 1.

Box 1. Summary of abbreviations and acronyms used in the text, figures and tables.

AIC	Akaike's information criterion
BFDV	Beak and feather disease virus
CIL	Lower confidence interval
CIU	Upper confidence interval
f	Inbreeding coefficient
GLM	Generalised linear model
GLMM	Generalised linear mixed model
HFC	Heterozygosity-fitness correlation
H:L	Heterophil to lymphocyte ratio
HL-HA	Haemolysis haemagglutination assay
ICC	Intra-class correlation coefficient
IT	Information theoretic
NAbs	Natural antibodies
PBFD	Psittacine beak and feather disease
PCV	Packed cell volume
PHA	Phytohaemagglutinin
RI	Relative importance
RRBC	Rabbit red blood cells
SH	Standardised heterozygosity
Sub-pop	Subpopulation
Supp	Supplementary fed
Non-supp	Non-supplementary fed
VIF	Variance inflation factor
ZIGLMM	Zero-inflated generalised linear mixed model

An outbreak of psittacine beak and feather disease (PBFD) in 2004/5 initiated the routine collection of blood samples from all nestlings in an effort to identify infection status of the causative agent (beak and feather disease virus, (BFDV)) and population-level prevalence. BFDV is a vertically and horizontally transmitted circovirus which is known to mostly affect sub-adults, many of which die of secondary infections associated with the onset of disease (Todd, 2004). Infection status was determined via PCR and visualised on agarose gel for 45-day old nestlings between 2005 and 2009 (see Kundu et al., (2012) for a detailed methodological description).

5.2.2 Productivity and recruitment

Productivity and recruitment was summarised using all available nesting attempt records collected between 2005 and 2011. Data collected before this time was not included in these analyses due to the intensive nature of the conservation programme prior to this period and the effects that procedures such as cross-fostering, hand-rearing and reintroductions may have had upon productivity and recruitment. Recruitment for each annual cohort was determined by the number of fledglings from any given year which had subsequently been identified as adult individuals attempting to nest. Most individuals attempt to breed for the first time at three years old (although some as early as two) and therefore the recruitment data features a considerable time-lag. When summarising recruitment levels I only used the data for individuals produced during the three breeding seasons 2005, 2006 and 2007 in order to maximise the number of recruits detected. Whilst three years old is the usual age at first breeding, many individuals are considerably older when they attempt to breed for the first time and in very rare cases fledglings may disappear for many years and are assumed dead before being found occupying a nest site (MWF, 1996-2012). I therefore calculated recruitment of fledglings produced in 2008 but did not use these figures or results in any further analysis.

5.2.3 Predicting the probability of recruitment and BFDV infection status

I used data concerning nesting environment, gender, individual multilocus heterozygosity, individual inbreeding coefficient and BFDV infection status to identify important factors which predict recruitment probability of individuals which fledged between 2005 and 2007. I extended this dataset to include all individuals where infection status was available, irrespective of recruitment, in order to identify any environmental or genetic predictors of infection status. This dataset therefore included nestlings produced during five consecutive breeding seasons between 2005 and 2009.

5.2.4 Variables of immune function and haematological parameters; field protocol

During the two breeding seasons 2009/10 and 2010/11 I collected data concerning individual level immune function, haematological parameters and morphological measurements from all 45 day old nestlings. Wing and tail length were recorded in addition to body mass. To assess the response of individual nestlings to an experimental immune challenge I followed the protocol of Smits et al., (1999) by administering a small amount of the mitogenic lectin, phytohaemagglutinin (PHA) to individuals of half of all known broods (selected randomly). Briefly, 20µl of a 5:1 PHA (Sigma-Aldrich L8754) in phosphate buffer (PBS; Oxoid, Dulbecco A–BR0014G) solution was administered intradermally to the right wing-web of each nestling. At least three measurements of patagium thickness at the injection site were taken to the nearest 0.01mm using a pressure-sensitive micrometer (Silverline 282378) prior to PHA exposure and the average of these measurements was taken as the pre-injection thickness. After a period of 24 hours (±2 hrs) the tissue thickness at the injection site was measured in a similar manner and the response to the PHA challenge was recorded as the average post-injection measurement minus the average pre-injection measurement. S.J.T was responsible for all PHA measurements and administering PHA. Blood samples were collected via jugular venipuncture for all individuals to facilitate the assessment of cellular and humoral components of the immune system as described below. Blood was collected from PHA-challenged individuals during the post-injection measurement procedure. The reasons for collecting blood samples from these individuals after the PHA challenge were twofold; this reduced the handling time of each nestling during the same nest site visit and also gave us the opportunity of studying the physiological response to PHA by comparing cellular and humoral variables of immune function between those individuals which had received the PHA challenge and those which had not.

Each blood sample was divided three ways to facilitate a variety of subsequent analyses:

1. Fresh, whole blood was taken directly from the syringe and at least two blood slides were made using the wedge method (Campbell and Ellis, 2006, Clark et al., 2009). These were air dried and fixed in 100% methanol for staining and leukocyte profiling.

2. Two to three drops of whole blood were stored in absolute ethanol to enable DNA extraction for genotyping and disease profiling.

3. The remainder of the blood, ~500µl was transferred to a heparinised collection tube (Teklab H1230) and gently agitated to prevent coagulation to facilitate the assessment of humoral immune function by natural antibodies (NAbs) and complement mediated lysis using the haemolysis-haemagglutination (HL-HA) technique (Matson et al., 2005).

Samples were transferred back to the field laboratory where the blood smears were stained with Leishman's stain solution (Fisher PB05) following the manufacturer's guidelines. A small amount of the heparinised blood was used to derive individual packed cell volume (PCV) using a small haematocrit centrifuge. The remainder of each blood sample was centrifuged at approximately 8000rpm for five minutes before plasma was extracted and stored at -80ºC until it could be transported back to the UK.

5.2.5 Variables of immune function and haematological parameters; laboratory protocol

Blood smears were initially assessed with a microscope at x400 magnification and only the best quality slide for each individual was selected for leukocyte profiling based upon the appearance of smudge cells and monolayer consistency. Heterophils, lymphocytes, basophils, monocytes and eosinophils were differentiated following Ritchie et al., (1997), Campbell and Ellis (2006) and Clark et al., (2009) and the first 100 leucocytes from each slide were identified and counted at x1000 magnification. The number of heterophils and lymphocytes were used to derive the heterophil to lymphocyte ratio (H:L). S.J.T examined all blood smears blindly with respect to date of collection and individual identification.

Natural antibody levels and complement mediated lysis of foreign antigens was assessed using the general haemolysis-haemagglutination assay (Matson et al., 2005) for each nestling produced between 2009 and 2010. The assay was performed following the methods of Matson et al., (2005) with some modifications. Twenty-five microlitres of 1.0 M PBS (Oxoid, Dulbecco A – BR0014G) were added to all wells in columns 2-12 of a 96-well, U-bottom microplate (Sterilin 612U96). Twelve and a half microlitres of eight individual plasma samples were then added to columns 1 and 2 and a multi-channel pipette was then used to serially dilute the plasma solution between columns 2 and 11 resulting in dilutions ranging from 1:3 in column 2 to 1:59049 in column 11. Column 1 was used as a positive control given that it contained only plasma and column 12 as a negative control as it contained only PBS. In every third plate, a control of chicken plasma was used alongside that of parakeet nestlings to ensure assay consistency. Twelve and a half microlitres of a freshly prepared solution of 1% rabbit red blood cells (RRBCs) (Harlan Laboratories UK) in PBS were then added to each well of the microplate to initiate the assay. Plates were then incubated at 37ºC for 90 minutes before being scanned with a top-lit flatbed scanner in order to visualise and score the agglutination of RRBCs by natural antibodies. After a further 60 minutes at room temperature the plates were scanned for a second time in order to score the complement mediated lysis of RRBCs. Each individual nestling was therefore given two scores, one for agglutination and one for lysis, both of which corresponded to the last cell of the microplate in which each reaction was observed. S.J.T conducted all HL-HA assays blindly with respect to date of sample collection and individual identification.

5.2.6 Statistical analyses

All statistical analyses were performed within the programming environment R (R Development Core Team, 2012). Generalised linear models (GLMs) and generalised linear mixed models (GLMMs) were applied using the R package lme4 (Bates et al., 2010). For the years between 2005 and 2011 I investigated the effects of supplementary feeding on clutch size, chicks hatched and chicks fledged using separate GLMs including year as a fixed factor

to account for annual variation in breeding conditions. I was particularly interested in levels of productivity relative to 2005; the year an outbreak of PBFD occurred. Factorial GLMs were conducted with a Gaussian error structure and identity link with 'supplementary fed' added last as a two-level factor and included the interaction term of 'year ^{X} supplementary fed'. Sequential sums of squares (type I SS) were applied to account for the unbalanced nature of this data (Hector et al., 2010) and Tukey's HSD post-hoc tests for multiple comparisons were used to identify significant pairwise relationships.

I investigated the probability of fledgling recruitment between 2005 and 2007 using GLMMs with individual recruitment as a binary response variable in all models and nest ID fitted as a random factor to account for pseudoreplication attributable to the non-independence of siblings. A binomial error structure with a logit link function was applied. I conducted separate GLMMs to incorporate the effects of different sample sizes attributed to some variables predicted to be important factors associated with recruitment. It was not possible to genetically determine the sex or to derive PBFD infection status for all individuals and I therefore included these variables in separate models with reduced sample sizes. Initially, I investigated the effects of nesting environment on recruitment probability. Age of female parent (Dam age), hatch order, hatch day, standardised multilocus heterozygosity and brood size at fledging were all added as explanatory covariates with supplementary feeding as a two-level fixed factor (true or false). The second model included sex as a fixed factor and the interaction terms 'sex ^{X} supplementary fed' and 'sex ^{X} SH'. I then investigated the effects of BFDV infection using infection status as a fixed factor and the interactions with hatch order and SH before also investigating any interaction effects of infection status and sex.

I investigated the relationship between inbreeding coefficient (*f*) and standardised heterozygosity (SH) for all fledglings produced between 2005 and 2010 using a simple bivariate GLMM with Gaussian error structure and identity link. SH was used as the response variable and *f* as the single explanatory term with nest ID as a random effect. The

effects of inbreeding and genetic diversity on recruitment probability were then assessed separately as simple bivariate GLMMs for all fledglings produced between 2005 and 2007 using recruitment probability as a binary response with either *f* or SH as a single predictor variable. Binomial error structures and logit link functions were applied with nest ID as a random effect. The significance of each explanatory term was tested using a likelihood ratio chi-square test where the model including the explanatory covariate was tested against the null model.

Possible predictors of BFDV infection status were then investigated for all fledglings produced between 2005 and 2009. A GLMM was applied with a binary response variable indicating presence or absence of infection and therefore a binomial error structure and logit link were used. In this model I used the following explanatory variables: SH, nest type, hatch order, Dam age, supplementary fed, brood size at fledging, sex and year (using 2005 as the reference year) with the 'year ^{X} supplementary' fed interaction term.

Next, I investigated the effects that BFDV infection and experimental immunostimulation had on individual variables of immune function and haematological parameters among fledglings produced in 2009. I applied separate models to each measured component of immune function and each time used the same combination of explanatory variables; PHA challenged or not, BFDV infection status, and the interaction term. Where the response variables were packed cell volume (PCV), heterophil to lymphocyte ratio (H:L) and agglutination by NAbs a Gaussian error structure and identity were applied to GLMMs with nest ID added as a random effect to account for effects of a common nesting environment (and therefore parents) among siblings. Heterophil to lymphocyte ratio (H:L) was log-transformed to achieve residual normality. The significance of each predictor variable in the model was tested using likelihood ratio chi-square tests by testing the full model against the same model reduced by the predictor variable of interest. Where the response variables were counts of rare white blood cells or complement mediated lysis score I applied zero-inflated GLMMs (ZIGLMMs) using the glmmADMB package in R (Skaug et al., 2008) to account

for the zero-inflated distribution of these data, the difficulty in transforming such variables where true zeros are present (Martin et al., 2005, Bolker et al., 2009) and to avoid biased estimated parameters, standard errors and overdispersion (Zuur et al., 2009). Effects of individual inbreeding coefficient (*f*) on parameters of immune function were assessed for all fledglings produced between 2009 and 2010 which could be attributed an accurate *f* based on adequate pedigree data; i.e. where all four grandparents were known. Each immune function variable was modelled separately as before using GLMMs and ZIGLMMs where appropriate. In each model, *f* was the single explanatory predictor and its significance was tested using likelihood ratio chi-square tests.

Finally, I assessed the effects of various environmental, genetic, and morphological variables on each immune function and haematological parameter for fledglings produced in 2009 and 2010, again constructing separate models for each measured response variable using GLMMs and ZIGLMMs accordingly. Nest ID was added as a random effect term in all of these models and the variance explained by this was investigated by calculating intra-class correlation coefficients (ICC). The input variables and interaction terms for these models varied depending on the immune or haematological response variable in question. I used extensive exploratory analyses (Bolker et al., 2009, Zuur et al., 2010, Grueber et al., 2011a) including graphical inspection, correlation matrices and simple bivariate tests to identify the most appropriate set of explanatory covariates for each response in order that the final models were not overparameterised but included variables of interest and biologically plausible interaction terms. During this process I identified a low number of potentially influencing, outlying data points. The results presented here do not include any models where these data were found to significantly affect final model outcomes; they were eliminated and the models repeated.

Model selection, model averaging and multi-model inference

Where the GLMMs and ZIGLMMs included numerous explanatory input variables and predictor terms I adopted an information-theoretic (IT) approach to model selection (Burnham and Anderson, 2002, Johnson and Omland, 2004, Whittingham et al., 2006, Garamszegi, 2011). Unlike the stepwise approach to multiple regression IT analysis does not depend on a single best model derived from arbitrarily set significance levels to infer biological processes among inherently complex systems (Johnson and Omland, 2004, Garamszegi, 2011). By using Akaike's information criterion (AIC) (Akaike, 1973) all models within a candidate set, including a null model fitted only with an intercept, are directly compared based upon complexity and model fit (Burnham and Anderson, 2002). Models can therefore be ranked and evaluated according to their relative support from the observed data where the top ranking model represents the best fit (and the smallest AIC) and all subsequent models are scored according to their relative support using the difference in AIC (ΔAIC). Before producing the full candidate set of models I centred all the input variables to a mean of zero and a standard deviation of 0.5 following Gelman (2008) as this allows the standardisation of multiple predictor variables to a common scale therefore aiding the interpretation of parameter estimates of input variables measured on different scales (Gelman, 2008, Grueber et al., 2011a).

I used the R package MuMIn (Bartoń, 2009), to evaluate all candidate models in a given set and weight each one based on AIC_c (AIC adjusted for small sample size) and Akaike weights, and therefore each model was ranked with respect to goodness-of-fit and model selection uncertainty (Burnham and Anderson, 2002). I restricted the final model set before model averaging to all models where $\Delta AIC_c < 4$ in order to eliminate potentially implausible models with low AIC weights (Burnham and Anderson, 2002, Bolker et al., 2009, Grueber and Jamieson, 2011). Model averaging was then applied to the reduced model set to compute a weighted average of parameter estimates and their associated standard errors (SE). The relative importance of explanatory variables were then calculated by summing the

Akaike weights across all models in which the variable was present resulting in an estimate of probability that the variable of interest features in the best model. Averaged parameter estimates and unconditional SE are reported in all summary results of GLMMs and ZIGLMMs after model averaging.

5.3 Results

5.3.1 Summary of productivity and recruitment

Table 1 illustrates annual productivity of all known nesting attempts (breeding attempts where at least one egg was produced) between 2005 and 2011 and a summary of overall productivity based on pairs which are known to take supplemental food and those which are not.

Across all seven breeding seasons spanning 2005 to 2011, mean clutch size was not significantly affected by supplementary feeding ($F_{1,477}$ = 0.2.77, P = 0.10), there was no variation among years ($F_{6,478}$ = 120, P = 0.31) and no interaction effect ($F_{6,471}$ = 0.72, P = 0.63). There was no single effect of year on mean number of chicks hatched per clutch ($F_{6,478}$ = 1.81, P = 0.09) but supplementary fed pairs hatched on average 0.28 (CI 0.05-0.50) more chicks than non-supplementary fed pairs ($F_{1,477}$ = 6.00, P = 0.01). Additionally, there was a highly significant interaction effect of 'year ^{X} supplementary fed' ($F_{6,471}$ = 4.07, P = < 0.001). *Post-hoc* tests of multiple comparisons revealed that among supplementary fed pairs, the mean number of chicks hatched in 2005 was significantly lower than all years between 2007 and 2011 (2007: difference = 0.97, CI 0.15-1.78, P < 0.01; 2008: difference = 1.10, CI 0.29-1.91, P < 0.001; 2009: difference = 1.10 CI = 0.30-1.88, P < 0.001; 2010: difference = 0.93, CI 0.15-1.71; P < 0.01 2011: difference = 0.82, CI = 0.06-1.59, P < 0.02). The mean number of chicks hatched by non-supplementary fed pairs was not affected by year (all P values > 0.44). Supplementary feeding also had a significant effect on mean number of chicks fledged ($F_{1,477}$ = 21.5, P < 0.001) resulting in 0.53 (CI 0.31-0.76) more fledglings per nesting attempt. A significant effect of year on mean number of fledglings ($F_{6,478}$ = 2.62, P = 0.02)

was observed but there was no interaction effect ($F_{6,471}$ = 1.32, P = 0.24). *Post-hoc* tests revealed that the mean number of fledglings produced in both 2008 (difference = 0.61, CI 0.01-1.22, P = 0.04) and 2009 (difference = 0.61, CI 0.02-1.19, P = 0.02) was significantly greater than 2005 and that these differences were only apparent among nesting attempts of supplementary fed adults. Mean number of offspring recruited per brood was not significantly affected by year ($F_{2,174}$ = 0.23, P = 0.79) or supplemental feeding ($F_{1,173}$ = 0.64, P = 0.42). Similarly, mean number of offspring recruited per individual fledged was not significantly affected by year ($F_{2,115}$ = 0.47, P = 0.63) or supplemental feeding ($F_{1,114}$ = 0.23, P = 0.63).

5.3.2 Predictors of recruitment

Nesting environment, gender and disease status

Known nesting attempts recorded between 2005 and 2007 produced a total of 228 fledglings. Fifty-six of these were produced by non-supplementary fed adult pairs and 15 (27%) of these fledglings have subsequently been recruited into the breeding population. Supplementary fed pairs produced the majority 172 fledglings, 46 (27%) of which have subsequently been recorded as breeding individuals. Results of GLMMs to predict recruitment of individuals fledged from three consecutive years since 2005 are shown in Table 2. Nest ID did not explain significant variance in recruitment (ICC =0.16, 95% CI = -0.03-0.34). Sex was the only significant and reliable predictor of fledgling recruitment revealing that male individuals were more likely to be recruited into the breeding population than females. Exact binomial tests revealed that the sex ratio of individuals at fledging stage did not differ significantly from the hypothesised ratio of 50:50 (α = 0.05, P = 0.88), however the sex ratio of recruited individuals was significantly different from 50:50 (α = 0.05, P = 0.04) and therefore supports the evidence from the GLMMs that male fledglings have a higher probability of recruitment than females.

Table 3 summarises the numbers and proportions of individuals of known sex which fledged and were subsequently recruited into the breeding population, categorised by supplemental feeding. Among broods reared by breeding pairs which take supplemental food male fledglings were more likely to be recruited than females (χ^2 = 4.9, d.f = 1, P = 0.03), a pattern which was not observed among broods where parents relied exclusively on natural food resources (χ^2 = 0, d.f. = 1, P = 1). Figure 1 illustrates the sex ratio (where known) of supplementary fed fledglings and recruits for individuals produced during 2005, 2006 and 2007 and Figure 2 illustrates the difference in mean recruitment probability between male and female fledglings.

Initial exploratory analysis revealed that supplementary fed pairs breed earlier in the season than those which do not take supplemental food (mean hatch day of supplementary fed broods = 26, mean hatch day of non-supplementary fed broods = 34; t = 3.35, d.f. = 87.2, P = 0.001). I therefore repeated the model to investigate the effects of supplementary feeding, hatch day and gender on recruitment probability. This model consisted of probability of recruitment as a binary response variable, hatch day as an explanatory covariate and gender and supplemental feeding as two-level fixed factors. All first level interaction terms were included, nest ID was added as a random effect and binomial errors and a logit link were applied. Gender was confirmed as a significant predictor, revealing that male individuals were more likely to be recruited. In addition, I observed significant interaction terms indicating that both 'supplementary fed ^{X} hatch day' and 'sex ^{X} hatch day' affected recruitment probability (Table 4). Graphical examination of these significant interaction terms indicates that recruitment probability of supplementary fed individuals is positively associated with hatch day whilst that of non-supplementary fed individuals is negatively associated with hatch day (Figure 3). Furthermore, there also appears a contrasting relationship between hatch day and recruitment probability according to gender; recruitment probability of males is positively associated with hatch day and that of females negatively (Figure 4).

Recruitment was not predicted by BFDV infection status (Table 5a) but was revealed as a significant predictor when gender was introduced to the model (Table 5b). However, due to the unbalanced nature of the data the sample size used for this model was restricted due to the availability of individuals where both sex and infection status had been determined. Therefore the significance of this result is uncertain; reflected by the proximity of the upper confidence interval to zero.

Inbreeding and genetic diversity

Individual inbreeding coefficients (*f*) were calculated where all four grandparents were known within the pedigree for all fledglings hatched between 2005 and 2010 resulting in a total of 414 individuals (Figure 5). Mean inbreeding coefficient of all fledglings produced during this time was $f = 0.03$, ±0.06 and only 111 individuals were inbred ($f > 0$). A significant and negative relationship was observed between f and standardised heterozygosity ($\beta = -0.95$, ±0.17, $F = 32.98$, d.f. = 410, $P = < 0.001$) but f only accounted for 7% of the variation in SH (Figure 6). Of 228 fledglings produced between 2005 and 2007, inbreeding coefficients were determined for 156 individuals from 74 broods and SH was determined for 225 individuals from 118 broods. Neither f or SH were significantly associated with the recruitment probability of fledglings produced between 2005 and 2007 (Table 6).

5.3.3 Determinants of BFDV infection

Individual nestling infection status was available for a total of 428 fledglings produced between 2005 and 2009. None of the explanatory variables in the model significantly predicted individual infection status (Table 7). The variable with the highest relative importance factor was 'fledglings' which is equivalent to the brood size at fledging, indicating a trend that reflects greater probability of infection among individuals from larger broods. Of those individuals with available BFDV infection status (n = 428), sufficient pedigree data allowed inbreeding coefficients to be derived for 292 of them. Infection status

was not significantly associated with pedigree-derived inbreeding coefficient (β = -4.30, ±3.38, χ^2 = 1.77, P = 0.18) or standardized heterozygosity (β = -0.72, ±0.65, χ^2 = 1.25, P = 0.26) among these individuals.

5.3.4 Drivers of immune function and haematological parameters

Individual variation in haematological parameters and variables associated with immune function were investigated among nestlings produced between 2009 and 2010. Initial exploratory investigations of explanatory variables revealed a lack of strong correlations. The two pairs of predictor variables that revealed the highest correlation coefficients (r = 0.4) were; wing length and hatch order, indicating that wing length decreases with increasing hatch order, and clutch size and hatch day, suggesting that females which lay earlier in the season produce larger clutches. Variance inflation factors were all < 1.30, the highest being that for wing length (VIF = 1.29), providing further evidence of a lack of strong correlations among the continuous covariates. Owing to the unbalanced nature of this dataset, I investigated separately the effects of BFDV infection (infection status only available for 2009) and inbreeding (unknown or unidentified parents or grandparents in the pedigree thus reducing sample size).

Effects of immunostimulation

Infection with the circovirus BFDV is associated with immunosuppression and potentially fatal disease (PBFD) (Todd, 2000, Ritchie et al., 2003). Therefore, the effect that an active BFDV infection can have upon variables of immune function and haematological parameters was investigated for the 2009 data (Tables 8 and 9). Status of BFDV was assessed for 132 nestlings from 2009 with 31 individuals revealing an active infection, 60 of these individuals were also subjected to the PHA challenge. Only one haematological parameter was significantly associated with either BFDV status or PHA challenge; those individuals which were experimentally challenged with PHA revealed a significantly higher H:L ratio (Table 8). There were no significant effects on any of the haematological parameters of an

interaction between individual BFDV status and PHA challenge and the magnitude of the PHA response was not influenced by BFDV infection (BFDV negative: n = 46, mean PHA response = 0.55mm, ±0.41; BFDV positive: n = 14, mean PHA response = 0.57mm ±0.35. t = 0.16, d.f. = 24.79, P = 0.87).

Inbreeding and immune response

Individual inbreeding coefficients were not significantly associated with any of the haematological or immune function parameters for nestlings produced during 2009 and 2010 (Table 10).

5.3.5 Predictors of immune function variables and haematological parameters among nestlings produced during 2009 and 2010.

Response to experimental immune challenge with PHA.

The following input variables were used in a GLMM to predict the magnitude of response to immunostimulation by PHA: SH, body mass, hatch day, hatch order, brood size at fledging, year (two level factor of 2009 and 2010) and supplemental feeding (two level factor of true or false). I included the interaction term 'body mass ^{X} year ^{X} supplemental feeding' as initial graphical inspection suggested that effects of annual variation in body mass between supplementary fed and non-supplementary fed broods may be prevalent. Nest ID was included as a random effect and explained considerable variance in PHA response (ICC = 0.44, 95% CI = 0.22-0.63). Only three explanatory variables were included in the final model set indicating that the majority of predictors had little or no effect on response to PHA. The magnitude of PHA response was significantly greater in 2010 than in 2009 (Table 11). Supplementary feeding and SH were also present in the final model after model averaging but did not represent significant predictors of magnitude of PHA response.

Packed cell volume

Nest ID explained considerable variance in PCV (ICC = 0.51, 95% CI = 0.35-0.64). The set of explanatory variables was: SH, hatch order, sex, body mass, supplemental feeding, subpopulation (three level factor), brood size at fledging and PHA challenged (two level factor true or false). Supplemental feeding, sex and subpopulation were observed to significantly affect PCV (Table 12). I observed lower PCV values among male nestlings, lower values among nestlings from the 'Bel Ombre' subpopulation in comparison to the 'Brise fer' subpopulation and higher values among supplementary fed individuals. However, since the confidence intervals for all of these parameter estimates (and the parameter estimates themselves) were very close to zero the effects are considered to be minimal.

Heterophil lymphocyte ratio

The ratio of heterophils to lymphocytes was log-transformed to achieve residual normality and significant variance was not explained by the random effect of Nest ID (ICC = 0.13, 95% CI = -0.05-0.31). Explanatory covariates were: SH, body mass, hatch order, brood size at fledging, supplemental feeding, year and sex. I included the interaction effect of supplemental feeding and PHA challenged. Individual H:L ratio was significantly associated with experimental immune challenge; individuals which were experimentally challenged with PHA revealed a higher H:L ratio (Table 13 and Figure 7).

Eosinophil basophil and monocyte counts

I used separate ZIGLMMs to investigate the predictors of counts of eosinophils, basophils and monocytes among individual leukocyte profiles. The same set of predictor variables was used in each model and consisted of: SH, hatch order, body mass, wing length, brood size at fledging, PHA challenged, year, sex and supplementary fed. The interaction terms 'supplementary fed ^{X} PHA challenged', 'PHA challenged ^{X} sex' and 'PHA challenged ^{X} year' were included.

Nest ID (the random effect) did not explain a significant amount of variance in eosinophil counts (ICC = 0.13, 95% CI = -0.06-0.31) but did explain significant variance in counts of basophils (ICC = 0.36, 95% CI = 0.18-0.51) and monocytes (ICC = 0.37, 95% CI = 0.20-0.52). Sex was the only significant predictor of eosinophil counts revealing an increased proportion among male individuals (Table 14). The proximity to zero of the lower confidence interval for 'supplementary fed' indicates a tendency for supplementary fed individuals to produce more eosinophils. Year was the only significant predictor of basophil proportions; individuals sampled in 2009 were observed to have increased levels of basophils in comparison to those sampled in 2010 (Table 15). Several significant explanatory variables were observed to predict monocyte counts (Table 16); individuals from 2010 revealed higher proportions of circulating monocytes than 2009 and proportions among males were higher than females. Hatch day was significantly and negatively associated with monocyte counts; those hatched earlier in the season produced revealed a higher proportion. Older individuals within a brood also tended display increased monocyte counts. The interaction effect of 'PHA challenged ^{X} sex' was also a significant predictor. This was due to an increase in monocyte production among PHA challenged females, there was no similar increase among PHA challenged males.

Haemagglutination-haemolysis assay

The same set of predictors was used as explanatory covariates for both responses (agglutination and lysis) associated with the haemolysis-haemagglutination assay: SH, body mass, wing length, dam age, year, PHA challenged, sex and supplementary fed. Interaction terms 'PHA challenged ^{X} supplementary fed' and 'PHA challenged ^{X} sex' were included. The random effect of Nest ID explained a significant amount of variance in both lysis score (ICC = 0.35, 95% CI = 0.17-0.50) and agglutination score (ICC = 0.41, 95% CI = 0.20-0.57). The ZIGLMM to investigate complement mediated lysis revealed that individuals which were challenged with PHA also produced much higher lysis scores (Table 17 and Figure 8). Male individuals were capable of increased lysis compared to females (Figure 9) and a significant

interaction between the two (PHA challenged and sex) variables was observed which revealed that female, PHA challenged individuals were able to mount a significantly greater lysis reaction than male, PHA challenged individuals (Figure 10). I also observed a marginally significant effect of year indicating that individuals produced in 2010 were capable of a greater complement mediated lysis reaction to RRBCs (Table 17). Year was the only significant predictor of agglutination by NAbs with individuals produced in 2010 displaying greater ability for agglutination of RRBCs (Table 18).

5.4 Discussion

This study has revealed that the provisioning of supplemental food increases average productivity per breeding attempt, but that the probability of an individual fledgling being recruited into the breeding population is not influenced by parental supplementary feeding. The recruitment probability of male fledglings is higher than that of females, indicating the presence of differential post-fledging selection. Furthermore, recruitment probability was observed to be associated with date of hatch, but this association was secondarily dependent upon fledgling gender and supplementary feeding. Supplementary fed pairs bred earlier than non-supplementary fed pairs but fledglings from supplementary fed broods were more likely to survive from broods hatched later in the season. Among non-supplementary fed pairs, fledgling recruitment probability was higher among early-hatched broods. There was no evidence to suggest the existence of heterozygosity-fitness correlations (HFCs) and no evidence that inbreeding affected fitness or survival.

Productivity and recruitment

The issue of providing supplemental food is a contentious one and has been associated with negative effects including increased disease transmission (Dobson and Foufopoulos, 2001, Ewen et al., 2007, Robb et al., 2008), dependency (Jones and Reynolds, 2008) and other fundamental ecological processes which unsettle natural selection (Robb et al., 2008) potentially leading to selection of certain genetic characteristics (Frankham, 2008).

Nevertheless the provisioning of supplemental food has obviously increased productivity in this species but has not increased the probability of recruitment among individual fledglings. All individuals also forage in the wild and the provision of supplemental food is an attempt to make up for a shortfall in naturally available resources. The increase in number of supplementary fed pairs during this study suggests that supplemental food is vital to maintain the current level of population growth, however this resource is supplied *ad libitum* and it is not known to what extent individuals need this resource or use it because it represents 'a free lunch'. The outbreak of PBFD in 2004/5 was followed by very rapid evolution of the causative agent BFDV; genetic mutations which very quickly became fixed among the host population shortly after the outbreak (Kundu et al., 2012).

These results suggest that pair productivity in terms of brood size at hatching and fledging was severely reduced during 2005 but those effects were short lived (or at least continued with much reduced impact), this is most likely attributable to the disease outbreak and subsequent mutational variation affecting virulence. Of interest is the proportion of fledglings which were recruited during 2005, 2006 and 2007 indicating a relatively consistent pattern regardless of disease outbreak. Relating the productivity and recruitment of this species to others seems somewhat irrelevant, as there are no comparable examples of similar recovery programmes of island endemic psittacines and even comprehensive studies of similar wild parrots are rare. Data do exist concerning the breeding output of this species before intensive management began (Jones, 1987, Jones and Owadally, 1988, Jones and Duffy, 1993), however sample sizes are very low and many nest manipulations took place. Nevertheless, it was estimated that of 63 known breeding attempts between the 1970s and 1990s the average number of fledglings produced per breeding attempt was 1.0 (Jones, unpublished data), this study demonstrates that the contemporary population produces approximately 0.5 more fledglings per breeding attempt.

Predictors of recruitment

Recruited offspring are more likely to be male despite the parity of sex ratio among broods at fledging, suggesting that selection acts disproportionally against females after fledging, an effect which is mainly apparent among supplementary fed offspring. The fact that this skew is not observed among non-supplementary-fed recruits may well be a factor of low sample size in this group. This skew is unlikely due to nutritional effects as has been observed in the kakapo (*Strigops habroptila*) for example (Clout et al., 2002), and potentially associated with sex-biased philopatry (Gowaty, 1993). Results from Chapter 3 provide evidence to suggest that dispersal in this species is male-biased, and therefore local resource competition may also be sex-biased as females compete for limited nesting resources. Owing to the ongoing nest site monitoring regime female individuals (responsible for incubation and brooding young chicks) are far more likely to be detected in the breeding population than males and therefore this estimate of sex skew is likely to be conservative. Male-skewed sex ratios have been documented in many species of parrots for many reasons (Taylor and Parkin, 2008), it has been associated with the maintenance of genetic diversity for example, and the phenomenon is not a new one in this species. The documented sex skew in the 1970s and 1980s appeared to balance out in the 1990s as population numbers increased due to conservation intervention (MWF, 1996-2012). The previous skew was attributed to a random effect in a remnant population which would, historically have represented an even ratio (Jones and Duffy, 1993). However, this assumption is based upon the existence of 36 museum skins; 17 of which were female (Jones, 1987) and, for reasons noted above, perhaps the easiest sex to shoot! A potential explanation is that this species represents a cooperatively breeding system whereby the natural state is for many males to assist a single female in rearing offspring and there are numerous observations which tend to support this notion, especially among wild broods before intensive conservation efforts began (Duffy, 1994, MWF, 1996-2012). The availability of a constant food source and increased nesting

resources may well have lead to a situation where it was not necessary for many male 'helpers' to contribute to rearing a single brood.

Evidence from this study suggests that hatch date is an important factor in the observed difference in recruitment probability of males and females and this association is further affected by supplemental feeding. Hatch date was found to be significantly earlier among supplementary fed broods compared with non-supplementary fed broods. Fledgling survival of non-supplementary fed pairs is negatively correlated with hatch day; those fledged at the beginning of the breeding season are more likely to survive than those fledged from later broods. This pattern appears to be reversed among broods which take supplemental food and this contrasting relationship may in part explain the increase in probability of recruitment among males since they are also more likely to be recruited from late fledged broods and the majority of the breeding population are supplementary feeders. Laying-date has been documented as an important predictor of fledging success in many avian systems because individuals which produce chicks at the peak of seasonal food abundance are predicted to be the fittest (e.g. Lack, 1968, Van Noordwijk et al., 1995) and survival rates are predicted to be greater for individuals produced early in the season (Visser et al., 1998, Verhulst and Nilsson, 2008). Furthermore, species shift their breeding phenology in response to environmental fluctuations, climatic conditions and weather patterns (Cresswell and Mccleery, 2003, Visser and Both, 2005) to maximise reproductive success and sex biased post-fledging survival may also be attributed to adaptive seasonal variation (Husby et al., 2006).

No other reliable predictors of fledgling recruitment were observed, indicating that nesting environment, individual heterozygosity, BFDV infection status and management have little influence over the survival of fledglings.

BFDV infection

This study revealed that BFDV infection status was not predicted by any of the measured variables of immune function and was also not predicted by indices of genetic diversity or inbreeding. The prevalence of BFDV among nestlings was highest in 2005 than any other subsequent year where data was available, supporting the link between outbreak and reduced productivity. Infection status among nestlings was not predicted by any of the environmental or management factors and in fact the next most relevant predictor after 'year' was brood size at fledging, reflecting a tendency towards individuals from larger broods having an increase probability of infection. This result must largely be explained by the random effect of a larger brood increasing the chance that at least one individual will show infection. Individual infection status was not predicted by genetic diversity or inbreeding coefficient. In agreement with other studies I observed a weak but negative relationship between SH and *f* (Balloux et al., 2004, Slate et al., 2004, Taylor et al., 2010, Grueber et al., 2011b). Both of these factors revealed negative trends associated with probability of infection indicating conflicting patterns given that inbreeding was negatively associated with reduced heterozygosity.

Positive BFDV infection status among nestlings most likely represents a sub-clinical infection which may or may not lead to disease. PBFD is characterised by immunosuppression and feather dystrophy (Pass and Perry, 1984, Todd, 2000, Ritchie et al., 2003), however I observed no evidence to suggest that infection with BFDV among nestlings was associated with any of the measured indices of immune function. In fact, experimental exposure to PHA provoked a stronger response than infection with BFDV and resulted in a significant shift in leukocyte proportions among nestlings produced in 2009. Variables of immune function and haematological parameters were also not affected by a dual immune challenge, characterised by simultaneous BFDV infection and experimental immunostimulation. This perhaps indicates the irrelevance of determining individual BFDV status. It appears that in nestlings at least, BFDV infection status actually tells us very little;

it does not predict survival, it is not associated with environmental, genetic or management factors and it does not appear to affect indices of immune function (or at least the ones that were measured). These conclusions obviously depend on the assumption that the PCR test for the active BFDV virus is accurate and is not characterised by many false negatives or environmental contamination.

Variables of immune function

None of the indices of immune function or haematological parameters that I measured during this study were associated with either individual inbreeding coefficient or standardised heterozygosity in nestlings produced between 2009 and 2010. Is this lack of association in a bottlenecked, island-endemic species surprising? Perhaps not; given the publishing bias in favour of significant results concerning HFCs (Hansson and Westerberg, 2002, Balloux et al., 2004) we should not be surprised when they are absent in any system. In contrast, a significant positive relationship between magnitude of PHA response and SH was observed in *P. krameri* (Chapter 4) highlighting an important distinction between the two species. This distinction is perhaps explained by low variance in SH and indices of immune function among *P. echo* compared to *P. krameri* as a result of contrasting bottleneck proportions and levels of inbreeding.

Variables of immune function were not affected by BFDV infection but experimental PHA challenge did affect both cellular and humoral components of immune function. These results identified significant annual variation in variables of immune function characterised by an increase in response to PHA, increased monocyte proportions, increased innate humoral immune response and a decrease in basophil proportions among individuals produced in 2010 compared to 2009. Although detailed investigations of temperature and rainfall patterns were not incorporated into these models it was noted that the two breeding seasons represented contrasting levels of daily rainfall between September and December (mean 2009: 7.72mm ±1.66, mean 2010: 2.44mm ±0.52; $t = 2.99$, d.f. = 242, $P < 0.01$).

Therefore some differences may be explained by variations in physiological responses to weather conditions and these results suggest that immune responses may be stronger in dry compared to wet years. Significant physiological responses to experimental immunostimulation were observed but the magnitude of the PHA response was not associated with environmental, genetic or management factors. The PHA challenge resulted in increased individual H:L ratio and promoted increased complement mediated lysis of a foreign antigen which at least demonstrates a reactive immune system regardless of BFDV infection status.

Male birds generally display reduced immune responses in comparison to females (Folstad and Karter, 1992, Klein, 2000, McGraw and Ardia, 2005, Nunn et al., 2009) and this is generally attributed to circulating androgens and increased testosterone among males (Folstad and Karter, 1992). Evidence from this study however, suggests that male fledglings exhibit an increased humoral immune response and circulating levels of phagocytic monocytes when compared to females and this potentially gives them a selective advantage over females of the same age. Juvenile psittacines are known to be disproportionately (compared to adults) affected by PBFD (Ritchie et al., 1989, Ritchie et al., 2000, Todd, 2004) and this is also the case for this species (Jones and Merton, 2012). The sex skew of recruited fledglings may therefore, be explained if males are better equipped immunologically to tolerate or eliminate infection post-fledging. In monogamous species both sexes exhibit similar reproductive strategies and therefore selection pressures should also be similar on both sexes, however in polygynous species a difference in susceptibility between the sexes is often more obvious (Zuk, 1990, Zuk and McKean, 1996, Klein, 2000).

5.5 Recommendations for reintroduction

I revisit the four questions initially intended to help select which sub-adult individuals would give the greater chance of establishing a new, reintroduced population of endangered Mauritius parakeets upon their release into a disease landscape.

1. How is recruitment affected by environmental, management and genetic factors?

The only significant single predictor of recruitment is gender. I suggest that subsequent cohorts of individuals for release should reflect the probability that disproportionately more males will be recruited. Recruitment probability is also significantly affected by interactions involving gender and supplementary feeding.

2. Is PBFD status predicted by environmental, management and genetic factors?

Among nestlings of this species, no. It would appear that BFDV infection among these individuals is driven by factors other than those associated with heterozygosity, inbreeding, management and nesting environment and it is apparent that selection as a result of this disease occurs post-fledging. This result is perhaps not surprising given the highly infectious nature of the horizontally and vertically transmitted PBFD circovirus. Consequently, based on these results, infection status at the fledging stage is not an important consideration when selecting individuals for release.

3. How are indices of immune function affected by environmental, management and genetic factors?

Some evidence suggests that males displayed a stronger immune function. Inter-annual effects were noticed in many variables of immune function and this may be related to fluctuating weather conditions. Evidence from this study suggests that individuals hatched in dry years may be immunologically better equipped to tolerate parasite infection. Immune function variables were not affected by supplemental feeding, inbreeding or genetic diversity.

4. Can we identify which individuals would be more suitable for reintroduction based upon genetic and immunological factors?

Currently, no. Once data are available concerning the recruitment of the individuals included in this study that were sampled for immunological measures, significant immunological predictors of recruitment may emerge.

I have illustrated how hatch date can influence recruitment probability based on supplemental feeding and gender. Therefore, these factors may be useful in selecting juveniles which represent the greatest chance of establishing a founder population. These results suggest that the ideal release cohort would:

- be female biased, to compensate for male biased survival,
- comprise individuals from early-hatched, non-supplementary fed broods and late-hatched supplementary fed broods,
- comprise individuals hatched during dry years.

A fine balance must be struck between sustaining a viable population through conservation management and allowing natural selection to determine the optimal trajectory of an endangered species' evolutionary ecology. Nevertheless, this study has demonstrated how the probability of success of reintroductions can be enhanced by fine-tuning release strategies through the objective selection of individuals. Intensively-monitored natural field systems such as that of the Mauritius parakeet represent a gold-mine of information to help guide reintroduction strategies and through carefully designed research can provide a scientific and objective basis upon which to select individuals for reintroduction.

5.6 Figures and tables

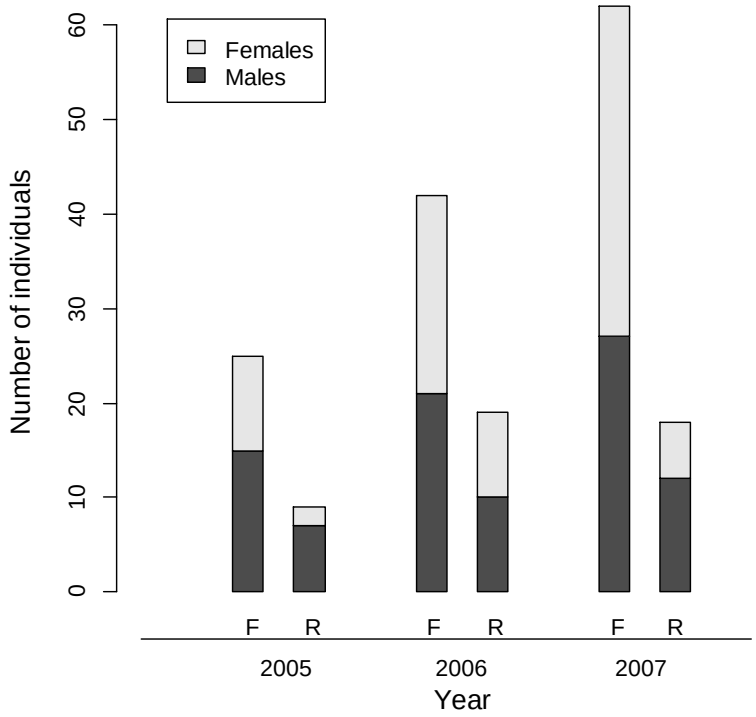

Figure 1. The number of individuals fledged (F) and subsequently recruited (R) from three consecutive breeding seasons divided by gender.

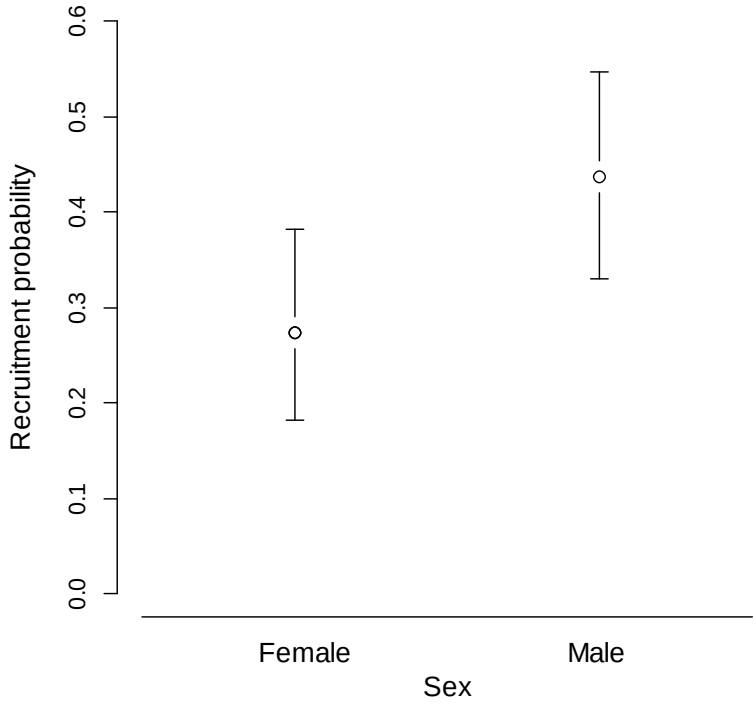

Figure 2. The recruitment probability of fledged individuals by gender with 95% CI calculated using the exact method.

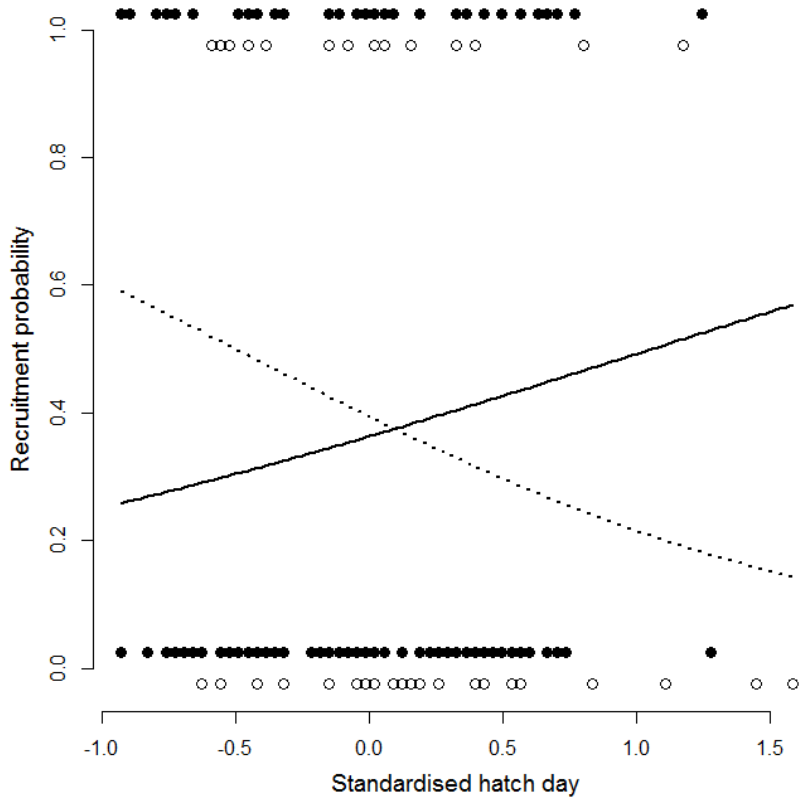

Figure 3. The recruitment probability of supplementary fed (solid line and filled circles) and non-supplementary fed (dashed line and open circles) individuals produced between 2005 and 2007 in relation to hatch day. Curves were fitted using the inverse logit function of the logistic equations from the GLMM in Table 4.

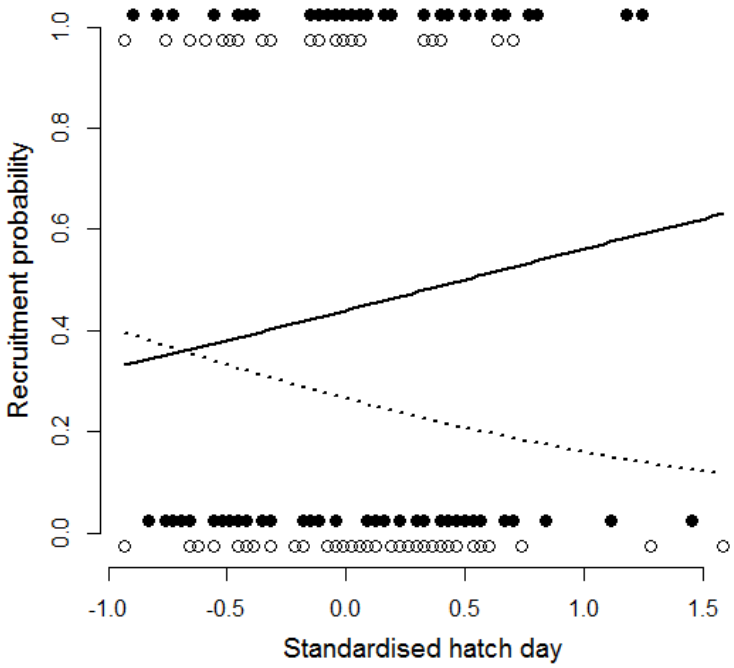

Figure 4. The recruitment probability of male (solid line and filled circles) and female (dashed line and open circles) individuals produced between 2005 and 2007 in relation to hatch day. Curves were fitted using the inverse logit function of the logistic equations from the GLMM in Table 4.

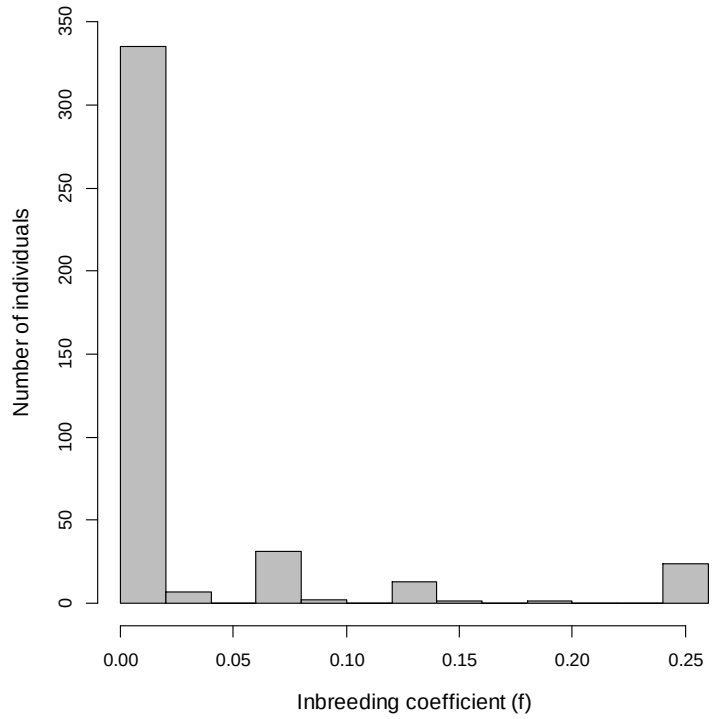

Figure 5. Histogram of individual inbreeding coefficient for all fledglings produced between 2005 and 2010 where all four grandparents were known.

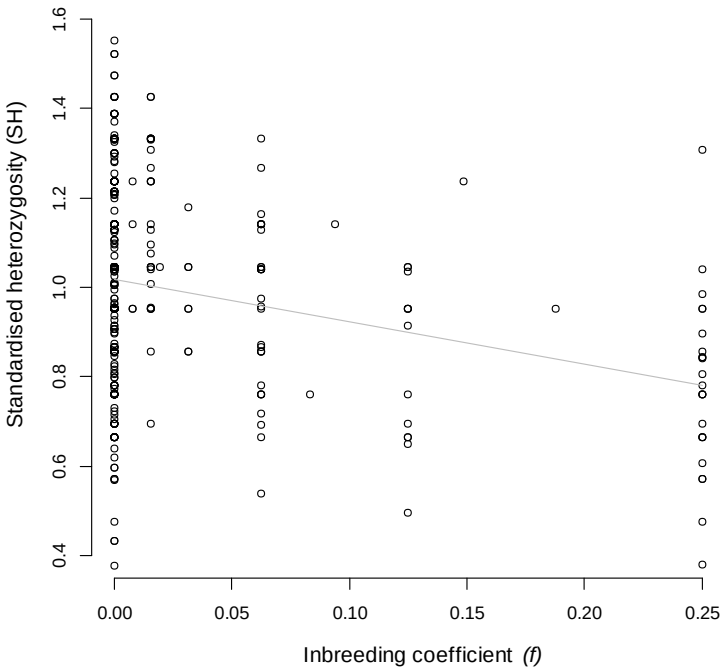

Figure 6. Relationship between pedigree-derived inbreeding coefficient and standardised heterozygosity for all fledglings produced between 2005 and 2010 where all four grandparents were known and genotype information was available (n = 411). Regression line from the GLM is shown (β = -0.95, ±0.17, F = 32.98, d.f. = 410, P = < 0.001, r^2 = 0.07).

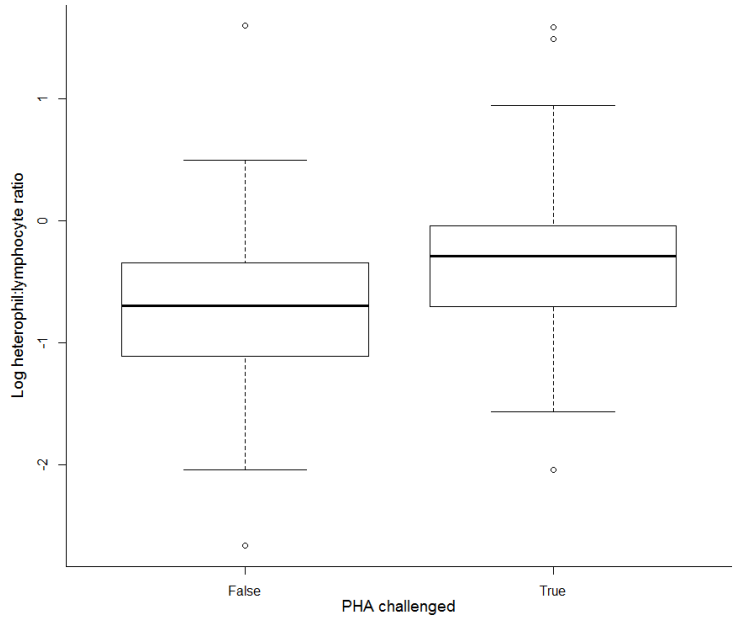

Figure 7. The ratio of heterophils to lymphocytes from leukocyte profiles among individuals divided by those which were (True) and were not (False) experimentally challenged with PHA.

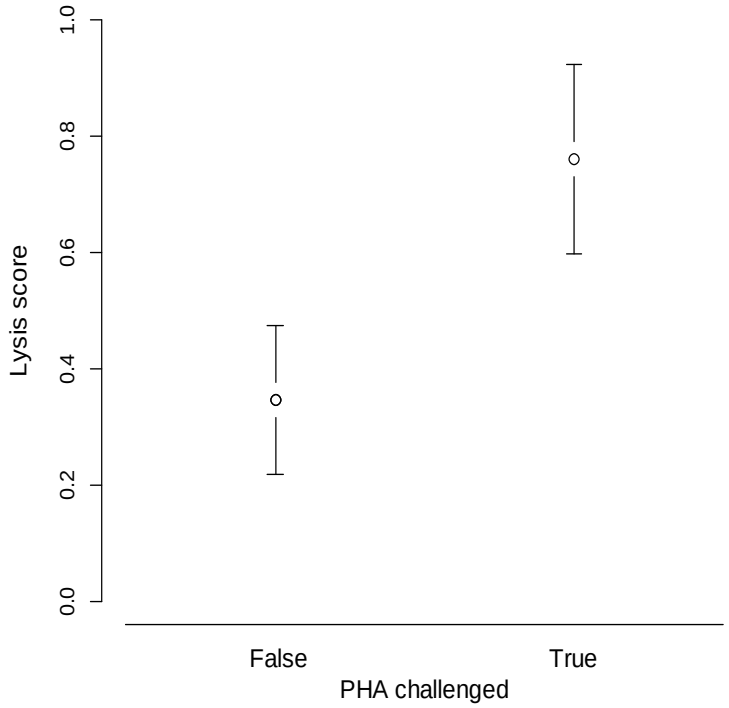

Figure 8. Mean complement mediated lysis scores and 95% CI among individuals in relation to experimental PHA challenge.

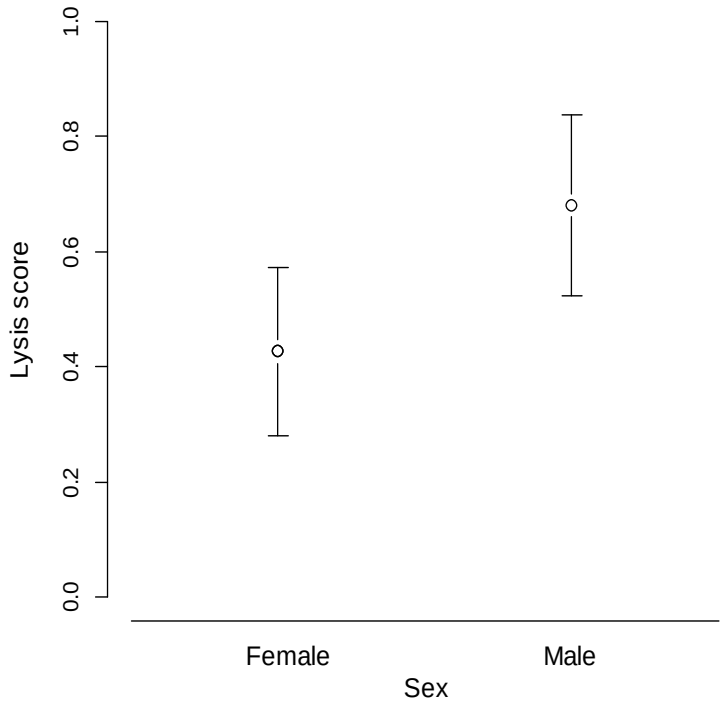

Figure 9. Mean complement mediated lysis scores and 95% CI for individuals in relation to gender.

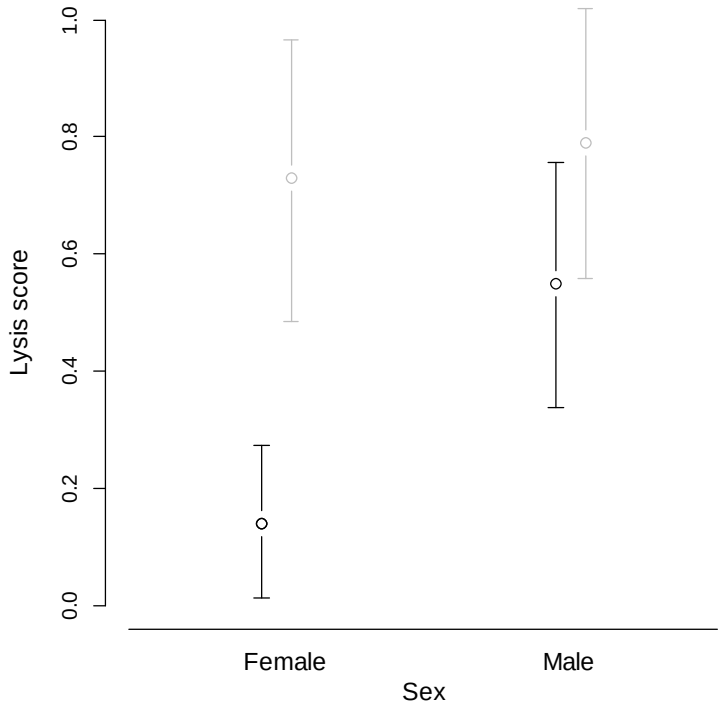

Figure 10. Mean complement mediated lysis scores of individuals based on gender and experimental immune challenge with PHA. Black bars represent those individuals which were not challenged and grey bars those which were.

Table 1 Summary of known nesting attempts between 2005 and 2011. Nesting attempt is defined as the number of clutches produced in a single breeding season including secondary clutches laid by females which recycle after the first clutch/brood fails. Percentages in brackets indicate the proportion of those nesting attempts where at least one of the breeding pair takes supplemental food. Values of total numbers of eggs, hatched, fledged and recruited individuals are taken from all known and monitored nesting attempts. Mean values represent minimum mean clutch size, eggs hatched, chicks fledged and recruited per nesting attempt. Bold text indicates overall figures and a summary of the outcomes of nesting attempts according to those pairs which take supplemental food (Supp) and those which do not (Non-supp).

Year	Nesting attempts	Total eggs	Mean clutch size	Total hatched	Mean hatched	Total fledged	Mean fledged	Total recruited	Mean recruited	Fledglings recruited
2005	51 (67%)	136	2.67 ±0.59	75	1.47 ±1.20	54	1.06 ±1.03	15	0.29 ±0.61	28%
2006	59 (71%)	158	2.68 ±0.60	109	1.84 ±1.10	72	1.22 ±1.15	21	0.36 ±0.64	29%
2007	67 (72%)	180	2.69 ±0.70	134	2.00 ±1.15	102	1.52 ±1.15	25	0.37 ±0.67	25%
2008	67 (75%)	186	2.78 ±0.67	139	2.07 ±1.06	112	1.67 ±1.15	19	0.28 ±0.48	17%
2009	78 (74%)	220	2.82 ±0.55	154	1.97 ±1.10	130	1.67 ±1.14	NA	NA	NA
2010	79 (78%)	216	2.73 ±0.71	152	1.92 ±1.07	124	1.57 ±1.06	NA	NA	NA
2011	84 (85%)	215	2.56 ±0.86	161	1.92 ±1.12	124	1.48 ±1.17	NA	NA	NA
Supp	**366**	**999**	**2.73 ± 0.69**	**724**	**1.98 ±1.10**	**592**	**1.62 ±1.14**	**46***	**0.37 ±0.72***	**27%***
Non-supp	**119**	**312**	**2.62 ±0.66**	**200**	**1.68 ±1.00**	**126**	**1.06 ±0.95**	**15***	**0.28 ±0.45***	**27%***
Overall	**485**	**1311**	**2.70 ±0.69**	**924**	**1.89 ±1.13**	**718**	**1.48 ±1.13**	**61***	**0.34 ±0.65***	**27%***

* Summarised recruitment figures do not include those from 2008.
NA, not applicable.

Table 2 Model averaged results of a GLMM to predict recruitment probability of fledglings from 2005, 2006 and 2007. Probability of recruitment was fitted as a binary response variable, nest ID as a random effect and binomial errors and logit link were applied.

Nest Environment	Explanatory variable	Estimate	SE	CIL	CIU	RI
N = 223, 116	Intercept	-1.17	0.18	-1.53	-0.81	N/A
	Hatch order	-0.44	0.35	-1.14	0.25	0.38
	SH	-0.43	0.35	-1.11	0.25	0.30
	Dam age	-0.33	0.37	-1.06	0.40	0.24
	Fledglings	0.26	0.40	-0.53	1.04	0.14
	Hatch day	0.13	0.36	-0.56	0.83	0.07
Sex	Explanatory variable	Estimate	SE	CIL	CIU	RI
N = 169, 105	Intercept	-0.70	0.19	-1.07	-0.33	N/A
	Sex (Male)	0.85	0.36	0.14	1.55	0.92
	SH	-0.61	0.37	-1.33	0.11	0.59
	Hatch order	-0.45	0.37	-1.17	0.27	0.35
	Supp (true)	0.10	0.44	-0.75	0.96	0.29
	Sex (male)*SH	0.75	0.82	-0.85	2.35	0.16
	Supp (true)*Sex (Male)	0.54	0.83	-1.08	2.17	0.03

Sample sizes indicated by N = individuals, clutch.

Table 3 Numbers of individuals of known gender which fledged between 2005 and 2007 and were subsequently recruited from supplementary fed (Supp) and non-supplementary fed (Non-supp) broods. Percentages in brackets represent the proportion of fledged individuals of each sex which were recruited into the breeding population.

	Fledged		Recruited	
	Male	Female	Male	Female
Supp	63	66	29 (46%)	17 (26%)
Non-supp	24	18	9 (38%)	6 (33%)
Total	87	84	38 (44%)	23 (27%)

Table 4 Relationships between supplementary feeding, hatch date and gender on fledgling recruitment probability. A GLMM was fitted with recruitment probability as a binary response variable and nest ID as a random effect. A binomial error structure and logit link was applied.

Sex, feeding and hatch day	Explanatory variable	Estimate	SE	CIL	CIU	RI
N = 169, 105	Intercept	-0.70	0.20	-1.09	-0.31	N/A
	Sex (male)	0.93	0.39	0.17	1.69	0.95
	Hatch day	-0.09	0.43	-0.93	0.76	0.67
	Supp (true)	-0.02	0.47	-0.93	0.89	0.54
	Sex (male) * Hatch day	1.70	0.85	0.03	3.38	0.52
	Supp (true) *hatch day	2.21	0.98	0.28	4.14	0.42
	Supp (true) * Sex (male)	0.33	0.94	-1.52	2.18	0.09

Sample sizes indicated by N = individuals, clutch.

Table 5 Results of GLMMS with binomial error structure and logit link to predict recruitment probability including variables of sex and BFDV infection status. Nest ID was added as a random effect.

a) PBFD	Explanatory variable	Estimate	SE	CIL	CIU	RI
N = 183, 107	Intercept	-1.11	0.19	-1.48	-0.75	N/A
	Hatch order	-0.63	0.39	-1.39	0.13	0.62
	PBFD (+)	-0.64	0.46	-1.54	0.26	0.53
	SH	-0.13	0.37	-0.86	0.59	0.25
	PBFD (+)*Hatch order	-0.58	1.00	-2.53	1.38	0.08
b) PBFD and Sex	Explanatory variable	Estimate	SE	CIL	CIU	RI
N = 141, 97	Intercept	-0.70	0.19	-1.08	-0.32	N/A
	PBFD (+)	-1.01	0.48	-1.95	-0.07	0.93
	Sex (Male)	0.62	0.38	-0.12	1.37	0.64
	SH	-0.49	0.40	-1.28	0.30	0.49
	Hatch order	-0.55	0.40	-1.34	0.24	0.52
	PBFD (+)*SH	1.11	1.02	-0.89	3.10	0.16
	PBFD (+)*Sex (Male)	-0.10	0.94	-1.95	1.75	0.11
	PBFD (+)*Hatch order	-0.44	1.02	-2.43	1.56	0.08

Sample sizes indicated by N = individuals, clutch.

Table 6 Effects of inbreeding (*f*) and standard heterozygosity (SH) on recruitment probability of fledglings produced between 2005 and 2007. Probability of recruitment was included in a generalised linear mixed model as a binary response variable with either *f* or SH as a single explanatory covariate. A binomial error term and logit link function was used with nest ID as a random factor. Significance of each explanatory covariate was tested using likelihood ratio tests.

Response variable	Explanatory variable	slope	SE	Chi	P
Probability of recruiting	F	1.62	3.79	0.12	0.73
Probability of recruiting	SH	-0.78	0.82	0.79	0.37

Table 7 Predictors of BFDV infection. Results of model averaged GLMMs using a binomial distribution and logit link with infection status as a binary response variable, nest ID was added as a random factor.

Response variable:	Predictor	Estimate	SE	CIL	CIU	RI
BFDV infection						
	Intercept	-0.65	0.36	-1.35	0.05	N/A
	Year 2006	-1.33	0.54	-2.39	-0.28	1.00
	Year 2007	-0.37	0.46	-1.28	0.53	1.00
	Year 2008	-2.28	0.57	-3.40	-1.16	1.00
	Year 2009	-0.75	0.44	-1.61	0.12	1.00
	Fledglings	0.56	0.32	-0.07	1.19	0.70
	Nest type (C)	-0.56	0.37	-1.29	0.17	0.55
	Supp (true)	0.55	0.43	-0.29	1.38	0.47
	SH	-0.31	0.28	-0.86	0.24	0.35
	Dam age	0.31	0.33	-0.33	0.95	0.29
	Hatch order	-0.03	0.30	-0.62	0.55	0.16

Table 8 Effects of BFDV status and PHA challenge on haematological components among nestling parakeets during 2009 using GLMMs with nest ID as a random factor, a Gaussian error structure and identity link. Significance of explanatory covariates was tested by likelihood ratio tests.

Response	Predictor	Slope	SE	χ^2	P
H:L	Challenged	0.56	0.17	11.02	0.00
	PBFD	0.09	0.19	0.24	0.62
	Interaction	-0.39	0.29	1.87	0.17
PCV	Challenged	0.00	0.01	0.32	0.57
	PBFD	-0.02	0.01	1.94	0.16
	Interaction	0.01	0.02	0.22	0.64
Agglutination	Challenged	-0.32	0.24	1.76	0.18
	PBFD	-0.54	0.27	4.01	0.05
	Interaction	0.48	0.41	1.19	0.27

Table 9 Effects of BFDV status and PHA challenge on indices of cell counts and complement mediated lysis using ZIGLMMs with a poisson distribution and log link; nest ID was added as a random factor.

Response	Predictor	Slope	SE	z	P
Eosinophil	Challenged	-0.36	0.20	-1.80	0.07
	PBFD	-0.27	0.28	-0.99	0.33
	Interaction	0.35	0.40	0.87	0.38
Basophils	Challenged	0.02	0.15	0.11	0.91
	PBFD	-0.06	0.20	-0.30	0.77
	Interaction	-0.16	0.31	-0.52	0.60
Monocytes	Challenged	-0.40	0.22	-1.85	0.06
	PBFD	-0.31	0.28	-1.11	0.27
	Interaction	0.32	0.41	0.77	0.44
Lysis	Challenged	0.61	0.36	1.69	0.09
	PBFD	0.60	0.48	1.26	0.21
	Interaction	-1.00	0.74	-1.35	0.18

Table 10 Effect of inbreeding coefficient on immune function variables and haematological parameters among nestlings produced during 2009 and 2010. Generalised linear mixed models with Gaussian error structure and identity link and ZIGLMMs (*) with poisson distribution and log link were used where response variables were cellular counts or complement mediated lysis. Significance of single explanatory covariates was assessed with likelihood ratio tests. All models contained nest ID as a random factor. Individual sample size for each model is shown in parentheses.

Immune/ haematological (response) variable	Explanatory variable	slope	SE	χ^2	P
PHA (89)	F	0.05	0.71	0.01	0.93
H_L (167)	F	0.09	0.96	0.01	0.93
PCV (171)	F	-0.07	0.07	0.99	0.32
Eosinophil* (167)	F	-1.37	1.26	0.94	0.33
Basophil* (167)	F	0.48	1.14	0.26	0.61
Monocyte* (167)	F	-1.12	1.52	0.41	0.52
Lysis* (168)	F	0.51	2.01	0.10	0.75
Agglutination (168)	F	0.38	1.53	0.06	0.81

Table 11 Factors affecting response to PHA.

Immune component	Explanatory variable	Estimate	SE	CIL	CIU	RI
PHA	Intercept	0.60	0.12	0.36	0.85	N/A
N = 116, 53	Year 2010	0.33	0.08	0.18	0.47	1.00
	SH	-0.22	0.16	-0.53	0.10	0.21
	Supp (true)	-0.13	0.10	-0.32	0.07	0.12

Table 12 Factors affecting PCV.

Immune component	Explanatory variable	Estimate	SE	CIL	CIU	RI
PCV	Intercept	0.38	0.00	0.37	0.38	N/A
N = 210, 106	Sex (male)	-0.02	0.01	-0.03	-0.01	0.43
	Sub-pop (BO)	-0.03	0.01	-0.04	-0.01	0.07
	Sub-pop (GG)	-0.02	0.01	-0.03	0.00	0.07
	Supp (true)	0.01	0.01	0.00	0.02	0.01

Table 13 Factors affecting H:L ratio.

Immune component	Explanatory variable	Estimate	SE	CIL	CIU	RI
H:L	Intercept	-0.51	0.05	-0.61	-0.40	N/A
N = 212, 102	Challenged (true)	0.43	0.10	0.23	0.63	1.00
	Fledglings	0.13	0.10	-0.06	0.33	0.13
	Year 2010	0.13	0.10	-0.07	0.33	0.13
	Supp (true)	0.12	0.11	-0.10	0.34	0.11

Table 14 Factors affecting eosinophil count.

Immune component	Explanatory variable	Estimate	SE	CIL	CIU	RI
Eosinophils	Intercept	0.68	0.21	0.27	1.09	N/A
N = 212, 102	Sex (male)	-0.34	0.16	-0.66	-0.02	0.98
	Supp (true)	0.32	0.19	-0.05	0.69	0.95
	Fledglings	-0.12	0.09	-0.31	0.06	0.57
	Challenged (true)	-0.30	0.26	-0.81	0.20	0.41
	Challenged*Sex	0.37	0.23	-0.09	0.83	0.33
	Year 2010	0.12	0.12	-0.11	0.35	0.29
	Challenged*Supp	0.35	0.30	-0.24	0.94	0.18
	Wing length	-0.07	0.13	-0.32	0.18	0.18
	SH	-0.03	0.12	-0.26	0.21	0.14
	Body mass	0.00	0.13	-0.25	0.26	0.14

Table 15 Factors affecting basophil count.

Immune component	Explanatory variable	Estimate	SE	CIL	CIU	RI
Basophils	Intercept	1.04	0.13	0.78	1.30	N/A
N = 212, 102	Year 2010	-0.79	0.11	-1.01	-0.57	1.00
	Body mass	0.19	0.10	-0.01	0.39	0.75
	Supp (true)	-0.07	0.17	-0.41	0.26	0.60
	Challenged (true)	-0.01	0.19	-0.40	0.37	0.55
	Sex (male)	-0.14	0.11	-0.35	0.07	0.45
	Hatch order	0.09	0.10	-0.10	0.29	0.30
	Challenged*Supp	-0.37	0.23	-0.82	0.07	0.24
	SH	0.01	0.11	-0.20	0.22	0.17
	Wing length	0.03	0.10	-0.17	0.24	0.16
	Challenged*Sex	0.04	0.21	-0.36	0.45	0.03

Table 16 Factors affecting monocyte count.

Immune component	Explanatory variable	Estimate	SE	CIL	CIU	RI
Monocytes	Intercept	0.20	0.18	-0.15	0.54	N/A
N = 212, 102	Year 2010	0.75	0.15	0.45	1.05	1.00
	Sex (male)	0.52	0.19	0.14	0.89	1.00
	Hatch day	-0.34	0.11	-0.56	-0.12	1.00
	Challenged (true)	0.24	0.22	-0.19	0.67	0.89
	Challenged*Sex	-0.52	0.21	-0.93	-0.11	0.89
	Hatch order	0.20	0.09	0.01	0.38	0.86
	SH	0.16	0.09	-0.02	0.34	0.63
	Wing length	0.17	0.10	-0.03	0.36	0.63
	Challenged*Year	0.32	0.22	-0.11	0.75	0.40
	Body mass	0.05	0.10	-0.15	0.26	0.18
	Supp (true)	-0.01	0.12	-0.24	0.22	0.14

Table 17 Factors affecting complement mediated lysis.

Immune component	Explanatory variable	Estimate	SE	CIL	CIU	RI
Lysis	Intercept	-2.06	0.46	-2.96	-1.16	N/A
N = 174, 94	Challenged (true)	1.54	0.47	0.61	2.47	1.00
	Sex (male)	1.27	0.48	0.32	2.21	1.00
	Challenged*Sex	-1.15	0.53	-2.19	-0.12	0.97
	Year (2010)	0.45	0.22	0.02	0.89	0.84
	Supp (true)	-0.24	0.26	-0.74	0.26	0.36
	Body mass	-0.26	0.22	-0.70	0.18	0.35
	Wing length	-0.17	0.21	-0.59	0.25	0.25
	Dam age	-0.05	0.21	-0.46	0.37	0.13
	SH	0.01	0.21	-0.39	0.42	0.13
	Challenged*Supp	-0.21	0.51	-1.20	0.78	0.03

Table 18 Factors affecting agglutination of foreign red blood cells.

Immune component	Explanatory variable	Estimate	SE	CIL	CIU	RI
Agglutination	Intercept	7.77	0.10	7.59	7.96	N/A
N = 174, 94	Year (2010)	0.42	0.19	0.05	0.80	0.74
	Supp (true)	-0.30	0.21	-0.71	0.10	0.24
	Body mass	0.20	0.17	-0.13	0.53	0.08
	Challenged	0.07	0.19	-0.30	0.44	0.05
	Dam age	-0.06	0.19	-0.43	0.31	0.05
	Sex (male)	0.10	0.15	-0.20	0.39	0.05
	Wing length	0.04	0.16	-0.27	0.35	0.04
	SH	0.00	0.16	-0.32	0.32	0.04

Chapter 6. Discussion

6.1 Summary

In **Chapter 2** I used common principal components analysis to investigate the interactions among variables of immune function at different organisational levels. This approach to decomposing the complexity of the immune system can be useful as a preliminary investigation. It cannot however, accurately identify subtle interactions at the individual level and is perhaps more suited to assessing species level variation.

Chapter 3 documented patterns of population level genetic diversity of the Mauritius parakeet from bottleneck proportions in the 1990s through a period of intensive management and recovery until 2010. Results suggested a lack of gene flow between subpopulations as a result of limited natural dispersal and an overall decreasing trend in population level genetic diversity measured with neutral microsatellite markers. Recommendations were made for the identification of suitable sites for artificial nestboxes in order to encourage natural dispersal and therefore gene flow.

Variables of individual-level immune function and multilocus heterozygosity between two sympatrically occurring congeneric species were investigated in **Chapter 4.** Nestlings of the invasive and continentally evolved Indian ringneck parakeet revealed increased immunocompetence when compared to the endangered, island-endemic Mauritius parakeet. Multilocus heterozygosity was not associated with any variables of immune function among Mauritius parakeets but was positively related to magnitude of response to PHA among Indian ringneck parakeets.

In **Chapter 5** I used existing data to summarise the productivity and recruitment of Mauritius parakeets during and after an outbreak of a highly infectious disease. I investigated potential genetic and environmental predictors of BFDV infection status and the effect that infection had upon variables of immune function. Infection status was not

predicted by multilocus heterozygosity and non of the immune function variables were affected by BFDV infection status. Finally, I used predictors of recruitment to identify individuals which, as part of a reintroduction attempt would be most likely to maximise establishment success and population persistence.

6.2 Background

The field of ecoimmunology has provided, and continues to offer, valuable insight concerning the ecological and evolutionary processes which combine to determine the fitness of individuals, populations and species in their natural environment. Researchers increasingly recognise the complexity of the immune system and the need to characterise immunocompetence using multiple measures whilst simultaneously evaluating specific parasite and pathogen pressures, effects of environmental conditions and evolutionary influences. Trade-offs between variables of immunity and a variety of other life history components including growth and reproduction for example have been demonstrated in birds (e.g. Knowles et al., 2008), and it is now widely accepted that heightened immune defences are energetically costly to a host and that these costs vary among individuals. The drivers of this variation are associated with environmental and genetic factors, both historical and contemporary (Schulenburg et al., 2009).

Further investigation into the genetic components of immune function represents an exciting future direction in ecological immunology. To date most studies incorporating variables of genetic diversity have done so by using neutral (and therefore non-selective) loci under the assumption that heterozygosity at these loci represents genome-wide diversity. However, functional immunological loci such as those associated with the major histocompatibility complex (MHC) are likely to be under constant selection and the effects of this are potentially exaggerated in small populations. Therefore, characterising diversity at these loci and identifying associated phenotypic variation offers even greater understanding of susceptibility to infectious agents in studies of avian ecoimmunology.

6.3 Contributions

6.3.1 Ecological immunology

An important contribution made by this thesis has been to combine valuable data collected as part of a long term conservation monitoring project and to integrate it with knowledge of individual immune function and disease infection status. Considering parasites and pathogens in studies of ecoimmunology is vital in interpreting the magnitude of individual immune function variables but often, for logistical reasons researchers must focus on those known to be important. This study has shown that an outbreak of highly infectious psittacine beak and feather disease (PBFD) affected productivity among breeding birds by reducing hatchability and nestling survival.

Since the outbreak of disease in 2004/5, the effects of beak and feather disease virus (BFDV) infection appear less obvious among nestlings and the determinants of individual infection status seem to be equally vague (Chapter 5). It is perhaps surprising that infection status is associated with none of the measures of immune function measured as part of this research (Chapter 6) when it is a documented and well-known immunosuppressant virus (e.g. Todd, 2000). One obvious explanation for this is that the virus assay was inaccurate, however, all positive results were confirmed via genome sequencing of the virus (Kundu et al., 2012) and therefore any inaccuracies were down to false negative results. Other possible explanations are that the lack of reaction to viral infection is due to less well developed immune systems of nestling birds when compared to adults (Clark et al., 2009), or that all individuals are equally exposed and that infection status derived by PCR is functionally uninformative. What seems to be apparent is that any selection as a result of infectious disease in this species occurs in the period after fledging and before sexual maturity.

6.3.2 Interspecies variation

At the species level, individual variation in immunocompetence is more likely to be detected in the presence of environmental, ecological and genetic heterogeneity and evidence presented in Chapter 4 suggests relatively low immunological heterogeneity among Mauritius parakeets compared to the superficially similar Indian ringneck parakeet. The presence of a phylogenetically similar and sympatrically occurring species represents an important comparison in this study and the contrasting relationships involving immune function and other life history variables between *P. krameri* and *P. echo* suggest a greater level of variability in immune function among the continentally evolved and invasive *P. krameri*.

Despite the lack of variation in multilocus heterozygosity (MLH) between these two species it is highly likely that the contemporary population of *P. krameri* on Mauritius is the result of multiple introductions (Jones, 1987) and since MLH is a poor indicator of inbreeding (Slate et al., 2004) actual levels of inbreeding are likely to be different. Unfortunately studies of inbreeding depression in small, endangered island populations often lack pre-bottleneck controls but evidence suggests that inbreeding depression can occur due to historic bottlenecks (Hale and Briskie, 2007a, Hale and Briskie, 2007b). The Mauritius parakeet has suffered a major range contraction due to the effects of deforestation, and having evolved on a small island with relatively small population size (in comparison to a continental species) is likely to have purged deleterious alleles as a result of inbreeding and loss of genetic diversity. This study however, reveals relatively low levels of contemporary inbreeding (Chapter 5). Comparing microsatellite diversity across species is not an ideal way of inferring historical bottlenecks but it is generally accepted that island populations display reduced genetic diversity in comparison to continentally evolved congenerics (Frankham, 1997, Jamieson et al., 2006). This trend may go some way to explain the difference in immune function variability among *P. echo* compared to *P. krameri* if immunogenetic variation has also been lost disproportionately as a result of island evolution, range

contraction and historic bottlenecks. Equally, evolutionary origins may explain this contrast in immune function variation if, as empirical and theoretical studies suggest (Matson, 2006, Matson and Beadell, 2010, Wikelski et al., 2004), immune responses among island taxa are 'relaxed' owing to reduced selection as a result of depauperate parasite communities when compared to continental hosts.

6.3.3 Conservation reintroductions

Conservation reintroductions frequently fail because consistent methodologies allowing objective assessment of the outcomes through appropriate post-release monitoring and *a priori* identification of targets are rarely applied (Armstrong and Seddon, 2008, Sutherland et al., 2010). The evidence presented in Chapter 5 illustrates the importance of post-release monitoring for two reasons: (i) outbreaks of disease are common in small, reintroduced populations and monitoring is vital to document these outbreaks and manage the threats they pose, and (ii) adequate post-release monitoring can provide useful data regarding how individuals might be selected for future reintroductions in order to maximise establishment success and population persistence. The ability to select individuals for reintroduction as demonstrated by the findings of this thesis represents a major step forward for future reintroduction attempts.

The value of opportunistic blood sampling of individuals cannot be understated and has been illustrated in Chapter 3. Small endangered populations often exist in a fragmented landscape and evaluating the genetic consequences of fragmentation and management provides conservation practitioners with the necessary information to maintain population genetic diversity in order to maximise long term viability. This study highlights the importance of considering natural evolutionary processes in reintroduction biology and the need to maintain and encourage dispersal wherever possible. Additionally, the effects and sustainability of post-release management should also be considered wherever possible. Techniques employed to increase population size, such as supplemental feeding must be

evaluated relative to the detrimental secondary effects which may occur such as increased population density and disruption of natural cues which determine breeding phenology. The ability to adapt to environmental fluctuations is vital for the long term persistence of reintroduced populations and therefore appropriate consideration must be given when evaluating management procedures.

6.4 Limitations and further research

Often, in studies of natural populations, investigators are limited in the range and scope of data that can be collected for logistical, practical and financial reasons. Data collected in association with reintroduction programmes are often concerned with answering specific questions which are frequently determined by short-term resource availability. As is apparent in this study this can often lead to temporally unbalanced datasets and a lack of continuity in monitoring efforts which culminate in interpretational and analytical challenges for researchers.

Immune responses involve highly complex interactions involving both innate and adaptive cellular and humoral components of the immune system but these interactions are poorly understood. Whilst it is tempting to try and combine these elements to derive an overall measure of immunocompetence, it is important that trade-offs within the immune system are considered and investigated despite the interpretational difficulty in an ecological context. This study would be improved with the incorporation of information regarding parental immunocompetence and particularly how maternal immune fitness might influence that of offspring. Interpretation of individual cellular immunity from leukocyte profiles is ideally made by counting the proportion of white blood cells in relation to red blood cells and this represents an important consideration for further analysis. Consideration of a larger suite of parasites and pathogens would also be desirable to improve the interpretation of certain immune function indices.

Analysis of temporal genetic trends could be greatly improved with a simulation of the loss of genetic diversity based upon a fully random mating population in order to provide a comparison to the observed results. Furthermore, an obvious improvement to this study would be to use the available microsatellite information to reconstruct the pedigree to account for the unknown ancestry of some individuals, thereby enhancing parentage assignment and enabling production of more detailed data concerning individual inbreeding coefficients.

Data is available concerning annual meteorological variables. Incorporating elements of temperature and rainfall for example, may offer a more complete picture regarding annual variation in productivity and recruitment and may also provide insight into the use of supplemental food by Mauritius parakeets in relation to the availability of naturally available resources.

6.4.1 Recommendations

A key recommendation based on the results of this study is further investigation into the use of supplemental food in order to accurately evaluate its benefits and any associated costs**.** Currently this resource is provided *ad libitum* and it is not known to what extent such provisioning is required to maintain population growth. The identification of individual-level consumption of supplemental food via the use of stable isotope analysis is the subject of a forthcoming research project funded by a successful application for funds to the Rufford Small Grants for Nature Conservation. This research is expected to be completed in 2013.

Future reintroductions of Mauritius parakeets to other areas of Mauritius or elsewhere as ecological replacements should be considered in the context of this study. Selecting individuals free of disease would be very difficult as this would require quarantine and extensive and repetitive disease screening. Using the results presented in this thesis conservation managers can now at least identify objectively those individuals more likely to successfully establish as part of a new population. The results presented in Chapter 5 reveal

that during the study period, male fledglings were more likely to be recruited into the breeding population than females. Supplementary feeding did not affect the probability of individual recruitment. Furthermore, probability of recruitment was associated with day of hatch; among supplementary fed broods the probability of individual fledgling recruitment decreased with increasing hatch day, the opposite relationship was observed among broods where adults did not take supplemental food.

An important future consideration for this species would be the identification of individuals produced during 2009/10 which are subsequently recruited into the breeding population. Although the results in this thesis revealed little variation in, or predictors of, individual immune function variables among nestlings, an important comparison should be made between those measured for immune function variables which survive to maturity and those which do not.

Technological advances offer exciting prospects for the future of ecological immunology. As it becomes relatively easier and less expensive to characterise MHC genetic diversity, more will be learned concerning the specific role of functional, immune-related genes in natural contexts and how this diversity is maintained and/or lost in small, free-living populations. Furthermore, it is likely that many as yet unknown genes contribute to the regulation of the immune system either directly or indirectly and the use of quantitative trait loci (QTL) mapping to identify such genes and their functionality will contribute to knowledge concerning immunity in natural populations.

Determining the prevalence of diseases among wild populations is of growing concern owing to the increasing awareness of emerging infectious diseases and this is another area where advancing technology offers the ecoimmunologist a valuable research avenue in the future. The application of microarray technology offers the opportunity to simultaneously detect a multitude of infectious agents from a single blood sample. Although in developmental infancy and concerned predominantly with domestic animal and human

health this method of detecting novel and unknown infectious agents is an intriguing advancement.

6.5 Conclusions

Inferring an individual's immune response or status in studies of ecological immunology is problematic, often there is very little knowledge of the current parasite and pathogen communities an individual is exposed to and often one can only determine prevalence of the parasites and pathogens that are actively targeted. Characterising immunity of wild populations is a challenge and often researchers have to cope with collecting data which may be difficult to handle statistically or prone to inaccuracy/issues of repeatability, i.e. blood cell counts of rare white cells and PHA challenge technique. In addition, determining disease prevalence often says little about the population-level impact and therefore the importance of simultaneously studying many different variables of immune function and the ultimate measure of fitness as measured by reproduction, survival and recruitment cannot be underestimated in studies of ecoimmunology.

This study has demonstrated the importance of post-release monitoring of reintroduced populations of endangered species and suggested how the success of reintroduction projects can be maximised by considering data collected from other long term monitoring initiatives. Ultimately the long term success of population reintroductions depends upon adaptive management and high-quality evidence based conservation.

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