Translocations and the 'genetic rescue' of bottlenecked populations

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Abstract

Many species around the world have passed through severe population bottlenecks due to anthropogenic influences such as habitat loss or fragmentation, the introduction of exotic predators, pollution and excessive hunting. Severe bottlenecks are expected to lead to increased inbreeding depression and the loss of genetic diversity, and hence reduce the long-term viability of post-bottlenecked populations. The objective of this thesis was to examine both the consequences of severe bottlenecks and the use of translocations to ameliorate the effects of inbreeding due to bottlenecks.

Given the predicted increase in probability of inbreeding in smaller populations, one would expect inbreeding depression to increase as the size of a population bottleneck decreases. Determining the generality of such a relationship is critical to conservation efforts aimed at minimising inbreeding depression among threatened species. I therefore investigated the relationship between bottleneck size and population viability using hatching failure as a fitness measure in a sample of threatened bird species worldwide. Bottleneck size had a significant negative effect on hatching failure, and this relationship held when controlling for confounding effects of phylogeny, body size, clutch size, time since bottleneck, and latitude. All species passing through bottlenecks of $\sim$100–150 individuals exhibited increased hatching failure. My results confirm that the negative consequences of bottlenecks on hatching success are widespread, and highlight the need for conservation managers to prevent severe bottlenecks.

In many endangered species, preventing bottlenecks is no longer an option as populations have already declined to a level where urgent action is required to mitigate the negative effects of inbreeding and ensure their long-term viability. In the past, two approaches have been used with some success: (1) the introduction of outbred individuals into inbred populations, and (2) the augmentation of inbred populations through the release of captive-reared individuals. However, both approaches have limitations. For example, in many threatened species, there are no outbred populations left to use as a source for introducing new individuals into inbred populations. Similarly, captive populations may not be available, and if they are, individuals may also be inbred and adapted to captivity, and perhaps less likely to survive in free-living conditions. I therefore experimentally

tested whether reciprocal translocations between different inbred populations could be an alternative technique to mitigate the negative effects of inbreeding and restore levels of genetic variation once a species or population has passed through a bottleneck.

First, I conducted a laboratory experiment using inbred lines of the fruit fly *Drosophila melanogaster*. I used founding populations of just one male and one female to create replicate inbred lines in two different strains of fruit flies. After two generations of inbreeding, I found that crossing individuals between the two bottlenecked strains reversed the effects of inbreeding and led to increases in overall breeding success and survival that persisted into the second generation of hybrid offspring. In contrast, crosses within each strain (but between different replicate lines) resulted in only slight improvements in some fitness components, and this positive trend was reversed in the second generation. The results of this experiment suggest that inbred populations can be used as donors to reduce the effects of severe population bottlenecks and 'rescue' an endangered species from inbreeding depression but that the effect is strongest if there are some initial genetic differences between donor populations.

To confirm whether 'genetic rescue' through the use of inbred populations can be used in a free-living animal, I repeated the above experiment in a natural setting by conducting reciprocal translocations between two severely bottlenecked and isolated South Island robin *Petroica australis* populations. Both populations had been founded by just five birds each and showed signs of inbreeding depression. I found significant increases in mean levels of heterozygosity in the hybrid offspring (crosses between the two populations) compared to inbred control offspring. Similarly, allelic richness increased significantly in both populations within the first year after the translocation. The significant increase in genetic diversity was accompanied by increases in overall levels of fitness. Hybrid birds experienced increased levels of both survival and recruitment into the breeding population, and sperm quality improved significantly in hybrid males compared with inbred males. Finally, I found a significant increase in one aspect of cell-mediated immunity in hybrid individuals. The results of the field study using robins confirm the pattern found in the laboratory with fruit flies and highlight that inbred populations should not be discounted as potential donors for genetic rescue when outbred populations are unavailable.

In conclusion, the finding that the negative effects of inbreeding increase with the severity of the population bottleneck experienced provides added impetus for conservation biologists to ensure endangered species do not pass through severe bottlenecks. For species or populations that are

already affected by inbreeding depression and have no outbred populations left to act as a source for the introduction of new genetic stock, the results of both the laboratory and the wild experiment confirm the potential value of translocations between different inbred populations of endangered species as a tool to mitigate the negative effects of inbreeding. In order to ensure the long-term viability of any threatened species or population, however, it is essential to realise that genetic interventions in form of reciprocal translocations need to be complemented with other management strategies aimed at the restoration or conservation of suitable habitat.

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Chapter 1

General Introduction

The loss and fragmentation of habitat due to direct and indirect anthropogenic influences constitutes one of the major threats to biodiversity (Foley et al. 2005, Sala et al. 2000). Illegal hunting (e.g. Leader-Williams et al. 1990), the introduction of exotic predators (e.g. Savidge 1987), and pollution (e.g. France and Collins 1993) have further exacerbated the rapid decline and isolation of many wild plant and animal populations (e.g. Butler and Merton 1992, Groombridge et al. 2001, Pimm et al. 2006), highlighting the importance of addressing the effects of reduced genetic diversity and inbreeding on population viability.

The effect of population bottlenecks on the fitness of a species is clearly illustrated by the black robin *Petroica traversi*, which was once widespread throughout the Chatham Islands but declined rapidly due to habitat loss and predation by introduced mammals (Butler and Merton 1992). By the 1880s, black robins were confined to Little Mangere Island, which can only support 20–30 individuals at any one time. By 1976, merely five individuals (one breeding female) remained (Butler and Merton 1992). The current population of approximately 200 individuals is therefore derived from a single breeding pair. The level of genetic variation at minisatellite loci in the black robin has been found to be among the lowest recorded for any avian species in the wild, and has been attributed to the extended period of time in which the black robin persisted as a single small population (Ardern and Lambert 1997). Similarly, the Mauritius kestrel *Falco punctatus* declined to a low of four known individuals (one pair) in 1974 as a consequence of habitat loss and pesticide contamination (Safford and Jones 1997, Groombridge et al. 2000). Following intensive management, the species has recovered to 500–800 individuals and is still increasing (Ewing et al. 2008).

Nonetheless, levels of heterozygosity and allelic diversity remain low (Groombridge et al. 2000), and the rates of inbreeding and loss of genetic variation in this species are among the highest yet documented in a wild vertebrate population (Ewing et al. 2008). As with robins and kestrels, the Florida panther *Puma concolor coryi* has also passed through a severe population bottleneck. This population was once part of a continuous, widespread population in the south-eastern USA, but habitat loss, fragmentation, and human persecution caused a severe range and demographic contraction more than a century ago (Pimm et al. 2006). Numbers declined to less than 30 individuals in the 1980s (Hedrick and Fredrickson 2010, Roelke et al. 1993), and the occurrence of genetic defects, such as cryptorchidism (unilateral or bilateral undescended testicles), poor semen quality, and heart defects, increased. These reproductive and congenital abnormalities have been associated with a severe reduction in genetic diversity (Barone et al. 1994, Culver et al. 2008, Hedrick and Fredrickson 2010, Roelke et al. 1993).

The prevalence of population bottlenecks is now widespread across most plant and animal taxonomic groups. In birds, it is estimated that more than 12% of species worldwide are now considered endangered and the percentage is even higher in mammals (BirdLife International 2004, Hilton-Taylor, C. (compiler) 2000). Furthermore, the number of species considered threatened in most groups is increasing over time, indicating that population bottlenecks, reduced genetic diversity, inbreeding and population viability will be an ongoing problem for the foreseeable future. For conservation managers, understanding the negative effects of bottlenecks, in terms of their genetic and fitness consequences, and how to mediate those effects, are key to preventing further species extinctions.

As a consequence of population bottlenecks, founder effects and random genetic drift (Nei et al. 1975), small populations risk the loss of genetic variation and this in turn may not only decrease fitness, but also limit their ability to respond to future environmental challenges, such as climatic extremes, pollutants, pests, and novel pathogens (Amos and Balmford 2001, Frankham 1995, Frankham et al. 2010, Lande 1988, 1995), and thus increase their probability of extinction (Frankham et al. 1999, Newman and Pilson 1997). Similarly, inbreeding depression, defined as the reduction in fitness of offspring resulting from the mating of closely related individuals, can negatively affect the viability of threatened species that are reduced to a number of fragmented and bottlenecked populations (Charlesworth and Charlesworth 1987, Crnokrak and Roff 1999, Hedrick and Kalinowski 2000, Keller and Waller 2002, Kristensen et al. 2010, O'Grady et al. 2006). During

a bottleneck event, alleles that occur at low frequencies may disappear completely, thereby reducing the number of alleles per locus (i.e. allelic diversity; Allendorf 1986, Frankham et al. 1999, Fuerst and Maruyama 1986), and consequently the evolutionary potential of populations. Inbreeding, on the other hand, affects fitness by increasing homozygosity, and therefore the expression of recessive (or partially recessive) detrimental alleles (Charlesworth and Charlesworth 1987, 1999, Lande 1988). Inbred individuals that are relatively homozygous at loci influencing fitness may therefore experience increased mortality and reduced reproductive success (Bensch et al. 2006, Charlesworth and Charlesworth 1999, Keller and Waller 2002, Olano-Marin et al. 2011). It has been suggested that bottlenecks and ensuing inbreeding could purge deleterious alleles by exposing them to natural selection when in the homozygous state (Hedrick 1994); however, complete purging of the genetic load and elimination of inbreeding depression is unlikely to occur in natural populations (Keller and Waller 2002, see also Mikkelsen et al. 2010, but see Laws and Jamieson 2011), and empirical evidence suggests that despite the opportunity for purging, inbreeding depression occurs in many threatened species (e.g. Ballou 1997, Byers and Waller 1999, Frankham et al. 2001).

The effects of inbreeding depression on fitness components such as fertility, lifetime reproductive success, survival, sperm quality, and immunocompetence are well documented (Amos et al. 2001, Billing et al. 2012, Brekke et al. 2010, Charlesworth and Willis 2009, Charpentier et al. 2008, Crnokrak and Roff 1999, Grueber et al. 2010, Keller 1998, Kruuk et al. 2002, Liberg et al. 2005, Mackintosh and Briskie 2005, Madsen et al. 1996, O'Brien et al. 1985, Räikkönen et al. 2009, Swinnerton et al. 2004, but see van de Casteele et al. 2003). For instance, several studies have found a negative relationship between parental genetic similarity and survival in mammals (Ralls et al. 1979, Stockley et al. 1993), and hatching success among birds (Bensch et al. 1994, Kempenaers et al. 1996). Likewise, a number of studies have found inbreeding or heterozygosity effects on ejaculate quality (Asa et al. 2007, Gage 2006, Gomendio et al. 2000, Roldan et al. 1998), and a correlation between poor semen quality and reproductive success has been documented in several species (Gomendio et al. 2000, Malo et al. 2005). Moreover, an association between low levels of genetic variation and disease outbreaks has been found in several wild populations of endangered species (e.g. O'Brien et al. 1985, Roelke et al. 1993, Thorne and Williams 1988). Studies on bird species that have gone through severe bottlenecks have shown that these birds are immunocompromised and therefore potentially more susceptible to the introduction of novel pathogens (Hale and Briskie 2007, Tompkins et al. 2006). Inbreeding can also reduce effective population size through the distortion of sex ratios (Frankham 1995, Worthington Wilmer et al. 1993, but see Frankham and

Wilcken 2006). Finally, the negative effects of inbreeding have been shown to increase with the severity of the population bottleneck (Briskie and Mackintosh 2004, Heber and Briskie 2010, see chapter 2).

Given the problems associated with population bottlenecks, it is not surprising that a number of attempts have been made to try to reverse the consequences of inbreeding by restoring genetic diversity (i.e., 'genetic rescue') through the introduction of new individuals. The translocation of outbred individuals into inbred populations of the same species (intraspecific hybridisation) has proven to be an effective tool to improve population viability by enriching depauperate gene pools and mitigating the negative effects of inbreeding (Fredrickson et al. 2007, Johnson et al. 2010, Madsen et al. 1999, Richards 2000, Westemeier et al. 1998, see also Frankham et al. 2011, Hedrick and Fredrickson 2010, Weeks et al. 2011). A greater prairie chicken *Tympanuchus cupido pinnatus* population in south-eastern Illinois, for example, had declined from 2,000 individuals in 1962 to fewer than 50 individuals in 1994. The decrease in population size was accompanied by a decline in both genetic diversity and fitness, as measured in terms of fertility and egg hatchability. The translocation of outbred individuals into the inbred Illinois population, however, led to a rapid increase in egg viability and population size (Westemeier et al. 1998). Table 1.1 on page 23 summarises some of the attempts where threatened or near threatened species and populations have been augmented to alleviate genetic problems (see supporting information in Frankham et al. (2011) for additional examples).

The hybridisation of related species (interspecific hybridisation) has shown similar effects on the viability of at least one endangered species (Tompkins et al. 2006). This, however, raises concerns about the genetic swamping of populations (Allendorf et al. 2001, Frankham et al. 2010) and the inherent risks of outbreeding depression (Edmands 2007, Frankham et al. 2011, Goldberg et al. 2005, Huff et al. 2011), which could work contrary to the desired effects by breaking up either locally adapted or co-adapted gene complexes (Hendry et al. 2007, Reid et al. 2003). The augmentation of inbred populations using outbred individuals of the same species, however, depends on the availability of suitable outbred donor populations. The applicability of this technique is therefore limited by the fact that an increasing number of endangered species survive only as a series of small, fragmented populations, with each likely subject to some loss of genetic variation and elevated levels of inbreeding.

Table 1.1: Examples of species or populations that have been augmented through the translocation of outbred or captive inbred individuals to mitigate genetic problems. See supporting information in Frankham et al. (2011) for additional examples.

Common name	Scientific name	Bottleneck size	Method	Outcome	Source
Mammals					
African lion	*Felis leo*	5	Introduction of genetically differentiated individuals	Increase in litter size and cub survival	Frankham (2009), Trinkel et al. (2008)
Bighorn sheep	*Ovis canadensis*	12	Introduction of individuals from 2 outbred populations	Increased reproductive success, survival, and 5 fitness-related traits	Hogg et al. (2006)
Florida panther	*Puma concolor coryi*	< 30	Introduction of individuals from the closest natural populations from Texas	Decline in frequency of deleterious traits, increased survival and population density	Hedrick and Fredrickson (2010)
Mexican wolf	*Canis lupus baileyi*	7	Re-introduction of captive individuals descending from 3 captive lineages founded with a total of 7 wolves	Increased reproductive success	Hedrick and Fredrickson (2010)
Black-footed ferret	*Mustela nigripes*	< 10	Re-introduction of inbred individuals from captivity, periodical augmentation using captive inbred individuals	Rapid population establishment and growth, maintenance of genetic diversity	Wisely et al. (2008)

(Continued on next page)

Table 1.1: Continued from previous page

Common name	Scientific name	Bottleneck size	Method	Outcome	Source
Birds					
Greater prairie chicken	*Tympanuchus cupido pinnatus*	< 50	Introduction of individuals from large, genetically diverse populations	Increase in egg viability	Westemeier et al. (1998)
Red-cockaded woodpecker	*Picoides borealis*	4	Introduction of individuals from 4 donor populations	Increase in the number of breeding groups and population size	U.S. Fish and Wildlife Service (2003)
Reptiles					
Swedish adder	*Vipera berus*	4	Introduction of individuals from a large, genetically diverse population	Increase in survival and recruitment, population size and genetic diversity	Madsen et al. (1999)
Plants					
Button wrinklewort	*Rutidosis leptorrhynchoides*	118	Manual pollen transfer using geographically distant source individuals	Increase in fertilisation success	Pickup and Young (2008)
Marsh grass of Parnassus	*Parnassia palustris*	< 100	Manual pollen transfer using geographically using source individuals from a different metapopulation	Increase in seed set	Bossuyt (2007)

In theory, even the exchange of individuals between different inbred populations should increase population viability, provided that the donor populations used for the translocations harbour different alleles (Charlesworth and Charlesworth 1999, Lynch 1991, Tallmon et al. 2004), by suppressing the expression of deleterious recessive mutations at loci that were previously homozygous (Charlesworth and Charlesworth 1999, Crow 1993, Edmands 2007, Lynch 1991). This leads to the prediction that crosses between two inbred populations should have a fitness advantage compared to within-population offspring, as they are more likely to be heterozygous for deleterious recessive alleles that cause inbreeding depression in the homozygous state (Falconer and Mackay 1996, Ingvarsson and Whitlock 2000, Whitlock et al. 2000). Despite the potential importance of population bottlenecks and inbreeding to the long-term management of endangered species, these theoretical models have not been tested in wild populations using a systematic and replicated approach. In a laboratory experiment using the fruit fly *Drosophila melanogaster*, Spielman and Frankham (1992) found that the introduction of a single immigrant into inbred populations lead to an increase in fitness (as measured by a competitive index measure). Similarly, Bijlsma et al. (2010) showed that genetic rescue using inbred individuals as donors can be effective to increase the fitness of inbred populations: crosses between inbred lineages of *Drosophila* led to substantial increases in pupal survival. However, in both experiments, the individuals used for the translocation were sourced from the same base population (Bijlsma et al. 2010, Spielman and Frankham 1992).

In some cases, the introduction of captive-reared inbred individuals into wild, depauperate populations has led to increased population viability, suggesting genetic rescue may be possible with inbred donor populations. The highly endangered black-footed ferret *Mustela nigripes*, for instance, passed through a bottleneck of < 10 individuals, and by the late 1980s, this species was extinct in the wild (Wisely et al. 2008). After the re-introduction using inbred captive-reared individuals, the newly established populations were periodically augmented using individuals from the same captive source population. Genetic diversity could thereby be maintained at levels equivalent to those found in the source population. Without augmentation, however, allelic diversity declined drastically and a deterioration of phenotypic traits (limb and overall body size) occurred (Wisely et al. 2008, but see Cain et al. 2011).

Similarly, the Mexican wolf *Canis lupus baileyi*, an endangered subspecies of the grey wolf, was thought to be extinct in the wild in the 1980s, and all Mexican wolves alive today originate from three captive lineages founded by a total of seven wolves (Hedrick et al. 1997). These lineages

were merged in 1995, and starting in 1998, a wild population was re-established through the release of captive-bred Mexican wolves (Fredrickson et al. 2007). In the re-introduced population, large fitness increases in terms of litter size and survival were observed in wolves with ancestry from two or more lineages, compared to individuals with ancestry from only one lineage (Fredrickson et al. 2007). Nonetheless, the augmentation of inbred populations using captive-reared individuals may have severe limitations, as captive populations are often not available, and if they are, captive individuals may also be inbred and perhaps adapted to captivity and less likely to survive in free-living conditions. The aim of this thesis is therefore to determine whether—in the absence of outbred donor populations of the same species—'genetic rescue' using only highly bottlenecked and inbred wild populations as donors can be implemented successfully to increase population viability.

Outline of the thesis

The main chapters of my thesis (chapters 2–6) have been written as independent manuscripts for submission to scientific journals. Cross-referencing and repetition to some extent was therefore inevitable. The chapters are organised in the format required by current scientific journals with abstract, introduction, material and methods, results, discussion and reference sections. Although I write in first person, note that some of the work in this thesis was done in collaboration with members of my supervisory committee and other researchers. However, in all cases I led the research and will be senior author on any resulting publication.

Chapter 2 explores the relationship between the size of the bottleneck that a species or a population went through, and the level of egg hatching failure, a fitness measure that is known to be susceptible to inbreeding depression in birds (Bensch et al. 1994, Spottiswoode and Møller 2004). Given the predicted increase in probability of inbreeding in smaller populations, one would expect inbreeding depression to increase as the size of a population bottleneck decreases. Determining the generality of such a relationship is critical to conservation efforts aimed at minimising inbreeding depression among threatened species. This chapter, authored by my senior supervisor, Associate Professor James V. Briskie, and myself, has been published in the international peer-reviewed journal Conservation Biology (Heber and Briskie 2010, Volume 24(6), pp. 1674-1678).

In chapter 3, I investigate the effects of crossing inbred populations of differing origin as a method of 'genetic rescue' for endangered species lacking outbred donor populations using repli-

cated experimental lines of inbred fruit flies *Drosophila melanogaster*. The objective of this study was to test whether the exchange of individuals between inbred populations reduces levels of inbreeding depression when controlling for potentially confounding effects of the environment. Due to the fast generation time and ease of rearing of fruit flies, I was able to conduct several replicates of the experiment, an option that is often not feasible when studying wild animal populations. This work was done in collaboration with Dr. Luis Apiolaza, School of Forestry, University of Canterbury, Christchurch, and has been published in the peer-reviewed open access journal PLoS ONE (Heber et al. 2012, Volume 7(8), p. e43113).

As the impact of hybridisation on fitness depends not only on the level of parental genetic similarity, but also on effects of the environment, I applied this laboratory experiment to wild populations of the South Island robin *Petroica australis*. For this purpose, I conducted experimental translocations between two severely bottlenecked and isolated robin populations located on two islands, Allports and Motuara, in the Marlborough Sounds, South Island, New Zealand. I assessed the effects of the translocations on genetic diversity (chapter 4) and a number of fitness components (chapters 5 and 6) by comparing hybrid individuals (crosses between the two populations) with inbred control individuals of the same age. More specifically, chapter 5 investigates the effects of the translocation on fitness measures such as breeding success, survival and recruitment rates, fertility and sperm quality, and parental care, whereas chapter 6 focuses on the strength of the immune system and susceptibility to a range of pathogens. Chapter 4 was done in collaboration with Professor Dr. Bart Kempenaers from the Department of Behavioural Ecology and Evolutionary Genetics at the Max Planck Institute for Ornithology in Seewiesen, Germany. For chapter 5, I collaborated with Bart Kempenaers and Dr. Arvind Varsani from the School of Biological Sciences, University of Canterbury, Christchurch. Bart Kempenaers, Arvind Varsani, and Dr. Eloise Jillings from the Institute of Veterinary, Animal and Biomedical Sciences at Massey University contributed to chapter 6.

The thesis concludes with a general discussion (chapter 7), summarising the main findings of the five data chapters and evaluating the benefit of reciprocal translocations between inbred populations as a management tool in the conservation of threatened species.

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Chapter 2

Population bottlenecks and increased hatching failure in endangered birds

Abstract: *Severe bottlenecks are expected to lead to increased inbreeding depression and reduce the long-term viability of post-bottlenecked populations. I tested the relationship between bottleneck size and population viability using hatching failure as a fitness measure across 51 threatened bird species around the world. Bottleneck size had a significant negative effect on hatching failure, and this relationship held when controlled for confounding effects of phylogeny, body size, clutch size, time since bottleneck, and latitude. All species passing through bottlenecks of ∼100–150 individuals exhibited increased hatching failure. My results confirm that the negative consequences of bottlenecks on hatching success are widespread, and highlight the need for conservation managers to prevent severe bottlenecks.*

2.1 Introduction

Anthropogenic influences such as habitat loss, introduced predators, and excessive hunting have forced many species through severe population bottlenecks (e.g. Groombridge et al. 2001). As bottlenecks reduce effective population size, inbreeding becomes more likely as gene flow between populations is interrupted and matings between relatives become more frequent (Boessenkool et al. 2007, Frankham et al. 2010, Edmands 2007). Decreased effective population size may lead to reduced genetic diversity, with the extent of loss depending on both the severity of the bottleneck and the rate of post-bottleneck growth (Nei et al. 1975). The resulting negative fitness consequences, termed inbreeding depression, include reductions in fertility and problems in embryogenesis (Keller and Waller 2002).

Increased hatching failure is one common effect of inbreeding in birds (Bensch et al. 1994, Spottiswoode and Møller 2004). Hatching failure averages $\sim$10% in non-inbred birds (Koenig 1982), but can be several times higher in inbred populations (e.g. Jamieson et al. 2003). Given the predicted increased risk of inbreeding in smaller populations, it might be expected that hatching failure should increase with the severity of bottleneck size. This pattern was confirmed by Briskie and Mackintosh (2004), who found an inverse correlation between bottleneck size and hatching failure among both native and introduced species in New Zealand. Species that passed through bottlenecks below $\sim$150 individuals had levels of hatching failure 2–4 times higher than those subject to less severe bottlenecks (Briskie and Mackintosh 2004). However, it is unclear if elevated levels of hatching failure among New Zealand birds are due to genetic factors alone, or if increased failure is also due to local habitat effects or the insular environment (Marr et al. 2006).

In this chapter, I investigate whether there is a general relationship between the severity of bottlenecks and levels of hatching failure in endangered birds around the world. Determining the generality of such a relationship is critical to conservation efforts aimed at minimising inbreeding depression among endangered species. I also use this relationship to estimate the minimum bottleneck size required to avoid hatching failure increasing beyond that observed in non-bottlenecked populations.

2.2 Material and methods

2.2.1 Data collection

I collected data on rates of hatching failure in 51 threatened bird species around the world. Species were distributed across 31 families and 14 orders, and the size of the bottleneck each passed through ranged between 4 and 20,000 individuals (see table 2.2 on p. 47). Hatching failure was estimated as the proportion of eggs incubated to term that failed to hatch, excluding failure from desertion, predation, or adverse weather. Eggs that fail to hatch under this definition therefore reflect either infertility or embryonic death, both of which are expected to increase with inbreeding (Jamieson and Ryan 2000). Bottleneck size was defined as the lowest population size recorded in a species. As levels of consanguineous matings were unavailable for most species, I assumed that inbreeding increased with the severity of a bottleneck.

I identified bottlenecked species from the World Bird Database of Threatened Species from BirdLife International and the International Union for Conservation of Nature Red List (IUCN 2008). Most of the species in my data set are endangered species in which the entire world population has passed through a bottleneck, but I also included data from isolated populations that had passed through a bottleneck even if other populations of the same species remain common (e.g. Chatham Island tomtit *Petroica macrocephala chathamensis*, Hawaiian stilt *Himantopus mexicanus knudseni*), including my own data from one isolated population of South Island robin *Petroica australis*. Data on hatching failure and bottleneck size was gathered from the literature and through personal communication with researchers.

For each bottlenecked population, I collected data on hatching failure, clutch size, body mass, absolute latitude, and time that had passed between the lowest population size and when data on hatching failure were recorded (see table 2.2 on p. 47). Hatching failure might be expected to increase with clutch size if species with small clutches are under more intense selection to prevent infertility. Similarly, hatching failure might vary with body size if larger species live longer and are under less intense selection to minimise infertility than short-lived species breeding only once or twice. Latitude was used as an indicator for climate zone, which could affect hatching failure in terms of prevailing temperature and day length. For example, bird species breeding at higher latitudes could have an advantage by having more daylight hours during which to forage and therefore minimise periods of egg neglect that could increase embryo mortality. Latitude was measured

as the mid-point between the northern and southern latitude extremes of the breeding range of a species. I used the absolute value of latitude, ignoring whether north or south of the equator, as there is no reason to expect birds in different hemispheres to vary in hatching success. Time since lowest population size could also affect hatching failure if selection removes infertile individuals from the post-bottlenecked population such that species recently passing through a bottleneck may not have had time to purge infertile individuals from the population. As the exact year of smallest population size was unavailable for all species, I used the number of decades since the bottleneck for the analysis. Decade number was calculated as the number of decades between the decade during which minimum population size occurred until the last year of collection of hatching failure data. A value of 0 was assigned to species declining at the time of data collection. One might expect that the number of generations since minimum bottleneck might be a more appropriate metric of potential purging, but this is unknown for most species. As larger species generally take longer to mature and have greater longevity, the possible effects of differences in generation time are controlled somewhat by the inclusion of body mass in my analyses.

2.2.2 Data analyses

The effects of population size on hatching failure were analysed using a general linear model (GLM) with Type II (adjusted) sums of squares (to control for any multicollinearity) with bottleneck size as the predictor variable and percent hatching failure as response. I started with a maximal model also including average clutch size, body mass, absolute latitude, and decades since bottleneck, and the interactions between these variables. I then simplified the model by removing non-significant parameters until no further reduction in residual deviance was observed (measured using the Akaike Information Criterion, AIC; Akaike 1973). I considered removing one species (kagu *Rhynochetos jubatus*), which almost qualified as a statistical outlier due to its large leverage. However, the removal of the kagu did not affect my main conclusions and it was retained in the analysis reported here. After examining the variances and distribution of residuals, I $\log_e$ transformed percent hatching failure and bottleneck size to meet the assumptions of the parametric model. The analysis was conducted in R version 2.10.1 (R Development Core Team 2008).

To control for effects of shared ancestry I calculated the phylogenetically independent contrasts on the values of bottleneck size and percent hatching failure with CAIC 1.2 (Purvis and Rambaut 1995). I constructed a phylogeny using Hackett et al. (2008) for taxa at the ordinal level, and

Sibley and Ahlquist (1990) and Dickinson (2003) to resolve relationships between taxa below the family level. Unresolved relationships were left as polytomies. Branch lengths were unknown for the entire phylogeny so I assumed either that branch lengths were equal, or that they were proportional to number of species in taxa (Purvis and Rambaut 1995). Both assumptions gave the same results and I report only the latter here. I then performed a linear regression of log hatching failure contrasts on log bottleneck size contrasts. The regression was forced through the origin. Finally, I tested assumptions following the recommendations in Purvis and Rambaut (1995). There were no significant relationships between the absolute values of the contrasts and the variance of the raw contrasts (all $p > 0.47$), suggesting that the assumption that residual variation has the same mean and variance around the regression line was met. The removal of the kagu (a potential outlier) also did not change the results of the contrast analysis.

2.3 Results

Hatching failure varied from 0% to almost 64% across species (see table 2.2 on p. 47). The best-fitting model retained only log bottleneck size as a significant predictor. In contrast, none of the other control variables (clutch size, body mass, latitude, and time since bottleneck) provided a significant reduction in residual deviance, and they were removed during model simplification (table 2.1). Log bottleneck size had a significant negative effect on percent hatching failure ($F_{1,49} = 22.97$, $p < 0.001$), such that populations passing through smaller bottlenecks had higher levels of hatching failure (figure 2.1a on p. 45). Below a bottleneck of approximately 100–150 individuals, all species experienced levels of hatching failure greater than the 10% seen in non-endangered birds (Koenig 1982). The effect of bottleneck size on hatching failure remained highly significant after controlling for phylogeny ($F_{1,44} > 23.98$, $p < 0.001$; figure 2.1b).

It may be argued that the above relationship was driven by the island species in this dataset; however, bottleneck size retained its significant effect on hatching failure rate when analysing the data (using GLMs with the same procedures as above) separately for island ($F_{1,37} = 10.01$, $p = 0.003$) and continental species ($F_{1,10} = 23.90$, $p < 0.001$). The effect of bottleneck size on hatching failure also remained significant after controlling for phylogeny when analysing island and continental species separately ($F_{1,33} = 8.03$, $p < 0.01$, and $F_{1,10} = 13.12$, $p < 0.01$, respectively).

Table 2.1: Summary of the GLM results of the maximal model (response = hatching failure) with Type II (adjusted) sums of squares before model simplification. Note that the best-fitting model only retained log (bottleneck).

Control variable	df	SS	MS	F-value	p-value
Clutch size	1	0.259	0.259	0.502	0.483
Body mass	1	0.969	0.969	1.877	0.178
Latitude	1	0.012	0.012	0.023	0.881
Time since bottleneck	1	0.009	0.009	0.017	0.896
Log (bottleneck)	1	10.245	10.245	19.838	0.00006
Residuals	45	23.239	0.516		

2.4 Discussion

My study confirms that there is a significant inverse relationship between bottleneck size and hatching success across a wide taxonomic range of bird species. Furthermore, this pattern affected both island and continental species, and there was no indication that my results were confounded by shared ancestry, clutch size, time since bottleneck, absolute latitude, or body mass. Bottleneck size had a strong effect on hatching failure, and below bottlenecks of $\sim$100–150 individuals, increased hatching failure was universal. This suggests that conservation biologists need to avoid bottlenecks below this value if they are to prevent increased hatching failure.

A reduction in hatching failure might be expected following a severe bottleneck if selection removes infertile individuals (Hedrick and Kalinowski 2000). However, I found no evidence for a decrease in hatching failure over time, though this result should be treated with caution as I used a relatively crude estimate of time since bottleneck, which did not account for differences in generation time among species. For example, the kakapo *Strigops habroptilus* breeds at intervals of 3–5 years while the black robin *Petroica traversi* breeds annually. If selection against infertile individuals is more intense among robins as a result of their shorter generation times, this may confound analyses based on calendar years. It is also possible that the time since most species passed through a bottleneck has been too short for selection to reduce hatching failure, and further work is needed to determine how quickly this might occur.

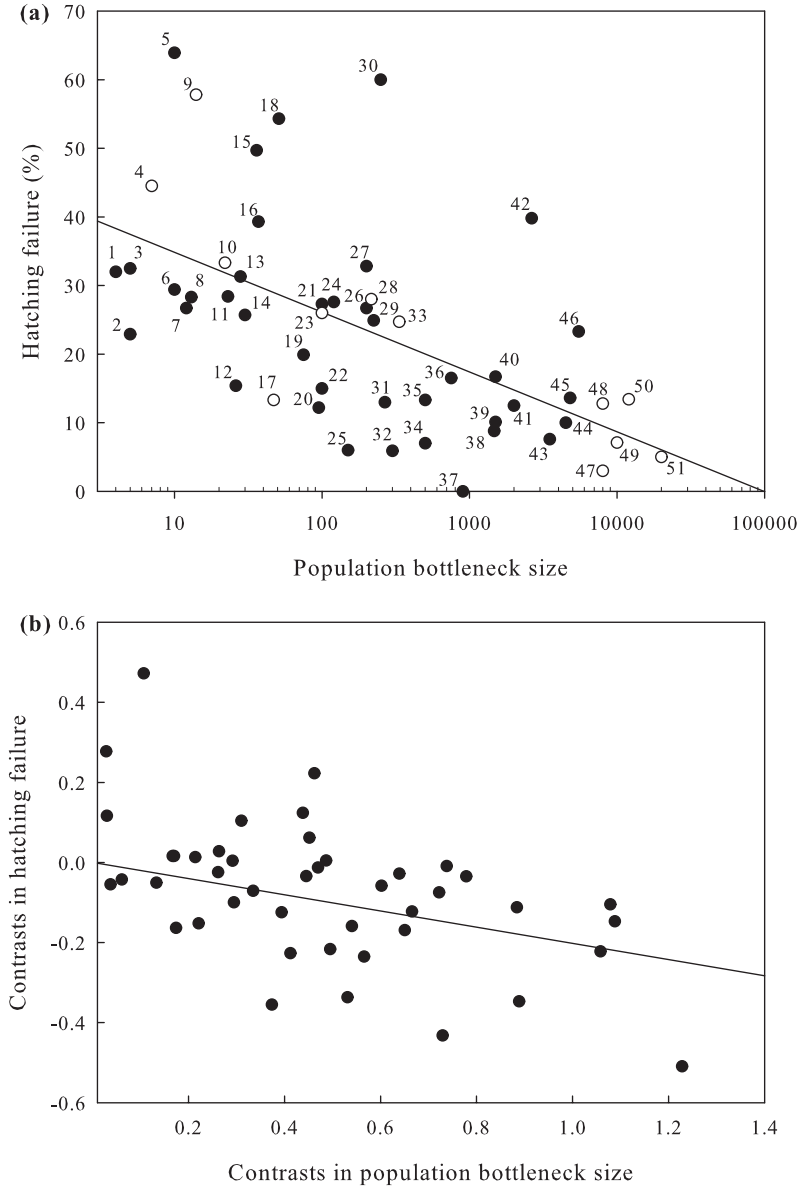

Figure 2.1: **a)** Relationship between bottleneck size and percent hatching failure in birds (open circles: continental species, filled circles: island species). For clarity, hatching failure is plotted on linear scale and bottleneck size on logarithmic scale although both were log transformed in the analyses. Species are: (1) *Falco punctatus*, (2) *Petroica traversi*, (3) *Petroica australis*, (4) *Nipponia nippon*, (5) *Sterna nereis*, (6) *Columba mayeri*, (7) *Anas laysanensis*, (8) *Amazona vittata*, (9) *Grus americana*, (10) *Gymnogyps californianus*, (11) *Himantopus novaezelandiae*, (12) *Acrocephalus sechellensis*, (13) *Terpsiphone corvina*, (14) *Branta sandvicensis*, (15) *Pterodroma cahow*, (16) *Philesturnus carunculatus*, (17) *Atlapetes pallidiceps*, (18) *Strigops habroptilus*, (19) *Anas nesiotis*, (20) *Thinornis novaeseelandiae*, (21) *Haematopus chathamensis*, (22) *Pterodroma magentae*, (23) *Tympanuchus cupido pinnatus*, (24) *Porphyrio*

hochstetteri, (25) *Neophema chrysogaster*, (26) *Himantopus mexicanus knudseni*, (27) *Myadestes palmeri*, (28) *Centrocercus minimus*, (29) *Foudia rubra*, (30) *Otus insularis*, (31) *Gallirallus owstoni*, (32) *Pterodroma axillaries*, (33) *Dendroica kirtlandii*, (34) *Anas aucklandica*, (35) *Philesturnus rufusater*, (36) *Petroica macrocephala chathamensis*, (37) *Rhynochetos jubatus*, (38) *Caprimulgus noctitherus*, (39) *Coenocorypha pusilla*, (40) *Charadrius obscurus aquilonius*, (41) *Haematopus unicolor*, (42) *Loxioides bailleui*, (43) *Procellaria parkinsoni*, (44) *Anarhynchus frontalis*, (45) *Megadyptes antipodes*, (46) *Eudyptes pachyrhynchus*, (47) *Vireo atricapilla*, (48) *Aphelocoma coerulescens*, (49) *Picoides borealis*, (50) *Charadrius montanus*, and (51) *Dendroica chrysoparia*. **b)** Relationship between contrasts in bottleneck size and contrasts in hatching failure.

Increased hatching failure might entail a substantial fitness cost for a small, short-lived species with few breeding attempts, but be less costly for a larger, longer-lived species in which opportunities for multiple breeding attempts might mitigate higher failure in a given breeding attempt (Spottiswoode and Møller 2004). Thus, larger species might be less vulnerable to the negative consequences of bottlenecks than smaller species. Nonetheless, I found no relationship between body mass and hatching failure, and thus large body size alone does not appear to prevent some degree of inbreeding depression.

Although the exact threshold above which inbreeding depression can be avoided is likely to vary among fitness traits, I found that increases in hatching failure were associated with bottlenecks of $<$ 100–150 individuals. This figure is consistent with the estimate of $\sim$150 individuals by Briskie and Mackintosh (2004) for birds in New Zealand. Although Briskie and Mackintosh (2004) included both native and introduced species in their study, it is not clear if increased hatching failure was due to bottlenecks or local environmental conditions. For example, differences in life history traits of island birds, such as body size and longevity, could change hatching failure independently of bottleneck effects (Clegg and Owens 2002). However, I found that bottleneck size increased hatching failure in both island and continental species, indicating that the pattern is unlikely to be just a product of local environmental conditions. Instead, severe bottlenecks appear to have the potential to increase reproductive failure in any species. My world-wide survey of birds that have passed through bottlenecks confirms that any management practices that result in severe bottlenecks, such as may occur in many translocation schemes (Griffith et al. 1989), are likely to lead to increased hatching failure and may limit the ability of a population to recover.

Table 2.2: Dataset including information on population bottleneck size, hatching failure (%), body mass (kg), absolute latitude, average clutch size, and decades passed since the minimum bottleneck for the bird species examined in this chapter.

#	Species	Scientific name	Bottleneck size	Hatching failure (%)	Body mass (kg)	Latitude	Average clutch size	Decades since bottleneck	Source
1	Mauritius kestrel	*Falco punctatus*	4	32.0	0.230	20	3	1	Jones (1987)
2	Black robin	*Petroica traversi*	5	32.5	0.022	44	2	1	Merton, D., pers. comm.
3	South Island robin (Motuara Is.)	*Petroica australis*	5	22.6	0.035	41	2	4	Heber, S., unpubl. data
4	Crested ibis	*Nipponia nippon*	7	44.5	1.600	30	3.5	2	Hildyard (2001), Xi et al. (2001)
5	New Zealand fairy tern	*Sterna nereis*	10	29.4	0.070	36	2	1	Parrish and Pulham (1995), OSNZ nest record cards
6	Pink pigeon	*Columba mayeri*	10	63.9	0.290	20	1.5	1	Bunbury (2006)
7	Laysan duck	*Anas laysanensis*	12	26.7	0.460	25	4	9	Dill and Bryan (1912), Reynolds (2002)
8	Puerto Rican amazon	*Amazona vittata*	13	28.3	0.275	18	3	3	Wunderle et al. (2003), Stehn, T., pers. comm.
9	Whooping crane (captive)	*Grus americana*	14	57.8	6.000	44	2	7	Canadian Wildlife Service and U.S. Fish and Wildlife Service (2007)
10	California condor	*Gymnogyps californianus*	22	33.3	7.700	34	1	3	California Condor Recovery Program (2009), Kiff, L., pers. comm.
11	Black stilt	*Himantopus novaezelandiae*	23	28.4	0.220	44	4	2	Maloney, R., pers. comm.
12	Seychelles warbler	*Acrocephalus sechellensis*	26	15.4	0.015	4	1	4	del Hoyo et al. (2006), Komdeur (1994)
13	Seychelles Paradise-flycatcher	*Terpsiphone corvina*	28	31.3	0.018	4	1	4	Currie et al. (2003, 2005)
14	Nene, Hawaiian goose	*Branta sandvicensis*	30	25.7	2.150	20	3	3	Banko et al. (1999), Smith (1952)
15	Bermuda petrel	*Pterodroma cahow*	36	49.7	0.250	32	1	5	Wingate, D., pers. comm.

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Table 2.2: Continued from previous page

#	Species	Scientific name	Bottleneck size	Hatching failure (%)	Body mass (kg)	Latitude	Average clutch size	Decades since bottleneck	Source
16	South Island saddleback	*Philesturnus carunculatus*	37	39.3	0.070	43	2.5	4	Hooson, S., pers. comm.
17	Pale-headed brush-finch	*Atlapetes pallidiceps*	47	13.3	0.032	3	2	1	Peraza (2009), Krabbe, N.K., pers. comm.
18	Kakapo	*Strigops habroptilus*	51	54.3	2.000	46	3	1	Merton, D., pers. comm.
19	Campbell Island teal	*Anas nesiotis*	75	19.9	0.500	52	3.5	1	Williams, M., pers. comm.
20	Shore plover	*Thinornis novaeseelandiae*	95	12.2	0.060	44	3	5	Davis (1994), OSNZ nest record cards
21	Chatham oystercatcher	*Haematopus chathamensis*	100	27.3	0.600	44	2	1	Schmechel and O'Connor (1999), OSNZ nest record cards
22	Chatham taiko	*Pterodroma magentae*	100	15.0	0.475	44	1	0	Imber, M., pers. comm.
23	Greater prairie chicken	*Tympanuchus cupido pinnatus*	100	26.0	0.800	36	11	1	Westemeier et al. (1998)
24	Takahe	*Porphyrio hochstetteri*	120	27.6	3.000	40	2	1	Jamieson and Ryan (2000)
25	Orange-bellied parrot	*Neophema chrysogaster*	150	6.0	0.045	43	4.5	0	Brown and Wilson (1984) Brown et al. (1985) Holdsworth (1997)
26	Hawaiian stilt	*Himantopus mexicanus knudseni*	200	26.7	0.200	20	4	6	Robinson et al. (1999), Goebel, K., and Uyehara, K., pers. comm.
27	Puaiohi	*Myadestes palmeri*	200	32.8	0.040	20	2	0	Kuehler et al. (2000)
28	Gunnison sage-grouse	*Centrocercus minimus*	216	28.0	2.000	39	8	0	Stiver et al. (2008)
29	Mauritius fody	*Foudia rubra*	224	24.9	0.018	20	3	1	Cristinacce, A., pers. comm.
30	Seychelles scops-owl	*Otus insularis*	250	60.0	0.155	4	1	3	BirdLife International (2008), Currie et al. (2004*b*), Currie et al. (2004*a*)
31	Guam rail	*Gallirallus owstoni*	267	13.0	0.230	13	3.5	1	Brock and Beauprez (2000)
32	Chatham petrel	*Pterodroma axillaris*	300	5.9	0.200	44	1	1	Gummer, H., pers. comm.
33	Kirtland's warbler	*Dendroica kirtlandii*	334	24.7	0.014	44	4.5	1	Walkinshaw (1983)

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Table 2.2: Continued from previous page

#	Species	Scientific name	Bottleneck size	Hatching failure (%)	Body mass (kg)	Latitude	Average clutch size	Decades since bottleneck	Source
34	Auckland Island teal	*Anas aucklandica*	500	7.0	0.500	50	3.5	18	Dumbell (1986), Williams (1986), Williams (1995)
35	North Island saddleback	*Philesturnus rufusater*	500	13.3	0.070	38	2.5	9	Lambert et al. (2005), OSNZ nest record cards
36	Chatham Island tomtit	*Petroica macrocephala chathamensis*	750	16.5	0.011	44	3	0	Powlesland et al. (2001), Powlesland, R., pers. comm.
37	Kagu	*Rhynochetos jubatus*	900	0.0	0.860	21	1	0	Salas and Letocart (1997)
38	Puerto Rican nightjar	*Caprimulgus noctitherus*	1,470	8.8	0.036	18	2	0	del Hoyo et al. (1999), Vilella (1995), Vilella, F.J., pers. comm.
39	Chatham snipe	*Coenocorypha pusilla*	1,500	16.7	0.080	44	2	9	Miskelly (1996), OSNZ nest record cards
40	New Zealand dotterel (North Island)	*Charadrius obscurus aquilonius*	1,500	10.1	0.160	38	3	2	OSNZ nest record cards
41	Variable oystercatcher	*Haematopus unicolor*	2,000	12.5	0.725	40	2.5	11	Baker (1973), OSNZ nest record cards
42	Palila	*Loxioides bailleui*	2,640	39.8	0.035	20	2	0	Banko et al. (2002)
43	Black petrel	*Procellaria parkinsoni*	3,500	7.6	0.700	36	1	0	Imber (1987)
44	Wrybill	*Anarhynchus frontalis*	4,500	10.0	0.060	44	2	0	Marchant and Higgins (1993),OSNZ nest record cards
45	Yellow-eyed penguin	*Megadyptes antipodes*	4,800	13.6	5.400	44	2	0	Darby and Seddon (1990)
46	Fiordland crested penguin	*Eudyptes pachyrhynchus*	5,500	23.3	4.000	47	2	0	Cassady St. Clair, C., pers. comm.
47	Black-capped vireo	*Vireo atricapilla*	8,000	12.8	0.009	34	4	0	Grzybowski (1995)
48	Florida scrub-jay	*Aphelocoma coerulescens*	8,000	3.0	0.074	28	3	1	Bowman and Woolfenden (2001), Woolfenden and Fitzpatrick (1996)

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Table 2.2: Continued from previous page

#	Species	Scientific name	Bottleneck size	Hatching failure (%)	Body mass (kg)	Latitude	Average clutch size	Decades since bottleneck	Source
49	Red-cockaded woodpecker	*Picoides borealis*	10,000	7.1	0.047	31	3	0	Jackson (1994), LaBranche and Walters (1994)
50	Mountain plover	*Charadrius montanus*	12,000	13.4	0.103	37	3	0	Knopf and Wunder (2006), Miller and Knopf (1993)
51	Golden-cheeked warbler	*Dendroica chrysoparia*	20,000	5.0	0.010	31	4	0	Ladd and Gass (1999), Reidy et al. (2008)

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Chapter 3

A test of the 'genetic rescue' technique using bottlenecked donor populations of *Drosophila melanogaster*

Abstract: *I produced replicated experimental lines of inbred fruit flies* Drosophila melanogaster *to test the effects of crossing different inbred populations as a method of 'genetic rescue' for endangered species lacking outbred donor populations. Two strains differing in the origin of the founders were maintained as isolated populations in a laboratory environment. After two generations of controlled full-sib matings, the resulting inbred fruit flies had significantly reduced breeding success and survival rates. However, crosses between the two bottlenecked strains reversed the effects of inbreeding and led to increases in breeding success and survival that persisted into the second generation of hybrid offspring. In contrast, crosses within each strain (but between different replicate lines) resulted in only slight improvements in some fitness components, and this positive trend was reversed in the second generation. This experiment highlights the potential value of translocations between different inbred populations of endangered species as a tool to mitigate the negative effects of inbreeding, but this benefit may depend upon the origin of the populations. My results also confirm the importance of maintaining adequate levels of genetic variation within populations and that severely bottlenecked populations should not be discounted as possible donors in genetic rescue programs for endangered species.*

3.1 Introduction

Anthropogenic influences such as habitat loss and fragmentation, the introduction of exotic predators, excessive hunting, and pollution have forced many species through severe population bottlenecks. Decreased effective population size during a bottleneck can lead to increased inbreeding and the loss of genetic diversity, which both adversely affect population viability (Brook et al. 2005, England et al. 2003, Frankham et al. 2010, Kristensen et al. 2010, Newman and Pilson 1997, O'Grady et al. 2006). The translocation of outbred individuals into bottlenecked populations has been shown to mitigate the negative effects of inbreeding and to restore genetic variability (e.g. Ingvarsson 2002, Madsen et al. 2004, Westemeier et al. 1998). For example, the introduction of outbred individuals led to a rapid increase in the fitness of wild inbred populations of both greater prairie chickens *Tympanuchus cupido* (Westemeier et al. 1998) and European adders *Vipera berus* (Madsen et al. 1999). Consequently, the 'genetic rescue' of endangered animals through the translocation of outbred individuals has become more frequent in recent years (Armstrong 1995, Johnson et al. 2010, Weeks et al. 2011).

The use of genetic rescue as a management tool depends on the availability of suitable outbred donor populations. However, for many endangered species there are no outbred populations left to act as a donor. Instead, many endangered species survive only as a series of small, fragmented populations, with each likely subject to some loss of genetic variation and increased levels of inbreeding. Theoretical models suggest that by crossing individuals from one inbred population with those of a second inbred population, the severity of inbreeding depression should decrease in the hybrid offspring (Edmands 2007). Such an effect might be expected if recessive deleterious alleles in one population become masked by alleles in the second population, and vice versa (Charlesworth and Charlesworth 1999, Lynch 1991). Experiments with fruit flies *Drosophila melanogaster* and houseflies *Musca domestica* support the prediction that immigration of individuals into inbred lines can lead to rapid improvements in fitness traits such as viability, productivity and survival (Bryant et al. 1999, Spielman and Frankham 1992, see also Newman and Tallmon 2001, but see Holleley et al. 2011). In one of the few studies to use inbred donors in the genetic rescue of a wild animal, Fredrickson et al. (2007) translocated inbred Mexican wolves *Canis lupus baileyi* to both captive and reintroduced populations of this species. As only three captive lineages of Mexican wolves survived from a total founding population of seven animals, no outbred individuals were available as donors. Despite low levels of genetic variation and fixed deleterious alleles within each lineage,

crosses between lines experienced increases in the proportion of live births, litter size, and survival of offspring (Fredrickson et al. 2007).

Despite the apparent success of the genetic rescue technique using inbred donors in lab and field studies, the general effectiveness of using inbred individuals as donors is not clear, nor whether the suitability of inbred donors varies with their source. In some species, prospective donor populations may share a recent common ancestry with a recipient population (as is the case with many daughter populations created through the translocation of individuals to found new populations for conservation purposes), and may not be differentiated enough to introduce new genetic variation. Alternatively, a donor population may be so differentiated (as may be the case for two subspecies or geographically isolated populations) that it may lead to a deterioration of fitness traits, in a process termed outbreeding depression (Edmands 1999, 2007, Frankham et al. 2011, Goldberg et al. 2005, Hedrick and Fredrickson 2010, Lynch 1991, Marr et al. 2002, Marshall and Spalton 2000, Tallmon et al. 2004, Weeks et al. 2011). The objective of this study was therefore to test whether the exchange of individuals between inbred populations reduces levels of inbreeding depression, and if the effectiveness of any change depends on the source of the donor population. To address this question, I conducted replicated experimental crossings within and between two artificially inbred strains of the fruit fly *Drosophila melanogaster* to test changes in the viability of the hybridised population.

3.2 Material and methods

3.2.1 Inbreeding method

Two strains of *Drosophila melanogaster* originating from different parts of the world (Wild type Oregon-R, USA, and Slg14-15, Sweden) were used to create inbred lineages. Both source populations were maintained in cages supporting > 500 individuals with overlapping generations. Despite the maintenance of large populations, stocks of fruit flies are known to lose genetic variation, with the degree of loss increasing with greater periods of time in captivity (Briscoe et al. 1992). Although I did not measure levels of genetic variation in each population directly, high levels of fertility and survival among individuals suggest neither was suffering inbreeding depression. I then created replicate inbred lines within each strain through two generations of full-sib matings. From each line, offspring were collected as virgins, and one full-sib pair was randomly chosen as parents for

the next generation. Each pair was housed in separate vials to prevent outbreeding (vials measured 75 mm x 25 mm x 25 mm). All eclosed young were removed twice a day to ensure virgins were used for the next generation. The inbreeding procedure was stopped after two generations, as both populations experienced problems with reproductive success and survival. Seven replica of full-sib pairs within each strain were started; however, three replica in the Slg14-15 strain and four replica in the Wild type Oregon-R strain were lost due to complete reproductive failure (see crosses in figure 3.1). The extinction of 7/14 (50%) of inbred lines is consistent with an expected increase in the risk of extinction with increased inbreeding (Brook et al. 2005).

All cultures were maintained on standard commercial medium (Formula 4-24 instant medium, blue, Carolina Biological Supply Company, North Carolina, USA) with a supplement of live yeast. The stocks were kept in an incubator at $25 \pm 1.0\,^{\circ}\mathrm{C}$, and a 12:12 hour light:dark photoperiodic cycle. The position of vials within the incubator was re-randomised on a regular basis.

3.2.2 Crossing experiments

Four inbred replica of the Slg14-15 strain (hereafter, Slg) and three inbred replica of the Wild type Oregon-R strain (hereafter, Wt) survived for two generations. The flies from these seven lines (named Wt1, Wt2, Slg1, Slg2, etc.) were used for replicate crossing (= "genetic rescue") experiments. To test the effects of hybridising individuals from inbred lines on the fitness of offspring, I paired inbred flies within each strain, but between different replica (e.g. Slg5 x Slg7, 20 pairs; e.g. Wt4 x Wt5, 27 pairs). The resulting offspring are referred to as F1 within-strain hybrids. I also paired inbred flies from one strain with inbred flies from the other strain (e.g. Slg6 x Wt7; 27 pairs), to test the effects of hybridising inbred individuals from differing strains on the fitness of their offspring. These are termed F1 between-strain hybrids (Slg-Wt; see figure 3.1). With these crossings, I tested whether inbred populations founded from different source populations could act as genetic rescue donors.

To determine the persistence of fitness effects from hybridising inbred lines, a second generation of hybrids (F2) was bred within each F1 within-strain and between-strain hybrids. Within each of the three groups (Slg within-strain hybrids, Wt within-strain hybrids, and Slg-Wt between-strain hybrids), F1 hybrids were either paired with other F1 hybrids of the same group (hybrid-hybrid matings; Slg: 19 pairs, Wt: 29 pairs, Slg-Wt: 29 pairs), or with inbred flies of the same strain (hybrid-inbred matings; Slg: 23 pairs, Wt: 33 pairs), or of both strains in the case of the between-

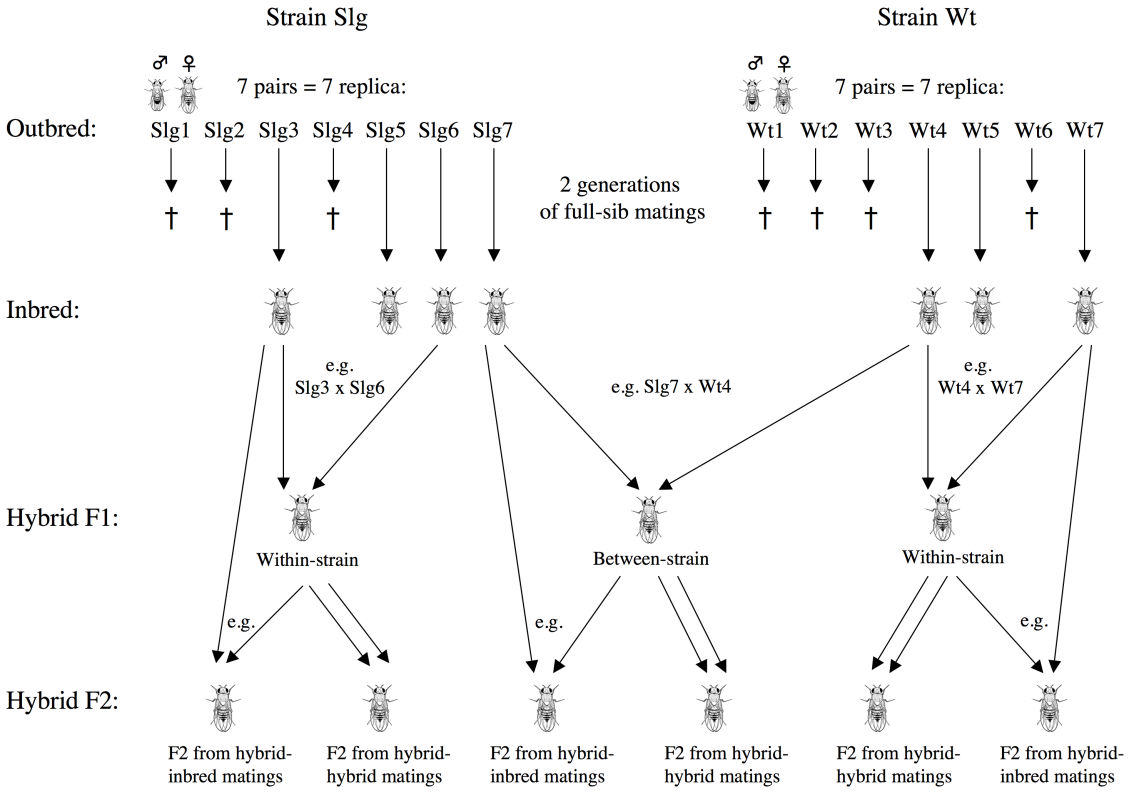

Figure 3.1: Diagram of the crossing experiment. Crosses (†) identify replica lost during the process of inbreeding. In the between-strain F2 hybrids, offspring from hybrid-inbred matings resulted either from matings of F1 hybrids with Slg inbred or Wt inbred flies (only matings between F1 between-strain hybrids and Slg inbred flies are shown for simplicity).

strain crosses (Slg-Wt: 30 pairs), resulting in six groups in the F2 hybrid generation (F2 hybrids resulting from either hybrid-hybrid or hybrid-inbred matings in each strain, Slg, Wt and Slg-Wt, respectively; see figure 3.1). Pairs from the original source populations were used as controls (37 pairs). The final number of pairings in each group varied due to the death of some flies during the course of the experiment.

Both reproductive success and survival are fitness measures vital to the persistence of populations. Breeding success and daily survival rates were therefore assessed for pairs and individuals in each group (inbred, F1 hybrid, F2 hybrid, and original).

3.2.3 Breeding success

Each pair was put into a clean vial with fresh medium and allowed to mate and oviposit for 96 h. Eggs were counted using a Wild Heerbrugg M3 stereomicroscope upon removal of the adults. Daily emergence of male and female adult progeny was counted twice a day until eclosion stopped. The total number of pupae and the number of not eclosed pupae were counted. In terms of absolute reproductive output, only the average number of eggs laid per pair is reported here, as the absolute numbers of pupae formed and adults eclosed depend on the number of eggs laid. The proportion of eggs that developed into pupae and pupae that developed into adults was calculated and used as measures of reproductive success. Pairs that did not lay any eggs were excluded from the calculations.

3.2.4 Survival

Upon eclosion, flies were counted and sexed under CO_2 anesthesia. Males and females were then transferred to new same-sex vials with standard instant medium, with a total of 20 flies per vial. Vials were checked daily up to a maximum of 8 days to record the number of dead flies. Flies still alive after 8 days were censored in the analysis (see below) to account for the end of the observation period.

3.2.5 Data analyses

As measures of breeding success did not differ significantly between the Slg and Wt strains (Wilcoxon Signed Rank tests; number of eggs laid: $p = 0.13$, proportion of eggs that pupated: $p = 0.61$, proportion of pupae that eclosed: $p = 0.12$), the two strains were pooled to facilitate comparison with the between-strain hybrids. This resulted in two groups in the first generation (hybrid F1 within-strain (Slg and Wt) vs. hybrid F1 between-strain (Slg-Wt)). As mean trait values of reproductive success of hybrid-hybrid and hybrid-inbred pairs were not significantly different in both F2 within-strain and F2 between-strain crosses (all credible intervals include 1), I pooled this data, resulting in two groups of F2 hybrids (hybrid F2 within-strain and hybrid F2 between-strain). I therefore compared a total of six groups of varying inbreeding status (inbred, hybrid F1 within-strain, hybrid F2 within-strain, hybrid F1 between-strain, hybrid F2 between-strain, and original).

Data from the breeding experiments were fitted using an ‘animal model’ (Henderson 1984),

which is a generalised linear mixed model that expresses phenotypic observations y_i as a function of an additive genetic component a_i. This model can accommodate the pedigree of the individuals through the use of a relatedness matrix. In addition to the genetic component, the model included a group effect with the six levels of inbreeding status. The number of eggs laid was analysed using a Gaussian distribution with an identity link function, while the proportion of eggs that pupated (pupae/eggs) and the proportion of pupae that eclosed (adults/pupae) were analysed using a binomial distribution with a logit link function. Inference for the animal model relied on a Bayesian framework, using Integrated Nested Laplace Approximations (INLA) to calculate the marginal posteriors for all parameters (Rue et al. 2009). Marginal posteriors were summarised using the posterior mean and 95% credible intervals. Model fitting was performed using "AnimalINLA" (Holand et al. 2011), a package for the R statistical software system (version 2.13.2, R Development Core Team 2011).

As survival did not differ significantly between the Slg and Wt strains (Wilcoxon Signed Rank test, p = 0.72), the two strains were pooled to facilitate comparison with the between-strain hybrids. Data for each fruit fly in the survival trial consisted of the time (in days) until exit from the study (either by death or by censoring), a censoring indicator specifying whether an individual survived until the end of the experiment or not (0 = individual survived and died at an unknown time in the future, 1 = individual died during the course of the experiment), and the group (inbred, hybrid F1 within-strain, hybrid F2 within-strain, hybrid F1 between-strain, hybrid F2 between-strain, and original) that the individual belonged to as factor. I analysed the survival data using the *survreg* function within the "survival" package in the R statistical environment (version 2.13.2, R Development Core Team 2011, Therneau 2011). I first fitted a parametric model assuming constant hazard of death (exponential error distribution) with censoring (as a number of individuals died at an unknown time after the end of the experiment). I then compared the model to a parametric model based on the Weibull distribution (assumption of age-specific non-constant hazard), which was a significant improvement ($p < 0.0001$). This model was simplified by pooling the survival rate of F2 offspring resulting from hybrid-hybrid matings with that of F2 offspring resulting from hybrid-inbred matings, as there was no significant difference between the survival rates in within-strain (Slg and Wt) and between-strain hybrids (Slg-Wt; p = 0.973 and p = 0.405, respectively). The simpler model was not significantly worse compared to the initial model (p = 0.08). I calculated effect sizes and adjusted p-values for the model estimates with the add-on R package "multcomp" (Hothorn et al. 2008). Adjusted 95% confidence intervals (CIs) were computed by hand using the adjusted p-values. The use of p-values adjusted for the multiple comparisons, and hence of ad-

justed 95% confidence intervals, is justified due to the large sample size used in the survival trial (n = 4,226, Nakagawa 2004). Daily survival probability was calculated using a modified Mayfield method (Mayfield 1961).

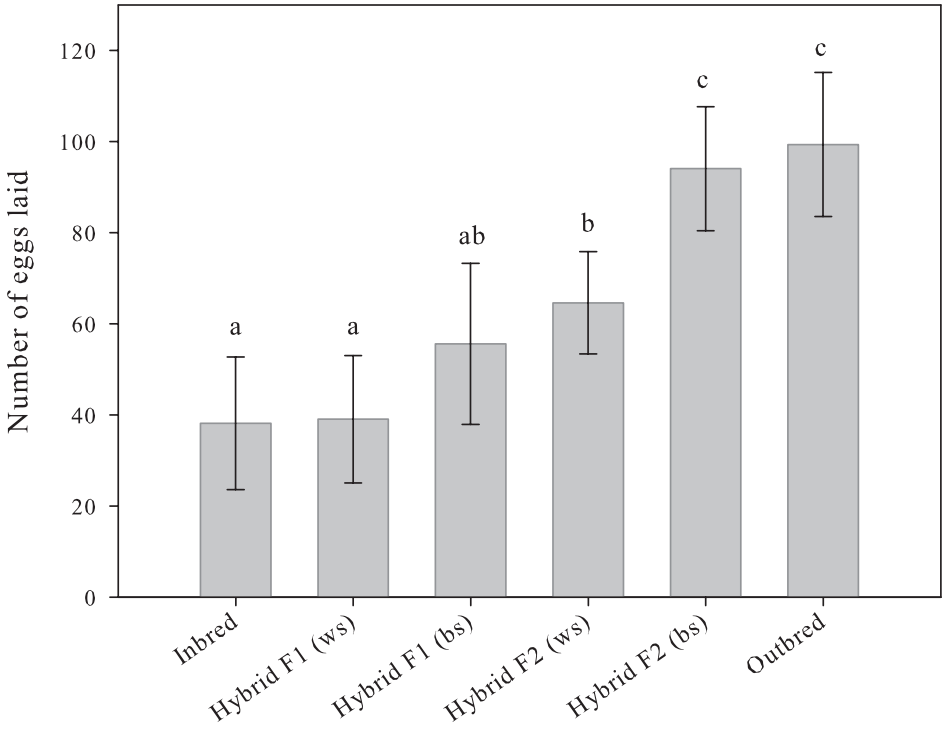

Figure 3.2: Absolute numbers of eggs laid (and 95% credible intervals) for inbred, hybrid, and original groups of *Drosophila melanogaster* (ws: within-strain crosses, bs: between-strain crosses). Different letters indicate significant differences between groups. Pairs that did not lay any eggs are excluded from the calculations.

3.3 Results

3.3.1 Breeding success

Inbreeding within each line lead to a significant reduction in the absolute numbers of eggs laid (figure 3.2; see table 3.1 for mean breeding values and 95% credible intervals). Exact values of the effect sizes of the pairwise contrasts for the average number of eggs laid, and their 95% credible intervals can be found in table 3.2.

Table 3.1: Mean breeding values and 95% credible intervals (CIs) for absolute numbers of eggs laid, proportion of eggs that pupated, and proportion of pupae that eclosed for inbred, hybrid, and original groups of *Drosophila melanogaster* (ws: within-strain crosses, bs: between-strain crosses). Pairs that did not lay any eggs are excluded from the calculations.

Trait	Cross	N (pairs)	Mean	Lower 95% CI	Upper 95% CI
Eggs	Inbred	44	38.17	23.60	52.71
	Hybrid F1 (ws)	47	39.10	25.08	53.05
	Hybrid F1 (bs)	27	55.59	37.94	73.27
	Hybrid F2 (ws)	101	64.61	53.37	75.85
	Hybrid F2 (bs)	62	94.06	80.45	107.64
	Original	37	99.34	83.53	115.15
Pupae/eggs	Inbred	44	0.77	0.57	0.89
	Hybrid F1 (ws)	47	0.81	0.64	0.91
	Hybrid F1 (bs)	27	0.76	0.54	0.89
	Hybrid F2 (ws)	100	0.78	0.62	0.89
	Hybrid F2 (bs)	61	0.93	0.85	0.97
	Original	37	0.95	0.87	0.98
Adults/pupae	Inbred	44	0.94	0.91	0.97
	Hybrid F1 (ws)	47	0.98	0.96	0.99
	Hybrid F1 (bs)	27	0.99	0.97	0.99
	Hybrid F2 (ws)	101	0.96	0.94	0.98
	Hybrid F2 (bs)	62	0.97	0.96	0.98
	Original	37	0.98	0.96	0.99

Egg number increased progressively from inbred pairs to the first and second generation of hybrid between-strain pairs and reached a maximum in pairs from the original population (table 3.1). The increase observed in F1 between-strain hybrids was not significant compared to inbred individuals (credible intervals include zero; table 3.2 and figure 3.2). However, F2 between-strain hybrids exhibited significantly higher values than inbred flies and F1 between-strain hybrids (credible intervals do not include zero; table 3.2 and figure 3.2), and did not significantly differ from original pairs (i.e., the number of eggs laid was comparable to that in flies from the original population). In contrast, the number of eggs laid in the hybrid F1 within-strain group was virtually identical to values recorded for inbred pairs (figure 3.2). The number of eggs laid increased in the second generation of within-strain hybrids, and was significantly higher than in inbred flies, but significantly lower than in flies from the original population, and thus had an intermediate status between inbred and original pairs. In other words, both within-strain and between-strain hybrids produced an in-

creased number of eggs, but the increase was more pronounced in between-strain hybrids than in within-strain hybrids. Only F2 between-strain hybrids were comparable to flies from the original population in terms of the absolute number of eggs laid (figure 3.2).

Table 3.2: Pairwise contrasts (effect size and 95% credible intervals) for absolute numbers of eggs laid for inbred, hybrid, and original groups of *Drosophila melanogaster* (ws: within-strain crosses, bs: between-strain crosses). Differences are significant if the 95% CIs do not include 0.

Trait	Comparison	Effect size	Lower 95% CI	Upper 95% CI
Eggs	Inbred–Hybrid F1 (ws)	−0.90	−22.62	20.82
	Inbred–Hybrid F1 (bs)	−17.57	−42.92	7.80
	Inbred–Hybrid F2 (ws)	−26.40	−45.91	−6.91
	Inbred–Hybrid F2 (bs)	−55.85	−77.46	−34.30
	Hybrid F1 (ws)–Hybrid F1 (bs)	−16.67	−41.55	8.24
	Hybrid F1 (ws)–Hybrid F2 (ws)	−25.50	−44.13	−6.94
	Hybrid F1 (bs)–Hybrid F2 (bs)	−38.28	−62.88	−13.89
	Hybrid F2 (ws)–Hybrid F2 (bs)	−29.45	−47.61	−11.22
	Inbred–Original	−61.17	−84.78	−37.57
	Hybrid F1 (ws)–Original	−60.27	−83.41	−37.12
	Hybrid F1 (bs)–Original	−43.60	−70.18	−17.04
	Hybrid F2 (ws)–Original	−34.77	−55.50	−14.05
	Hybrid F2 (bs)–Original	−5.32	−28.10	17.43

The proportion of eggs that developed into pupae in inbred flies decreased by 18% compared to flies from the original population (table 3.1). In contrast, the proportion of pupae that hatched was relatively high in all groups, ranging between 94% in inbred flies and 98% in flies from the original population (table 3.1). Effect sizes of the pairwise odds ratios (and 95% credible intervals) for the proportion of eggs that pupated and pupae that hatched are illustrated in figure 3.3 (exact values are listed in table 3.3 on p. 70; note that in odds ratios, differences are significant if the 95% credible intervals do not include 1, as opposed to pairwise contrasts, where differences are significant if the 95% CIs do not include 0).

In between-strain hybrids, pupating success in the F1 generation was virtually identical to that found in inbred flies; however, there was a significant increase in pupating success in the F2 generation and levels were not significantly different to those recorded in flies from the original population (figure 3.3, table 3.1, and table 3.3 on p. 70). Pupae eclosing success increased significantly in both the F1 and F2 generations of between-strain hybrids and was comparable to eclosing levels in flies from the original population.

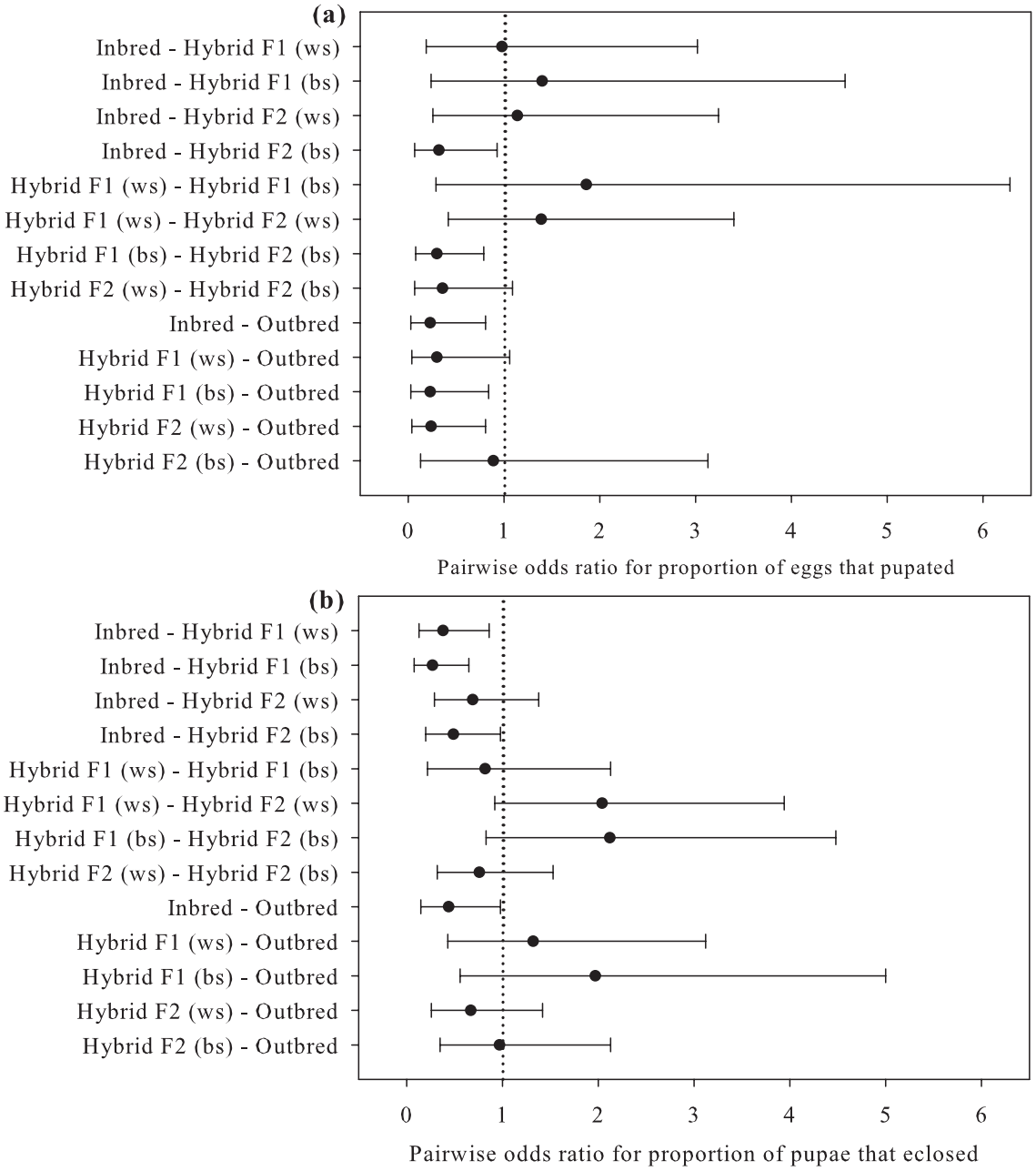

Figure 3.3: Pairwise odds ratios (effect size and 95% credible intervals) for proportion of eggs that pupated and proportion of pupae that eclosed for inbred, hybrid, and original groups of *Drosophila melanogaster* (ws: within-strain crosses, bs: between-strain crosses). In odds ratios, differences are significant if the 95% credible intervals do not include 1.

Table 3.3: Pairwise odds ratios (effect size and 95% credible intervals) for proportion of eggs that pupated and proportion of pupae that eclosed for inbred, hybrid, and original groups of *Drosophila melanogaster* (ws: within-strain crosses, bs: between-strain crosses). In odds ratios, differences are significant if the 95% credible intervals do not include 1.

Trait	Comparison	Odds ratio	Lower 95% CI	Upper 95% CI
Pupae/eggs	Inbred–Hybrid F1 (ws)	0.98	0.19	3.02
	Inbred–Hybrid F1 (bs)	1.40	0.24	4.56
	Inbred–Hybrid F2 (ws)	1.14	0.26	3.24
	Inbred–Hybrid F2 (bs)	0.32	0.07	0.93
	Hybrid F1 (ws)–Hybrid F1 (bs)	1.86	0.29	6.28
	Hybrid F1 (ws)–Hybrid F2 (ws)	1.39	0.42	3.40
	Hybrid F1 (bs)–Hybrid F2 (bs)	0.30	0.08	0.79
	Hybrid F2 (ws)–Hybrid F2 (bs)	0.36	0.07	1.09
	Inbred–Original	0.23	0.03	0.81
	Hybrid F1 (ws)–Original	0.30	0.04	1.06
	Hybrid F1 (bs)–Original	0.23	0.03	0.84
	Hybrid F2 (ws)–Original	0.24	0.04	0.81
	Hybrid F2 (bs)–Original	0.89	0.13	3.13
Adults/pupae	Inbred–Hybrid F1 (ws)	0.38	0.13	0.86
	Inbred–Hybrid F1 (bs)	0.27	0.08	0.65
	Inbred–Hybrid F2 (ws)	0.69	0.29	1.38
	Inbred–Hybrid F2 (bs)	0.49	0.20	0.98
	Hybrid F1 (ws)–Hybrid F1 (bs)	0.82	0.22	2.13
	Hybrid F1 (ws)–Hybrid F2 (ws)	2.04	0.92	3.94
	Hybrid F1 (bs)–Hybrid F2 (bs)	2.12	0.83	4.48
	Hybrid F2 (ws)–Hybrid F2 (bs)	0.76	0.32	1.53
	Inbred–Original	0.44	0.15	0.98
	Hybrid F1 (ws)–Original	1.32	0.43	3.12
	Hybrid F1 (bs)–Original	1.97	0.56	5.00
	Hybrid F2 (ws)–Original	0.67	0.26	1.42
	Hybrid F2 (bs)–Original	0.97	0.35	2.13

In F1 within-strain hybrids, the proportion of eggs that pupated tended to increase compared to inbred flies, but this increase was not significant (however, it was also not significantly lower than in flies from the original population). In the F2 generation of within-strain hybrids, the proportion of eggs that pupated decreased to levels observed in inbred flies. Similarly, eclosing success of pupae increased in the F1 generation of within-strain hybrids and was virtually identical to eclosing levels in flies from the original population, but decreased in the F2 generation to levels that were intermediate between inbred and original flies (figure 3.3 on p. 69, table 3.1 on p. 67, and table 3.3).

3.3.2 Survival

Daily mortality probabilities and results of the survival analyses (predicted life span, effect size of the pairwise comparisons, adjusted 95% confidence intervals and adjusted p-values) are summarised in table 3.4. In the F1 generation of hybrids, both within- and between-strain hybrids experienced a significant increase in survival probability, with the effect being more pronounced in between-strain hybrids (figure 3.4, table 3.4). There was a slight reduction in survival probability from the F1 to the F2 generation of between-strain hybrids, but it was not significantly different from survival probability in individuals from the original population. The positive effect seen in F1 within-strain hybrids, however, did not persist into the F2 generation, where survival was reduced to levels observed in inbred flies (figure 3.4, table 3.4).

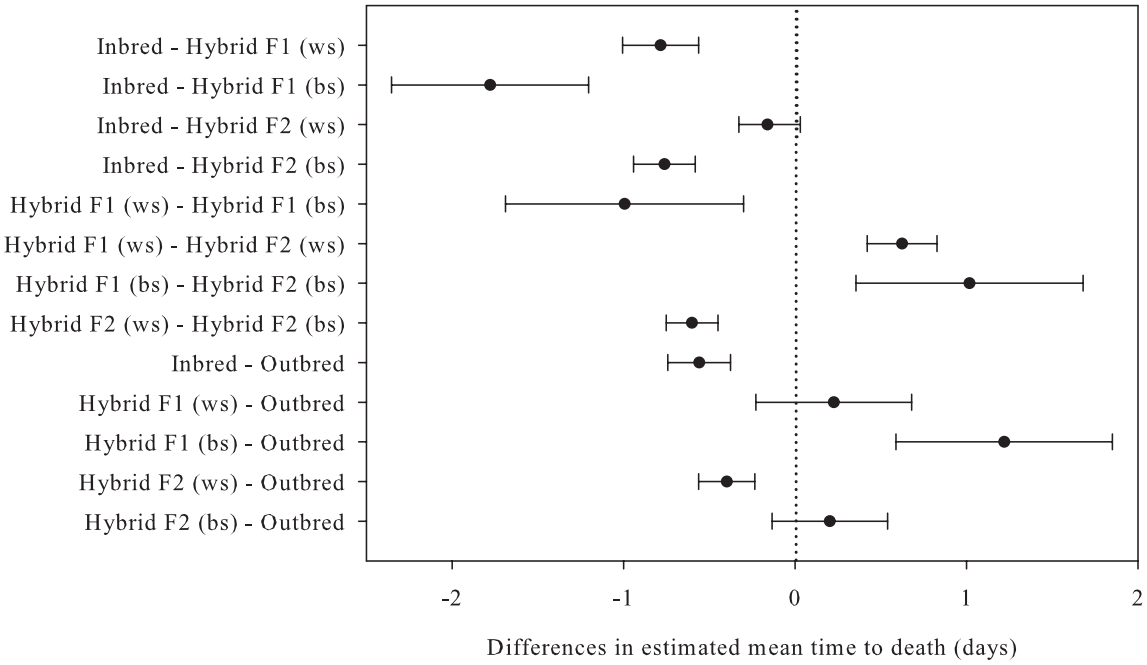

Figure 3.4: Effect size and adjusted 95% confidence intervals for pairwise comparisons of differences in survivorship between *Drosophila melanogaster* groups (ws: within-strain crosses, bs: between-strain crosses). Differences are significant if the 95% CIs do not include 0.

Table 3.4: Pairwise comparisons of mortality probabilities (as calculated using the Mayfield method), predicted life span, effect size and confidence intervals for the effect size (estimated using the *survreg* function) of *D. melanogaster* groups of varying level of inbreeding (ws: within-strain crosses, bs: between-strain crosses).

	Group	N	Daily mortality probability (%)	Predicted life span (days)	Effect size	Lower 95% CI (adjusted)	Upper 95% CI (adjusted)	Adjusted p-value
(1)	Inbred	332	5.8	8.06				
	Hybrid F1 (ws)	430	1.4	17.7	−0.785	−1.006	−0.563	<0.001***
(2)	Inbred	332	5.8	8.06				
	Hybrid F1 (bs)	405	0.2	47.8	−1.779	−2.353	−1.205	<0.001***
(3)	Inbred	332	5.8	8.06				
	Hybrid F2 (ws)	430	4.9	9.5	−0.161	−0.328	0.007	0.061
(4)	Inbred	332	5.8	8.06				
	Hybrid F2 (bs)	966	1.2	17.3	−0.762	−0.942	−0.582	<0.001***
(5)	Hybrid F1 (ws)	430	1.4	17.7				
	Hybrid F1 (bs)	405	0.2	47.8	−0.994	−1.689	−0.299	0.005**
(6)	Hybrid F1 (ws)	430	1.4	17.7				
	Hybrid F2 (ws)	1,586	4.9	9.5	0.624	0.420	0.828	<0.001***
(7)	Hybrid F1 (bs)	430	0.2	47.8				
	Hybrid F2 (bs)	966	1.2	17.3	1.017	0.355	1.680	0.003**
(8)	Hybrid F2 (ws)	1,586	4.9	9.5				
	Hybrid F2 (bs)	966	1.2	17.3	−0.601	−0.752	−0.450	<0.001***
(9)	Inbred	332	5.8	8.06				
	Original	507	2.0	14.1	−0.559	−0.742	−0.376	<0.001***
(10)	Hybrid F1 (ws)	430	1.4	17.7				
	Original	507	2.0	14.1	0.226	−0.229	0.680	0.33
(11)	Hybrid F1 (bs)	405	0.2	47.8				
	Original	507	2.0	14.1	1.220	0.589	1.851	<0.001***
(12)	Hybrid F2 (ws)	1,586	4.9	9.5				
	Original	507	2.0	14.1	−0.398	−0.562	−0.234	<0.001***
(13)	Hybrid F2 (bs)	966	1.2	17.3				
	Original	507	2.0	14.1	0.203	−0.135	0.540	0.24

Predictions of the mean age (in days) at death for *D. melanogaster* individuals in each group are based on extrapolations from the parametric *survreg* model. As a large proportion of the F1 between-strain hybrids were still alive at the end of the survival trial (and were thus censored),

the predicted life span is unrealistically long (see table 3.4). The predictions should therefore not be seen as absolute values, but rather in relation to one another (e.g. hybrid F1 between-strain individuals had a significantly longer life-span than individuals from any other group).

3.4 Discussion

As expected, the severe bottlenecks I induced in the two strains of fruit flies and the subsequent forced inbreeding led to a decline in individual breeding success and survival. Even after only two generations of inbreeding, half of my lines went extinct through reproductive failure. However, subsequent crossings within each strain of inbred flies (but between different replicate lines) as well as crossings between the two strains resulted in significant increases in survival and some measures of reproductive success. The positive effects of the crossing experiments were more pronounced in the between-strain hybrids, which increased even further in the F2 generation. Most importantly, between-strain hybrids exhibited a significant increase in both the absolute (number of eggs produced), and the relative reproductive output (proportion of eggs that developed into pupae, and proportion of pupae that eclosed). This was coupled with a marked increase in survival probability, which even exceeded the survival probabilities of individuals from the original populations. My results thus support the potential value of genetic rescue as a management tool for endangered species that survive only as a series of fragmented and bottlenecked populations.

For within-strain crossings, the results were of mixed nature. In terms of absolute reproductive output (number of eggs laid), the first generation of within-strain hybrids showed no increase compared to inbred flies. Significant positive effects were, however, observed in the second generation of within-strain crossings. Relative reproductive output (the proportion of eggs that developed into pupae, and the proportion of pupae that eclosed) tended to increase in F1 within-strain hybrids, but in F2 individuals this positive trend was reversed to levels measured in inbred flies. Similarly, survival probabilities increased significantly in F1 within-strain hybrids compared to inbred flies, but this positive effect did not persist into the second generation. Nonetheless, the increase in number of eggs laid observed in the second generation of within-strain hybrids constitutes a fitness improvement compared to inbred flies. Whether the improvement in fitness is sufficient to warrant the implementation of genetic rescue between populations of endangered species stemming from the same source population is not clear and would first require determining whether such crosses would

actually introduce any new genetic variation into the recipient populations. Given that a number of endangered species currently managed as discrete populations originated from the same single source population and yet show some genetic differentiation (e.g. black-footed ferret *Mustela nigripes*, Wisely et al. 2008), suggests further tests of the genetic rescue hypothesis using inbred populations descending from the same source would be worthwhile.

The relatively weak response I obtained to within-strain crosses contrasts with that obtained by Spielman and Frankham (1992). They found that even the introduction of a single immigrant into their fruit fly populations lead to an increase in fitness (as measured by a competitive index measure), even though the immigrants stemmed from the same base population. The difference with my results may be due to my use of laboratory strains while Spielman and Frankham (1992) used a wild caught population as their source from which to start inbred lines. As lab stocks of *Drosophila* have lower levels of genetic variation than their wild source populations (Briscoe et al. 1992), it is likely that more variation was present in their inbred lines than in my equivalent lines, and thus my within-strain crosses injected relatively little new variation in the donor populations. For some endangered species, which survive as only two or three populations that stem from only a single bottlenecked population, genetic variation is known be very low (e.g. black robin *Petroica traversi*, Ardern and Lambert 1997), and the use of crosses in such species may be similar to my use of lab stocks. Although I cannot determine why my results differ from this earlier study, without direct estimates of genetic variation present in potential donor populations, the prudent course of action would be to use donors not recently sourced from the same population as the recipients.

Although I did not quantify genetic variability, I assumed that the two original strains used in this study had some degree of genetic differentiation, given that they stem from different parts of the world. Under this assumption, I would therefore expect the fruit fly lines resulting from the full-sib matings to have relatively large differences in their allelic composition between the two strains. In contrast, within-strain hybrids (i.e., crosses of inbred flies of the same strain) were expected to be genetically similar. Nevertheless, even replica of the same strain were unlikely to be genetically identical, and thus deleterious alleles could still be masked by crossing flies from different replica within each strain. The subsequent exposure of deleterious alleles in the second generation of within-strain hybrids could then cause the reversal of the positive effects seen in the F1 generation (e.g. observed reduction in survival of F2 within-strain hybrids to levels similar to inbred individuals). As the biggest improvements in reproductive success and survival were observed in

between-strain hybrids (i.e., crosses of flies from two different strains that are likely to be genetically dissimilar), and these improvements persisted into the second generation, my observations are consistent with a concomitant increase in levels of genetic diversity in the hybrid offspring that persisted for at least two generations. However, an analysis of genotypes would be necessary to determine the exact mechanism for this fitness effect.

Outbreeding depression (reduced fitness in crosses between distantly related individuals) typically becomes apparent in the F2 generation of crosses, when the original parental gene combinations are split up by recombination processes such as chromosomal crossover and segregation, which can cause the disruption of extrinsic interactions between genes and the environment (e.g. of locally adapted gene complexes) or inherent interactions between genes (Edmands 1999, 2007, Marshall and Spalton 2000, Tallmon et al. 2004). Interestingly, some traits in my study (e.g. the proportion of eggs that developed into pupae) showed no change in the first generation of between-strain hybrids, but positive effects appeared subsequently in the second generation. One possible explanation for this observation is that it is due to a maternal effect – inbred mothers could potentially be less effective in provisioning for eggs compared to hybrid or outbred mothers. Regardless, the increase in fitness in the F2 compared to F1 between-strain hybrids indicates that the original populations used in this study were not genetically differentiated enough to induce outbreeding depression in the between-strain hybrid offspring. Nonetheless, when planning a translocation of an endangered species it would be important to choose source populations that adaptively match the population of concern (e.g. adapted to similar environments) in order to avoid outbreeding depression (see also Frankham et al. 2011, Hedrick and Fredrickson 2010, Weeks et al. 2011).

As previously found in other studies (e.g. Molina-Freaner and Jain 1993, Pimm et al. 2006), fitness differences between inbred and outbred populations are primarily due to survivorship differences. Similarly, my crossing experiments revealed that the most important improvement in fitness was survival probability. Although breeding success in the first generation of hybridisation showed only slight improvement in both within- and between-strain hybrids, when coupled with increased survival, this meant that individuals in hybrid populations had more opportunities to reproduce, and this result therefore carries important implications for the persistence of threatened populations. Some previous studies of genetic rescue using outbred populations as a source have found increased reproductive success in the recipient populations (e.g. Madsen et al. 1996, Westemeier et al. 1998). However, even if genetic rescue using inbred populations does not induce a similar increase on a

per breeding attempt basis, the technique may still provide management benefits if it increases adult survival and thus lifetime reproductive success.

The bottleneck that the two original populations used in this study were forced through (two generations of full-sib matings) was particularly severe. Although bottlenecks of this severity are unlikely to occur to the same extent in most natural populations, at least a few species have passed through bottlenecks that approach this level (e.g. black robin *Petroica traversi*: 1 pair, Jamieson et al. 2006, Mauritius kestrel *Falco punctatus*: 1 pair, Groombridge et al. 2000). For less critically endangered species, the effect of crossings on fitness might be less pronounced if they suffered a smaller loss of genetic variation from the outset, and it might therefore be valuable to test the effects of crossing individuals between populations of varying bottleneck size. Furthermore, the impact of hybridisation on fitness depends not solely on the level of parental genetic similarity, but also on effects of the environment. A way to investigate environmental effects in the laboratory could be to subject both inbred and hybrid individuals to changes in the environment, such as increases in temperature or salinity, or exposure to pathogens, and to test if the groups differ in their ability to respond to novel challenges (e.g. Bijlsma et al. 2010, Frankham et al. 1999).

The use of lab animals such as *Drosophila* provides a convenient model for studying the consequences of inbreeding and testing potential methods to remediate the negative effects (e.g. Bryant et al. 1999, Spielman and Frankham 1992). The objective of such studies, including mine, is to extrapolate these findings to more effectively manage populations of endangered organisms in the wild. However, caution is required in directly relating the results of lab studies to wild populations. The fruit flies in my study were provided with *ad libitum* food and a constant environment and, as far as I could tell, limited exposure to parasites and pathogens. Under such conditions, individuals with deleterious alleles may survive and reproduce that would otherwise not do so in the wild (Jiménez et al. 1994). This could lead to an over-estimate of the benefits of donors to an inbred population, especially if the benefit is small, as it is likely to be the case with donors equivalent to my "within-strain" lines. Furthermore, the long-term consequences of genetic rescue of wild populations with inbred donors from differing source populations are not clear. Other workers have conducted longer-term studies of the effects of immigration into laboratory populations of insects (Bryant et al. 1999, Newman and Tallmon 2001) and confirmed that the benefits can persist for more than three generations. Whether a similar pattern is seen in wild populations, in which some of the introduced alleles may be removed by selection, needs to be determined.

With the increasing number of species around the world passing through severe population bottlenecks, the results of my study provide an empirical demonstration of the immediate fitness benefits of hybridising different inbred populations. Although restricted to a laboratory environment, my results are consistent with the purported benefits of an earlier attempt to use inbred individuals to rescue wild populations of the Mexican wolf (Fredrickson et al. 2007). Whether these effects also hold in other populations of wild animals, and whether such benefits persist for more than a few generations needs to be tested. Nevertheless, the use of severely bottlenecked populations as donors to preserve even the most critically endangered species should not be disregarded in view of the potential benefits and the current rapid increase in the number of species that survive only as small and isolated populations vulnerable to both demographic and genetic stochasticity.

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Chapter 4

Effect of reciprocal translocations on the genetic structure of two inbred bird populations

Abstract: *I conducted reciprocal translocations between two bottlenecked and isolated populations of the South Island robin* Petroica australis *to investigate whether the genetic diversity of the populations changed after the introduction of new individuals. I found significant differences in mean levels of homozygosity by locus (HL) between inbred and hybrid birds on both islands, with hybrid birds exhibiting higher levels of heterozygosity. Similarly, average expected heterozygosity, allelic richness and the frequency of rare alleles all increased significantly in both populations within the first year after the translocation. The present study demonstrates that the reciprocal translocation between two isolated and inbred bird populations has likely enhanced both the short-term viability and the evolutionary potential of these populations by significantly increasing both levels of heterozygosity and allelic diversity. The results of this experiment highlight the possible value of translocations between different inbred populations of endangered species as a tool to mitigate the negative effects of inbreeding.*

4.1 Introduction

The continued decline of many animal and plant populations due to direct and indirect anthropogenic influences highlights the importance of addressing the effects of reduced genetic diversity and inbreeding on population viability. A loss in genetic variation as a consequence of population bottlenecks, founder effects and random genetic drift (Nei et al. 1975), may decrease fitness or limit the ability of a population to respond to future environmental challenges, such as climatic extremes, pollutants, diseases, pests, and parasites (Amos and Balmford 2001, Frankham 1995, Frankham et al. 2010). Similarly, inbreeding depression, defined as the reduction in fitness of individuals born to closely related parents, can reduce the viability of endangered species that are reduced to a number of fragmented and bottlenecked populations (Crnokrak and Roff 1999, Hedrick and Kalinowski 2000, Keller and Waller 2002, Kristensen et al. 2010). Fertility and survival, for example, are susceptible to inbreeding depression (Charlesworth and Charlesworth 1987, Charlesworth and Willis 2009), and sex ratios may also, albeit rarely, be distorted in inbred populations (Worthington Wilmer et al. 1993, but see Frankham and Wilcken 2006), greatly reducing the effective population size (Frankham 1995).

Genetic drift during a bottleneck event may cause alleles that occur at low frequencies to disappear completely, thereby reducing the number of alleles per locus (i.e., allelic diversity; Allendorf 1986, Frankham et al. 1999, Fuerst and Maruyama 1986). In contrast to genetic drift, inbreeding does not affect the frequency of alleles in a population; however, it can increase the proportion of homozygote individuals in a population (Armstrong and Cassey 2007). Inbred individuals that are relatively homozygous at loci influencing fitness may experience reduced fitness when deleterious recessive alleles are expressed (Charlesworth and Charlesworth 1999, Keller and Waller 2002). While allelic diversity is important for the long-term survival of a population by increasing its adaptability, sufficiently high levels of heterozygosity are essential to ensure a population's short-term viability (Allendorf 1986, but see England et al. 2003).

Considerable evidence supports the idea that the restoration of heterozygosity and allelic diversity by outcrossing enhances fitness-related characteristics (e.g. Reed and Frankham 2003, Slate and Pemberton 2002). Studies of heterozygosity-fitness correlations, for instance, have found that higher levels of heterozygosity are associated with decreased morphological variability and increased individual performance (Allendorf and Leary 1986, Mitton and Grant 1984, Mitton 1993, Yezerinac et al. 1992), and the validity of these findings has been supported by recent meta-analyses (Chap-

man et al. 2009, see also Frankham 2012, but see Britten 1996). In contrast, lower levels of heterozygosity are typically associated with decreases in fitness-related characteristics and individual performance (e.g. Charpentier et al. 2008).

In the management of endangered species, translocations of outbred individuals into inbred populations have been shown to increase the fitness of resulting offspring by restoring genetic diversity (Fredrickson et al. 2007, Johnson et al. 2010, Madsen et al. 2004, Richards 2000, Westemeier et al. 1998, see also Frankham et al. 2011, Hedrick and Fredrickson 2010, Weeks et al. 2011). However, the translocation of outbred individuals into inbred populations as a measure to restore genetic variation relies on the availability of suitable outbred donor populations, whereas for many endangered species, no such outbred populations are left (e.g. kakapo *Strigops habroptilus*, Jamieson et al. 2006). Instead, most endangered species survive only as a series of small, fragmented populations, with each likely subject to some loss of genetic variation and increased levels of inbreeding. Nevertheless, the exchange of individuals between two or more inbred populations should theoretically increase the number of alleles present in each population, provided that the donor populations used for the translocations harbour different alleles (Charlesworth and Charlesworth 1999, Lynch 1991, Tallmon et al. 2004). One would therefore expect that genetic diversity and levels of heterozygosity should increase following such a translocation and the severity of inbreeding depression decrease in the hybrid offspring (Edmands 2007). In the present study, I conducted an experimental translocation between two severely bottlenecked populations of the South Island robin *Petroica australis* to investigate whether genetic diversity of the recipient populations changed after the introduction of new individuals. My objective was to determine if genetic rescue could be used successfully to restore the genetic diversity of endangered species using only highly bottlenecked and inbred populations as donors.

4.2 Material and methods

4.2.1 Study populations

In 2008 and 2009, I conducted experimental translocations between two isolated and inbred populations of the South Island robin on Allports and Motuara islands, Marlborough Sounds, New Zealand. Both robin populations were founded in 1973 with only five individuals each to trial capture and transport methods to be used with the critically endangered Chatham Island black robin *Petroica*

traversi (Flack 1974, Armstrong 2000). The founders of each population originated from different parts of New Zealand (Flack 1974, Armstrong 2000). Henceforth, I refer to the descendants of the five founders on each island as "inbred" individuals, even though they might not be the product of recent within-family matings. At the time of the translocation, the two populations had been isolated for nearly four decades (i.e., for approximately 10 generations, Jamieson 2010) and were showing signs of inbreeding depression, such as reduced hatching success and problems with immune system function (Hale and Briskie 2007, Mackintosh and Briskie 2005). Although the islands differ in size (Allports Island: 16 ha, Motuara Island: 59 ha), the density of robins is similar on both islands (Allports: $\sim$60 adult individuals, $\sim$3.9 birds/ha; Motuara: $\sim$300 adult individuals, $\sim$5.1 birds/ha; SH, personal observation). A total of 31 female robins were exchanged between Allports and Motuara islands. In 2008, a total of 15 randomly chosen females were translocated from Allports to Motuara, and 10 females were translocated from Motuara to Allports. Due to the mortality of some translocated females (see below), an additional translocation of three females from each island was carried out in 2009 to ensure that > 5 females were recruited into each recipient population. For reference purposes, I refer to the year after the 2008 translocation as "1 year post-translocation", and the second year after the 2008 translocation as "2 years post-translocation", despite the follow-up translocation of six individuals in 2009. The number of individuals chosen for the experimental translocations was selected on the basis of leaving enough non-manipulated individuals on each island to act as controls as well as ensuring that enough females survived the translocation to breed. Only females were translocated in order to minimise disturbance to territorial boundaries that would occur if males were moved, and to ensure that new "mixed" pairs would form upon release (i.e., by not translocating pairs I forced translocated birds to re-pair with an individual from the other island). Post-release survival was similar on both islands (Allports: n = 7, 53.8%; Motuara: n = 10, 55.6%). This is lower than is normally found with translocation of this species (Jamieson 2010); however, all surviving females exhibited normal breeding behaviour. The direct offspring of the mixed pairs (translocated female with male native to the island) will hereafter be referred to as "hybrid F1", the offspring of hybrid F1 individuals as "hybrid F2", and the offspring of hybrid F2 individuals as "hybrid F3". Individuals were considered "hybrid F2" or "hybrid F3" if either one or both parents were hybrid F1 or F2, respectively (however, most of these individuals were backcrosses: only one hybrid F1 x hybrid F1 pair formed during the study period).

To obtain estimates of genetic variability in each island population prior to the translocation (2008), and 1 and 2 years after the initial translocation (2009 and 2010, respectively), I collected

blood samples from 658 individuals ($>$ 90% of adult individuals and their offspring in each island population) in three consecutive years (before the translocation in 2008, and in 2009 and 2010). Blood samples were collected via brachial venipuncture, and approximately 10–30 μl of blood was stored in 1 ml of Queen's Lysis Buffer (0.01 M Tris-HCl, 0.01 M NaCl, 0.01 M Na-EDTA (pH 7.5), 1% (v/v) n-Lauroylsarcosine; pH 7.5; Seutin et al. 1991) at room temperature. A detailed description of the genotyping procedure is given in appendix A on p. 107.

4.2.2 Population genetic characteristics

I genotyped 658 individuals at 32 autosomal microsatellite markers (see table A.1 on p. 109). Deviations from Hardy-Weinberg equilibrium (HWE) and evidence of linkage disequilibrium (LD) were tested using the GENEPOP v4.1 software package (Rousset 2008) using the data from Allports Island subdivided into study years (pre-translocation, and 1- and 2 years post-translocation). Significance tests were conducted using Fisher's method, as implemented with the default parameters for the Markov chain protocol in GENEPOP. After false discovery rate (FDR) control of the p-values to correct for multiple comparisons, only locus Ind29 showed deviation from HWE and was removed from the analyses due to the presence of null alleles. Null alleles were also found in locus PC3, which was also excluded from further analyses. Locus Pau20 was excluded from the analyses as it was homozygous for all 658 individuals genotyped. Locus P2/P8 was used for sexing purposes. Apart from P2/P8, none of the loci showed evidence of sex linkage.

For the analysis of linkage disequilibrium, the sequence of each microsatellite marker was aligned to the zebra finch *Taeniopygia guttata* genome by performing a BLAT (BLAST-Like Alignment Tool) search at the University of California Santa Cruz Zebra Finch Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway?db=taeGut1) in order to determine its chromosome location. Of twenty-six pairs of loci located on the same zebra finch chromosome, 16 showed significant linkage disequilibrium after FDR. An analysis of the strength of the LD using the software LinkDos on the web (Garnier-Géré and Dillmann 1992), however, revealed that the linkage was commonly weak (correlations ranging from 0.185 to 0.446, see table A.1 on p. 109). A total of four microsatellite markers (Ind29, PC3, Pau20, and P2/P8) were thus excluded from further analyses, leaving 28 loci distributed over 13 chromosomes for the calculations of genetic parameters (see table A.1).

Individual levels of heterozygosity for all genotyped individuals (n = 658) were calculated using

the *GENHET* function in the R statistical environment (Coulon 2010, R Development Core Team 2011). I chose homozygosity by locus (HL) as the heterozygosity measure to compare between inbred and hybrid individuals and between population subsets (pre-translocation, 1- and 2 years post-translocation, and separately for each island), as it has been found to correlate better with the inbreeding coefficient and genome-wide heterozygosity than other indices of individual heterozygosity at multiple loci (Aparicio et al. 2006). The HL index varies between 0 (when all loci are heterozygous) and 1 (when all loci are homozygous); thus, the smaller the HL value, the more heterozygous an individual (Aparicio et al. 2006).

It has been argued that neutral loci (i.e., loci that are located in regions of the genome that are not transcribed into RNA, and therefore not expressed) are better predictors for the effects of inbreeding than functional, that is, expressed loci (Olano-Marin et al. 2010, 2011). I therefore classified the above 28 microsatellite markers as potentially "functional" or "neutral" by aligning their sequences to zebra finch expressed sequence tags (ESTs) using the Basic Local Alignment Search Tool (BLAST) on the National Centre for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov). Loci that were homologous to zebra finch ESTs were considered functional, whereas markers showing no homology to avian ESTs were considered neutral (see also Olano-Marin et al. 2010). Of the 28 markers considered, nine were classified as "functional" and 19 as "neutral"; all analyses were repeated separately for neutral and functional markers. There was a significant correlation of HL measured between the subsets of functional and neutral markers (r $HL_{functional}$–$HL_{neutral}$ = 0.335, $p < 0.0001$). Furthermore, models using $HL_{allmarkers}$ always had the lowest Akaike Information Criterion (AIC, Akaike 1973). I therefore only present results calculated using all 28 microsatellite markers.

All genotyped individuals, subdivided into four groups (inbred individuals originating from Allports Island, hybrid individuals on Allports Island, inbred individuals originating from Motuara Island, and hybrid individuals on Motuara Island), were included in the analysis when testing for genetic differences between inbred and hybrid individuals. Only adult breeding individuals (Allports Island: n = 138; Motuara Island: n = 274) were used to investigate the changes of genetic parameters pre- and post-translocation, as they constitute the effective population and potentially contribute to the future gene pool. These adult breeding individuals included the translocated females as well as inbred adults already present on the islands before the translocation. Expected heterozygosity (H_{exp}), observed heterozygosity (H_{obs}), allelic richness (A), and allele frequencies were calculated

for each population subset (pre-translocation, 1- and 2 years post-translocation), separately for each island, using FSTAT 2.9.3 software (Goudet 1995) to detect shifts in the genetic make-up of the populations over time (see table 4.2 on p. 94). The estimation of allelic richness using FSTAT 2.9.3 is based on the year with the smallest sample size for each island (number of individuals typed at all loci; Allports: n = 42, Motuara: n = 70), thus adjusting *A* for differences in sample size as proposed by Leberg (2002). Effective number of alleles per locus, F_{ST} (measure of inbreeding per population subset relative to that expected under random mating in the total population) and F_{IS} (deviation of the observed heterozygosity of an individual relative to the heterozygosity expected under random mating) values for each population subset were calculated using GenoDive 2.0 software (Meirmans and van Tienderen 2004, see table 4.2 on p. 94). F_{ST} values were also computed for Allports and Motuara Island separately for each year to investigate how differentiated the two island populations were prior to the translocation, and how population differentiation changed in the two years following the translocations. The significance of F_{ST} and F_{IS} values was assessed using 20,000 permutations. F_{ST} values computed with GenoDive 2.0 are equivalent to Weir and Cockerham's (1984) theta (θ).

The genetic structure of each population subset (pre-translocation, and one and two years after the initial translocation) was visualised separately for each island using the software STRUCTURE 2.3.2 (Pritchard et al. 2000, figure 4.1).

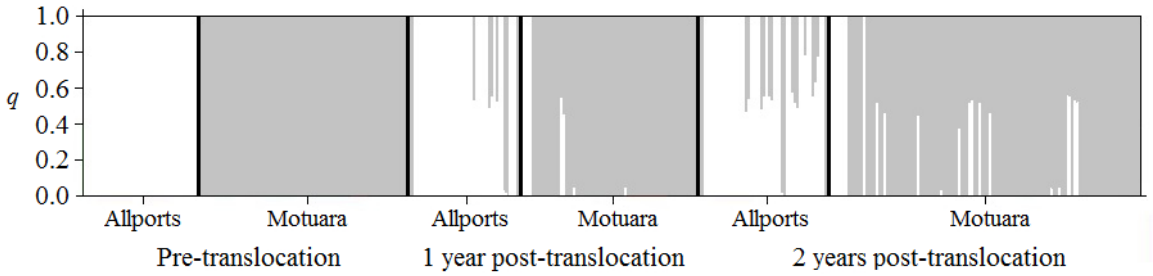

Figure 4.1: Genetic composition of the South Island robin population subsets prior to the translocation, and 1 and 2 years after the translocation, separately for Allports and Motuara Island from the replicate run of STRUCTURE for K = 2. Each individual is represented by a vertical line identifying the individual's estimated membership of the two island populations (as indicated by the individual membership coefficient *q*). Black lines separate years and islands (Allports: white; Motuara: grey).

4.2.3 Statistical analyses

All statistical analyses were conducted in the R statistical environment (version 2.13.1, R Development Core Team 2011). First, I tested whether there was a significant difference in mean HL levels between hybrids of the first generation (F1, offspring of translocated females and inbred males) and their offspring (F2, offspring of hybrid F1 birds; and F3, offspring of hybrid F2 birds) by performing a one-way ANOVA with "individual HL" as response and "group" (hybrid F1, hybrid F2, and hybrid F3) as predictor. I visually inspected whether the assumptions of the model were met using the *plot(model)* function. Multiple comparisons between the groups were performed using the *Tukey HSD* function. As there was no significant difference between F1, F2, and F3 hybrids (see results section 4.3), I pooled them into one group per island (Allports hybrids and Motuara hybrids), when testing for differences in levels of HL between inbred and hybrid individuals. Hence, a one-way ANOVA was performed to test the effect of the predicting factor "group" with four levels (inbred individuals on Allports, hybrid individuals on Allports, inbred individuals on Motuara, and hybrid individuals on Motuara) on the response variable "individual HL". Effect sizes, 95% confidence intervals (CIs), and adjusted p-values for the multiple comparisons were again computed using the *Tukey HSD* function.

Table 4.1: Output of the mixed effects model of year (pre-translocation/1 year post-translocation/2 years post-translocation), group (inbred/hybrid), and island (Allports/Motuara) on levels of HL after model simplification.

	Estimate ($\pm$ s.e.)	test	p-value
Intercept	0.251 ± 0.023	$t_{261} = 10.84$	< 0.0001
1 year post-translocation[1]	0.007 ± 0.002	$t_{143} = 3.727$	0.0003
2 years post-translocation[1]	0.009 ± 0.002	$t_{143} = 4.746$	< 0.0001
Inbred birds[2]	0.158 ± 0.024	$t_{143} = 6.515$	< 0.0001
Motuara Island[3]	0.032 ± 0.004	$t_{261} = 8.418$	< 0.0001

[1]reference = 2008 (pre-translocation)
[2]reference = hybrid birds
[3]reference = Allports Island

To test the effect of the translocation on the genetic structure of the populations on the two islands over time, I fitted a mixed model with "year" (pre-translocation, 1 year and 2 years post-

translocation), "island" (Allports and Motuara Island) and "group" (inbred and hybrid) as factors using the add-on R package "nlme" (Pinheiro et al. 2009). Individual bird identity was included as a random factor in order to avoid pseudo-replication, as many individuals were present in more than one year. The maximal model included the three factors and interactions between island and group, and island and year. Both interaction terms were non-significant (island*year: $F_{2,143} = 0.717$, p = 0.49; island*group: $F_{1,143} = 1.385$, p = 0.24), and were thus excluded during model simplification. The minimal model hence retained only the three main factors without their interaction (see table 4.1). Model simplification did not change the effect of any of the main factors. I calculated effect sizes and 95% confidence intervals for the model estimates with the add-on R package "multcomp" (Hothorn et al. 2008).

I assessed the normality of the error distribution of my data on expected heterozygosity, allelic richness, number of rare alleles per locus (defined as alleles with a frequency < 0.05), and frequency of rare alleles per locus using the Shapiro-Wilk test, and performed either paired t-tests or Wilcoxon Signed Rank tests, depending on whether the error distribution was normal or not, to test changes over time for each island separately.

4.3 Results

Within hybrid individuals, average levels of HL were lowest in the hybrid F1 generation (mean $\pm$ s.e.: 0.286 ± 0.014, n = 42), increased in the hybrid F2 generation (mean: 0.327 ± 0.023, n = 24) and were highest in the hybrid F3 generation (mean: 0.37 ± 0.017, n = 5). However, this increase was not significant (one-way ANOVA: hybrid F1 to hybrid F2: effect size = 0.041, 95% CI = -0.017 to 0.1, p = 0.22; hybrid F2 to hybrid F3: effect size = 0.043, 95% CI = -0.069 to 0.156, p = 0.63; and hybrid F1 to hybrid F3: effect size = 0.084, 95% CI = -0.024 to 0.192, p = 0.16; figure 4.2a on p. 93).

On both islands, there was a significant effect of "group" on levels of HL when comparing inbred with hybrid birds: on Allports Island, inbred birds had a mean HL $\pm$ s.e. of 0.418 ± 0.01 (n = 128), whereas hybrids had a mean HL of 0.282 ± 0.02 (n = 41; effect size = 0.136, 95% CI = 0.082 to 0.189, $p < 0.0001$; figure 4.2b on p. 93). Similarly, mean HL on Motuara Island was higher in inbred birds (mean: 0.457 ± 0.005, n = 459) than in hybrid birds (mean: 0.337 ± 0.02, n = 30; effect size = 0.12, 95% CI = 0.064 to 0.176, $p < 0.0001$). Mean HL of hybrid birds did not differ

significantly between the two islands (effect size = 0.055, 95% CI = -0.017 to 0.126, p = 0.20). Inbred individuals on Motuara, however, had a significantly higher level of HL than inbred birds on Allports Island (effect size = 0.039, 95% CI = 0.009 to 0.068, p = 0.005; figure 4.2b).

The mixed model revealed that the factor "group" explained most of the variance, indicating that mean HL levels were most strongly positively associated with inbred individuals in the two study populations (effect size = 0.158, 95% CI = 0.098 to 0.218; table 4.1 on p. 90 and figure 4.2c on p. 93). Mean levels of HL were also significantly higher on the larger island of Motuara (effect size = 0.032, 95% CI = 0.023 to 0.042) than on the smaller Allports Island. There was a significant, albeit small decrease in mean levels of HL from pre-translocation to 1 year post-translocation (effect size = 0.007, 95% CI = 0.002 to 0.011) and from pre-translocation to 2 years post-translocation (effect size = 0.009, 95% CI = 0.004 to 0.014). The difference between mean levels of HL one year after the translocation to two years after the initial translocation, however, was not significant (effect size = -0.002, 95% CI = -0.007 to 0.002; figure 4.2c).

F_{ST} values between the two island populations were highest prior to the translocation (F_{ST} = 0.273 $\pm$ 0.032, 95% CI = 0.212 to 0.333, p = 0.001), and decreased in the years 1 and 2 after the translocation (year 1: F_{ST} = 0.169 $\pm$ 0.023, 95% CI = 0.126 to 0.214, p = 0.001; year 2: F_{ST} = 0.129 $\pm$ 0.018, 95% CI = 0.095 to 0.164, p = 0.001, respectively), as the populations became more similar due to the mutual exchange of individuals.

4.3.1 Indices of genetic diversity per island population

A summary of the indices of genetic diversity per island population is given in table 4.2 on p. 94.

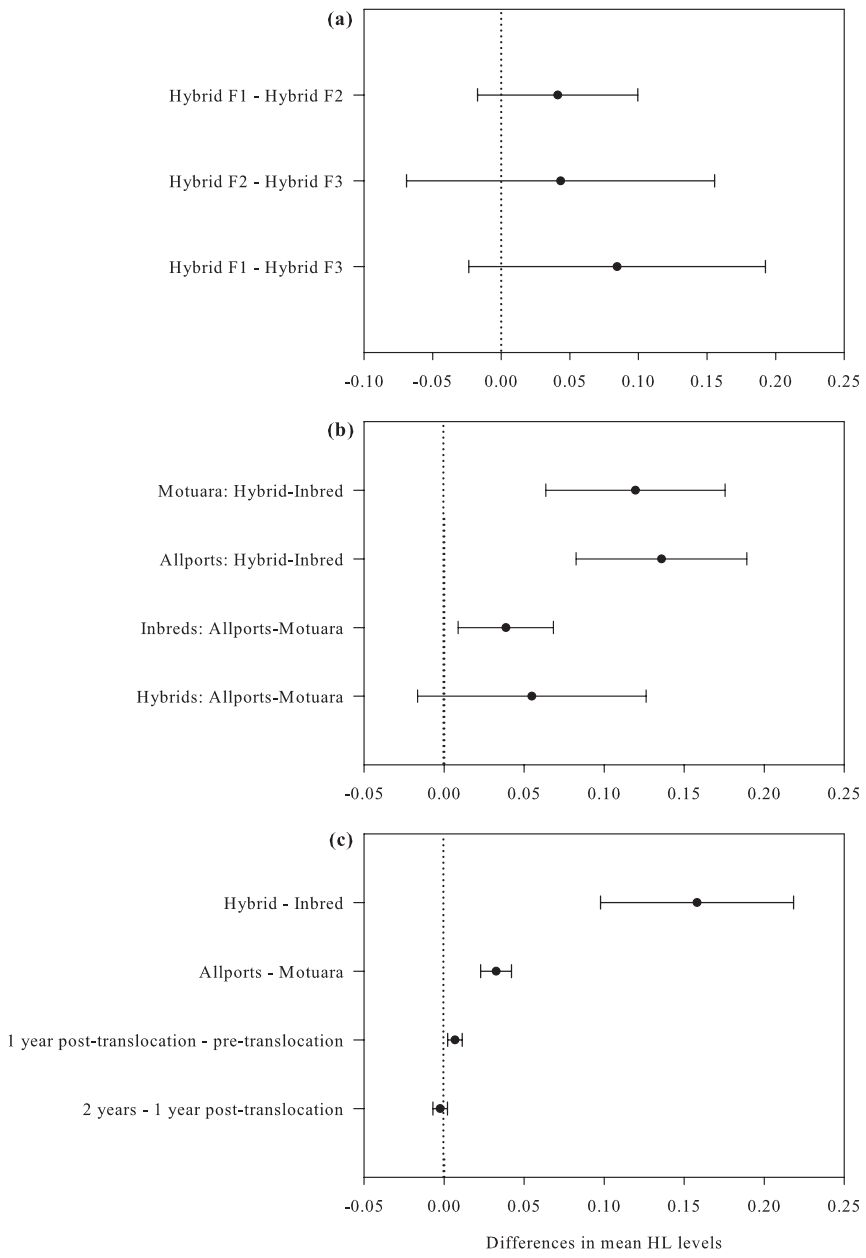

Figure 4.2: Effect size estimations of pair-wise differences in mean level of HL and their 95% confidence intervals: **a)** pair-wise comparisons between various hybrid groups (generations F1, F2, and F3); **b)** pair-wise comparisons between hybrid and inbred individuals, separately for each island, and within inbred and hybrid birds on Allports and Motuara islands, respectively; **c)** effect sizes for the factors "group", "island", and "year" on mean levels of HL.

Table 4.2: Genetic properties of two South Island robin populations pre- and 1 and 2 years post-translocation. F-statistics and effective number of alleles per locus were calculated using GenoDive 2.0 software (Meirmans and van Tienderen 2004); all other genetic information was calculated using FSTAT 2.9.3. (Goudet 1995).

										F_{ST}	
Island	Status	N	N alleles/ locus	Eff N alleles/ locus	Allelic richness	H_{exp}	H_{obs}	HL	F_{IS}	Pre-translocation	1 year post	2 years post
Allports	Pre-translocation	45	2.68	2.15	2.68	0.462	0.511	0.397	−0.092***	–	0.005	0.017***
	1 year post	43	3.79	2.44	3.78	0.518	0.535	0.366	−0.034		–	−0.006
	2 years post	50	3.79	2.55	3.76	0.527	0.554	0.346	−0.052**			–
Motuara	Pre-translocation	82	2.96	2.17	2.96	0.489	0.492	0.446	−0.019	–	0.001	0.006***
	1 year post	70	3.68	2.34	3.68	0.521	0.493	0.453	0.054***		–	−0.0001
	2 years post	122	3.75	2.41	3.70	0.527	0.507	0.439	0.036**			–

N alleles/locus: mean number of alleles sampled per locus
Eff N alleles/locus: effective number of alleles per locus
H_{exp}: expected heterozygosity
H_{obs}: observed heterozygosity
F_{IS} and F_{ST} values: **p < 0.01, ***p ≤ 0.001

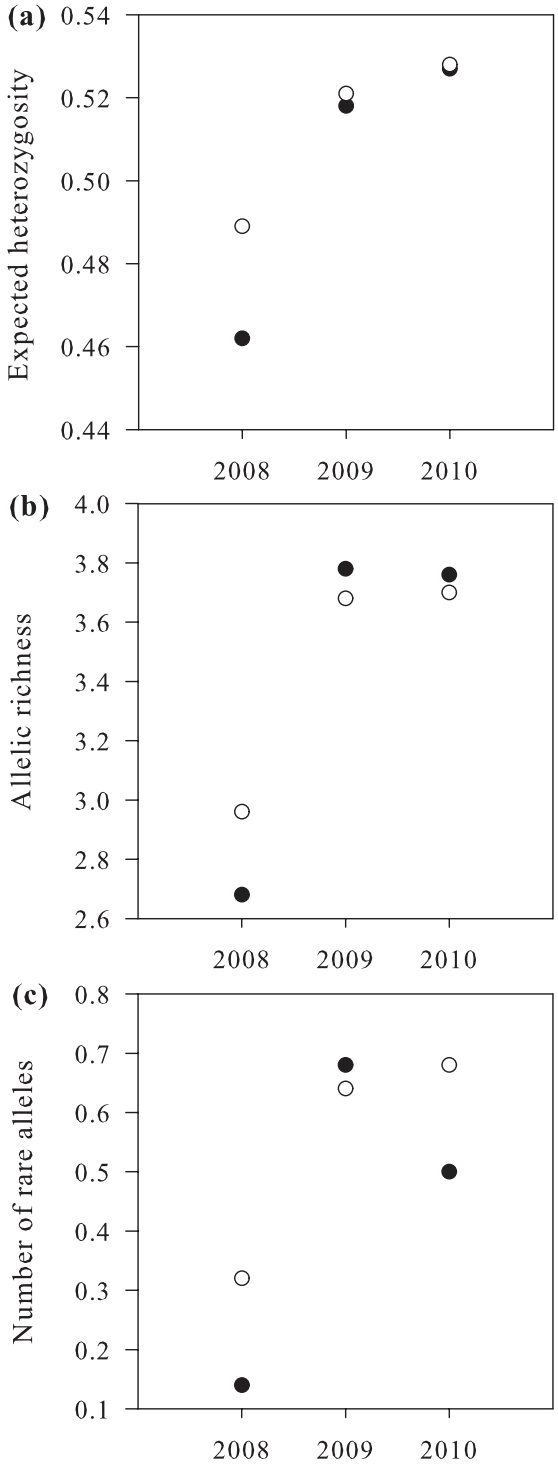

Figure 4.3: Changes in indices of genetic diversity over time in two island populations of South Island robin (filled circles, Allports Island; open circles, Motuara Island): **a)** mean expected heterozygosity, **b)** allelic richness, and **c)** mean number of rare alleles per locus.

4.3.1.1 Allports Island

The average expected heterozygosity ($H_{exp} \pm$ s.e.) for the 28 loci prior to the translocations was 0.462 ± 0.04 (range: 0 – 0.766). There was a significant increase one year after the initial translocation (mean: 0.518 ± 0.04, range: 0.046 – 0.805; $t = -5.015$, $p < 0.001$, d.f. = 27), but not following the second translocation (0.527 ± 0.04, range: 0.059 – 0.824; $t = -1.604$, $p = 0.12$, d.f. = 27; figure 4.3a on p. 95). Allelic richness was significantly higher one year after the initial translocation (mean: 3.78 ± 0.30) compared to prior to the translocation (mean: 2.68 ± 0.20; $z = 3.94$, $p < 0.001$), but did not change significantly from 1 year post- to 2 years post-translocation (mean: 3.76 ± 0.30; $z = -2.032$, $p = 0.063$; figure 4.3b). The mean number of rare alleles per locus (defined as alleles with frequency < 0.05) changed significantly from prior to the initial translocation (mean: 0.143 ± 0.07) to 1 year post-translocation (mean: 0.679 ± 0.17; $p = 0.01$), and slightly decreased from 1 year post-translocation to 2 years post-translocation, but this latter trend was non-significant (mean: 0.5 ± 0.17; $p = 0.35$; figure 4.3c). Likewise, the frequency of rare alleles increased significantly from before the translocation (mean: 0.006 ± 0.002) to 1 year after the translocation (mean: 0.027 ± 0.004; $p < 0.001$), but did not change significantly from one year to two years after the translocation (mean: 0.037 ± 0.005; $p = 0.3$).

4.3.1.2 Motuara Island

Prior to the translocations, average expected heterozygosity ($H_{exp} \pm$ s.e.) for the 28 loci on Motuara Island averaged 0.489 ± 0.03 (range: 0 – 0.772). There was a significant increase one year after the initial translocation (0.521 ± 0.03, range: 0.042 – 0.812; $t = -3.999$, $p < 0.001$, d.f. = 27), but not after the second translocation (0.527 ± 0.03, range: 0.032 – 0.825; $t = -0.889$, $p = 0.38$, d.f. = 27; figure 4.3a on p. 95). Allelic richness increased significantly from a mean of 2.96 ± 0.22 to 3.68 ± 0.33 following the translocation ($z = 3.372$, $p < 0.001$). There was no significant change in allelic richness following the second translocation (mean: 3.7 ± 0.31; $z = -0.706$, $p = 0.52$; figure 4.3b). There were no significant changes in the mean number of rare alleles per locus (alleles with frequency < 0.05) from prior to the translocations (mean: 0.321 ± 0.1) to 1 year post-translocation (mean: 0.643 ± 0.16; $p = 0.18$), or from 1 year post-translocation to 2 years post the initial translocation (mean: 0.679 ± 0.15; $p = 0.8$; figure 4.3c). The frequency of rare alleles per locus, however, increased significantly from pre-translocation (mean: 0.009 ± 0.003) to 1 year post-translocation (mean: 0.032 ± 0.006; $p = 0.0002$), but did not change significantly from 1 year

after the translocation to 2 years after the first translocation (mean: 0.04 ± 0.007; p = 0.31).

4.4 Discussion

Several case studies of natural populations have shown that gene flow from outbred populations into inbred populations can reverse the detrimental effects of inbreeding and increase fitness measures over the short-term (Arrendal et al. 2004, Bouzat et al. 2009, Fredrickson et al. 2007, Hogg et al. 2006, Johnson et al. 2010, Madsen et al. 1999, 2004, Trinkel et al. 2008, Vilá et al. 2002, Westemeier et al. 1998). Here, I demonstrate that—in the absence of outbred donor populations—I was able to significantly increase levels of genetic diversity in two highly bottlenecked and isolated robin populations through the reciprocal translocation of females between these two populations. As expected, I found significant differences in mean levels of homozygosity by locus (HL) between inbred and hybrid birds on both islands, with hybrid birds exhibiting lower levels of HL (i.e., higher levels of heterozygosity). Similarly, average expected heterozygosity, allelic richness and the frequency of rare alleles all increased significantly in both island populations within the first year after the translocation. In contrast, F_{ST} values decreased after the translocation, confirming that although genetic rescue using only inbred populations can increase genetic diversity, it will also decrease the genetic distinctiveness of these populations.

There was a non-significant trend towards a decrease in heterozygosity within hybrid birds over the generations: F1 individuals had higher heterozygosity levels than F2 individuals, which in turn had higher heterozygosity levels than F3 individuals. It is expected that this trend will continue from generation to generation, and it would therefore be necessary to determine whether the observed decrease in heterozygosity in the first three generations of hybridisation is associated with a corresponding decrease in fitness traits ("dilution" of heterosis effect). In a reintroduction study of the endangered and inbred black-footed ferret *Mustela nigripes*, Wisely et al. (2008) found that allelic diversity declined drastically and phenotypic deterioration was apparent after the periodical augmentation of one of the reintroduced populations ceased. In contrast, the periodical reinforcement with translocated individuals from captivity helped to maintain the genetic diversity in another black-footed ferret population, despite its persistently low population size (Wisely et al. 2008), suggesting that, for endangered species, a singular reciprocal translocation between inbred populations cannot be used to halt the erosion of genetic diversity (Bensch et al. 2006, Hagenblad et al. 2009).

This is especially true in spatially confined populations, such as island populations (see also Adams et al. 2011). Similar to populations that are maintained at a large size, populations that experience growth are less likely to lose genetic diversity than small populations, as the effects of genetic drift and inbreeding are less pronounced (Gilpin and Soulé 1986). However, a spatially confined population has no possibility to expand beyond carrying capacity, and will thus inevitably lose genetic variation. This is of particular importance for translocation schemes that involve translocations of endangered species to islands where introduced predators have been eradicated, such as in New Zealand (Armstrong and McLean 1995).

Notwithstanding questions about the need for ongoing management of small and isolated populations, my findings suggest that reciprocal translocations between inbred populations can certainly be used to decelerate the process of genetic erosion, and could thus be a valuable short-term measure to prevent a species from becoming extinct (see also Frankham et al. 2011, Hedrick and Fredrickson 2010, Weeks et al. 2011). According to this, the periodical introduction of new genetic stock should be part of a wider management scheme including other measures such as habitat restoration and predator control.

Post-release survival of translocated females on both islands was lower than that often reported for translocations (e.g. Armstrong 1995). This was possibly due to the fact that translocations often occur in form of a reintroduction into habitats where the species in question is no longer present or occurs only at low densities, whereas in reciprocal translocations, individuals are released into habitat with established territories and, as in the present study, with densities close to carrying capacity. The search for a new territory and mate following a reciprocal translocation can therefore be expected to entail increased levels of stress for the translocated individual, and it might be advisable to bear this in mind when deciding on the number of individuals used for a translocation.

Despite the comparatively low number of surviving females, the translocation caused a small, but significant increase in heterozygosity in the first year following the translocation, but there was no significant further increase from the first to the second year after the initial translocation. The majority of females were translocated between the islands in 2008, whereas the 2009 translocation consisted of only six individuals in total and did not provoke further increases in heterozygosity. The lower levels of heterozygosity found in inbred birds on Motuara compared to inbred birds on Allports Island are an unexpected finding and difficult to interpret from a genetic perspective. It is possible that these differences in the opposite direction to that predicted from the size of the two

populations are the result of the effects of early translocation history: despite of the initial translocations consisting of 5 birds to each island, the effective population size could have been lower on Motuara compared to Allports Island. Alternatively, some of the initial genetic founders on Motuara, but not on Allports, could have been eliminated by chance, i.e., the number of genetic founders might not correspond to the number of released founders (see also Biebach and Keller 2010, 2012). However, no data on the actual contribution of the founder robins exists. At the time of the translocations, mice *Mus musculus* were present on Allports, and Polynesian rats *Rattus exulans* (kiore) on Motuara Island (Flack 1974; introduced predators were only eradicated in 1991, Cash and Gaze 2000). A possible explanation for the higher levels of heterozygosity found amongst inbred birds on Allports is that the population could have grown at a faster rate due to less intense predation if mice have a smaller impact compared to rats, and therefore retained more genetic diversity. Furthermore, the founding individuals on Allports Island originated from a mainland site near Kaikoura, whereas the founders of the Motuara population originated from Nukuwaiata Island, and it is possible that the mainland robins had higher levels of genetic diversity to begin with.

Overall, mean population-wide heterozygosity was lower on the larger Motuara Island (59 ha), than on the smaller island, Allports (16 ha). Similarly, increases of expected heterozygosity (H_{exp}) over time were slightly less pronounced on Motuara Island compared to Allports Island (see figure 4.3b on p. 95), suggesting that translocations between inbred populations may be more beneficial to smaller populations than to larger ones. As I exchanged similar numbers of females between the two islands (18 females from Allports to Motuara, and 13 from Motuara to Allports), the proportion of hybrids relative to the size of the receiving population was considerably smaller on Motuara ($\sim$300 adults) than on Allports Island ($\sim$60 adults). The translocation was thus expected to have a smaller positive impact on Motuara Island due to the smaller migration rate, which is reflected in the lower levels of heterozygosity and the smaller changes in measures of genetic diversity over time on Motuara Island. Another possible explanation for the increased beneficial effects of the translocation on the smaller population is that the fixation load is expected to be larger in the smaller population due to random genetic drift, and the introduction of new individuals would be expected to have a greater impact on a population with higher fixation load (Frankham et al. 2010). However, the experimental design used in this study does not allow me to distinguish between these two mechanisms, as I exchanged a similar number of individuals between the islands. In order to separate the two effects, I would have had to translocate different numbers of individuals proportional to the size of the receiving population.

On both islands, both expected heterozygosity and allelic richness increased significantly one year after the initial translocation, but showed no further significant increase following the second translocation. Nonetheless, the significant increase of both measures of genetic diversity after the initial introduction of individuals demonstrates that both the short-term viability (increased heterozygosity) of the populations and their evolutionary potential (increased allelic diversity) improved as a result of the mutual exchange of individuals. While the number of birds introduced into the smaller population on Allports Island was large enough to cause a significant increase in both the number and frequency of rare alleles in the first year after the initial translocation, it did not have the same effect on the number of rare alleles on Motuara Island, probably due to the smaller proportion of introduced females. The frequency of rare alleles, however, also increased significantly in the first year after the initial translocation on Motuara. As the frequency of initially rare alleles increased in the second year following the first translocation, many of them were no longer considered rare (i.e., with a frequency of < 0.05 according to my definition) in the second year following the translocation.

To measure the degree of population differentiation, I calculated two types of F_{ST} values: the first one constitutes a measure of differentiation between the two island populations on Allports and Motuara, and thus represents a measure of spatial differentiation (spatial F_{ST}), even though I am investigating its change over time. As one would expect, the F_{ST} value between the two islands was highest before the translocation, and decreased over the two years following the initial translocation as the offspring of the translocated females increased in numbers and the populations on the two islands became genetically more similar. However, with only three years of study, it is not possible to predict whether this trend will continue in the future. Nonetheless, it is interesting to note that the trend is consistent during the study period. This raises questions about issues of homogenisation of populations and the ensuing decrease in overall diversity. The most serious effect of the reduction in differentiation between the populations is that it reduces the suitability of those same populations for operations of genetic rescue in the future. Some studies suggest that dividing populations into groups with limited gene flow between them can result in greater overall retention of variation than in an undivided population of the same total size, as different allelic variants will get fixed in each group (Ávila et al. 2011, Caballero et al. 2010, Fernández et al. 2008). It could therefore be valuable to establish the exact migration rate that is needed in order to maintain considerable differentiation between subpopulations but avoiding an excessive increase in inbreeding (Fernández et al. 2008).

The second type of F_{ST} values I computed represents a measure of the changes in genetic composition of the population on each island over time (temporal F_{ST}, see table 4.2 on p. 94). These temporal F_{ST} values showed the same trend on both islands: while there was no significant differentiation between the individuals present before the translocation and the individuals constituting the adult population one year after the initial translocation, both populations became significantly differentiated two years after the translocation compared to prior to the translocation. As negative F_{ST} values do not exist, the slightly negative value in both islands when comparing the population one year after the translocation to two years after the initial translocation should be interpreted as 0. These F_{ST} values are not significant and therefore indicate that on both islands, there was no major change in population differentiation from one to two years after the translocation.

On both islands, there was an excess of heterozygote individuals prior to the translocation, which is reflected in negative F_{IS} values (see table 4.2 on p. 94). On the smaller island, Allports, this observed excess is highly significant. As only breeding adults were used to investigate changes in indices of genetic diversity over time, one possible explanation for the heterozygote excess prior to the translocation is that comparatively more heterozygous individuals have a higher probability of survival, and that—even though being inbred—the adult population therefore exhibits higher than expected levels of heterozygosity. I tested this hypothesis by analysing the effect of individual HL on the probability of offspring to survive into adulthood using data on offspring produced on Allports and Motuara islands in 2008 and 2009 (n = 133), and the survival of these individuals to 2009 and 2010 (n = 56), respectively. Individual HL had a significant negative effect on survival in that more heterozygous offspring had a higher probability of surviving to age one ($p = 0.03$; see appendix B on p. 111 for detailed information on the analysis). This result supports the hypothesis that the heterozygote excess prior to the translocation is due to higher survivorship among more heterozygote individuals (see also Bensch et al. 2006).

In both island populations, the observed excess of heterozygote individuals decreased from prior to the translocation to one year after the translocation and even turned into a significant heterozygote deficiency on the larger island, Motuara (see F_{IS} values in table 4.2 on p. 94). This increase of homozygote individuals one year following the initial translocation is caused by a mechanism termed the Wahlund effect: even though I introduced only female individuals, I artificially created a subpopulation structure by including these translocated females into my calculations of genetic diversity, and as a result, an excess of homozygote individuals. It is unclear, however, why this

effect is more pronounced in the larger population, as the number of translocated females relative to overall population size is much lower there. As the hybrid individuals (i.e., the more heterozygote individuals) spread through the population, the Wahlund effect was reversed in the second year after the translocation, despite the additional exchange of six individuals between the two islands, which is reflected in the decrease of F_{IS} values. To get a more accurate estimate of the effect of the translocations on genetic diversity, it would be valuable to calculate these measures using only the hybrid and inbred offspring (offspring of mixed pairs and inbred control pairs), thus excluding all translocated females and the inbred parental population present before the translocation in order to take into consideration that some of the translocated females might fail to produce viable offspring and thus not contribute to the future gene pool. This, however, was not feasible in the present study due to small sample sizes. Nonetheless, the significant increase in levels of heterozygosity among hybrid birds compared to inbred birds provides evidence for the successful recruitment of new alleles into both populations. The long-term effects of the translocations can ultimately only be assessed by documenting the spread of the introduced alleles after an extended period of time.

Conclusions

The present study demonstrates that the reciprocal translocation between two isolated and inbred bird populations has likely enhanced both the short-term viability and the evolutionary potential of these populations by significantly increasing both levels of heterozygosity and allelic diversity. The effects were stronger in the smaller population, and it might therefore be necessary to adapt the number of individuals used for a translocation to the size of the receiving population. However, this obviously depends on the availability of individuals in the donor population, and might therefore not be feasible in populations of endangered species.

Isolated populations will inevitably accumulate inbreeding over the long-term (Jamieson 2010). The decrease in levels of heterozygosity even within the first three generation of hybrids draws attention to the fact that, for endangered species, a reciprocal translocation between inbred populations cannot be a one-off solution to counteract the erosion of genetic diversity; however, periodical introduction of new genetic stock in combination with other conservation measures (i.e., habitat restoration, predator control etc.) could well be a short-term measure to prevent a species from becoming extinct. Despite the increase of genetic variation observed after the reciprocal translocation of inbred individuals, the long-term viability of a population on the verge of extinction can obvi-

ously not be guaranteed unless the initial circumstances that led to the decline, such as habitat loss, are reversed (see also Bouzat et al. 2009). Considering the current rate of population fragmentation, the results of this study entail significant implications for the implementation of 'genetic rescue' as a conservation management tool in the preservation of endangered species.

Appendices

Appendix A

Microsatellite markers and genotyping

I genotyped a panel of 658 individuals at 32 autosomal microsatellite markers. DNA was extracted from blood samples using an E.Z.N.A. Blood DNA Kit D3392 (Omega Bio-Tek) according to the manufacturer's instructions. PCR was performed using a Qiagen Multiplex PCR Master Mix (# 206145). All primers used (see table A.1 on p. 109) had a concentration of 100 μM. Multiplex reactions were performed in 7 separate mixes with DNA concentrations ranging between 10 and 90 ng/μl following the manufacturer's recommendations: each 10 μl multiplex PCR contained 1 μl DNA, 5 μl of the 2x Qiagen Multiplex PCR Master Mix, 1 μl of the primer mix and 3 μl H_2O. For each of the 7 mixes, initial denaturation was performed at $95\,^{\circ}\mathrm{C}$ for 15 min, and final extension was achieved for 30 s at $60\,^{\circ}\mathrm{C}$.

The number of cycles, denaturation, annealing and extension settings varied between the mixes as follows (primer concentration in H_2O in a final volume of 500 μl will be indicated after each locus). In mix 1 (loci Pau09, 1.6 μl; Pau01, 1 μl; Pau04, 1.5 μl; and Pau20, 1.1 μl) the initial denaturing cycle was followed by 26 cycles of denaturation at $94\,^{\circ}\mathrm{C}$ for 30 s, $46\,^{\circ}\mathrm{C}$ annealing for 90 s, and $72\,^{\circ}\mathrm{C}$ extension for 60 s. For mix 2 (loci P2/P8, 2.5 μl; Ind28, 2 μl; TG11, 1.5 μl; ADCbm, 1.1 μl; Pau07, 1 μl; and Pau23, 1.5 μl), amplification was performed in 30 cycles of denaturation at $94\,^{\circ}\mathrm{C}$ for 30 s, annealing at $51\,^{\circ}\mathrm{C}$ for 90 s, and extension at $72\,^{\circ}\mathrm{C}$ for 1 min. For mix 3 (loci 4E8/Pca12, 6 μl; TG02, 1 μl; Pau26, 0.8 μl; Ppi2, 4 μl; Pau66, 0.8 μl; and Ind29, 5 μl), thermal cycling conditions after initial denaturation consisted of 28 cycles of denaturation at $94\,^{\circ}\mathrm{C}$ for 30 s, $52\,^{\circ}\mathrm{C}$ annealing for 90 s, and $72\,^{\circ}\mathrm{C}$ extension for 1 min. Mix 4 (loci POCC6, 0.5 μl; GCSW47, 1 μl; Mjg1, 1.5 μl; and Tgu1, 1.5 μl) had 29 cycles of denaturation at $94\,^{\circ}\mathrm{C}$ for 30 s, $54\,^{\circ}\mathrm{C}$ annealing for 90 s, and $72\,^{\circ}\mathrm{C}$ extension for 60 s. For mix 5 (loci Asμ15, 1.7 μl; CcaTgu21, 0.9 μl; Pau67, 0.6 μl; PC3, 1.3 μl; and Pau06, 0.9 μl), amplification was performed

in 29 cycles of denaturation at $94\,^{\circ}\mathrm{C}$ for 30 s, annealing at $55\,^{\circ}\mathrm{C}$ for 90 s, and extension at $72\,^{\circ}\mathrm{C}$ for 1 min. For mix 6 (loci Tgu3, 1 μl; TguEST09, 1.2 μl; and Escμ6, 3 μl), initial denaturation was followed by 28 cycles of denaturation at $94\,^{\circ}\mathrm{C}$ for 30 s, $56\,^{\circ}\mathrm{C}$ annealing for 90 s, and $72\,^{\circ}\mathrm{C}$ extension for 1 min. Finally, in mix 7 (loci Ase64, 0.7 μl; DkiB119, 0.7 μl; 4H2/Pca14, 2.9 μl; and 2F9, 1.2 μl) the initial denaturing cycle was followed by 28 cycles of denaturation at $94\,^{\circ}\mathrm{C}$ for 30 s, $57\,^{\circ}\mathrm{C}$ annealing for 90 s, and $72\,^{\circ}\mathrm{C}$ extension for 60 s.

1.5 μl of the PCR product was mixed with formamide containing the GeneScan 500 LIZ Size Standard, heat-denatured and resolved in POP4 polymer on an ABI 3130xl Genetic Analyser (Applied Biosystems, Inc.). Allele sizes were scored using Genmapper v4.0 software (Applied Biosystems, Inc.) and followed by manual inspection of all allele sizes. To improve the reliability of my results, 10% of individuals were genotyped more than once.

Table A.1: Primer sequences of the microsatellite markers of South Island robins used for the multiplex PCR assay. $^{a-p}$: The superscript identifies pairs of loci that are located on the same chromosome and showed significant linkage disequilibrium (LD) after FDR correction (common correlation: ^{a}: 0.289, ^{b}: 0.196, ^{c}: 0.414, ^{d}: 0.339, ^{e}: 0.185, ^{f}: 0.268, ^{g}: 0.32, ^{h}: 0.234, ^{i}: 0.302, ^{j}: 0.338, ^{k}: 0.263, ^{l}: 0.326, ^{m}: 0.366, ^{n}: 0.266, ^{o}: 0.362, ^{p}: 0.446); ^{1}: functional locus, ^{2}: neutral locus.

Marker	Primer sequence forward	Primer sequence reverse	Source
2F9^{1}	PET-GCATTTCTGGGCTGTAACAT	AAAGGACAATGTAATTGGTG	Townsend and Jamieson, unpublished
4E8^{2}	6FAM-TGTGGGAAACCAGAGGAAA	CAGGGGAAAAATAGAGAGGG	Lambert et al. (2005)
4H2^{e,2}	VIC-GTTTTGACCTACAGAGAGAG	GATAGGACAATCAACAGGCT	Lambert et al. (2005)
ADCbm^{a,1}	VIC-GATGTGAGTAACCAGCCACT	ATAACACAGGAGCGGTGA	Steinmeyer et al. (2009)
Ase64^{b,k,p,2}	6FAM-CCACCTTTCATACTGGGGAG	TTCAGCCAGTCAGTGTAGCC	Richardson et al. (2000)
Asμ15^{g,h,o,1}	6FAM-AATAGATTCAGGTGCTTTTTCC	GGTTTTTGAGAAAATTATACTTTCAG	Dawson, unpublished, Bulgin et al. (2003)
CcaTgu21^{1}	VIC-GGCAGACATGATTGCATCC	TCTCAGTGGTCATTGGAAAGTG	Olano-Marin et al. (2010)
DkiB119^{1}	6FAM-CATACAACTTCATGACTACCATAGCAC	TCCATAGTGACATAGAACGAGCTG	Dawson, unpublished, King et al. (2005)
Escμ6^{2}	NED-CATAGTGATGCCCTGCTAGG	GCAAGTGCTCCTTAATATTTGG	Hanotte et al. (1994)
GCSW47^{2}	VIC-GGCTTCTCTGGTTGCATGTC	ACAGTAATCCCCAGCCATCA	McRae et al. (2005)
Ind28^{2}	6FAM-CCCAGGAAGTATCCCAGAA	CCTCCAATGCTTTAGTGACC	Sefc et al. (2001)
Ind29^{b,c,d,2}	NED-TCAGGGAGCAAATCTCTACG	GGAAGAAGGCTGGGTAAAAT	Sefc et al. (2001)
Mjg1^{e,2}	PET-CCCGGGAAAGGCTTCGTCTTC	GGAGATTTTATATCGGTGGC	Li et al. (1997)
Pau01^{2}	VIC-TTAGAAGTGAAAGGCTTG	GAGGAATAAAAACAATGC	Townsend et al. (2012)
Pau04^{f,2}	PET-AATAAAGCAGATACTGAG	ACAGGTAAACCAGAGCAG	Townsend et al. (2012)
Pau06^{f,2}	NED-CTGAGGTTCAAAGTTTCC	ACCAGCCATCCTTATGC	Townsend et al. (2012)
Pau07^{g,2}	VIC-CTTTCCTTGACTGAAGTG	TTAGCTTCATTTCCAGTC	Townsend et al. (2012)
Pau09^{h,I,2}	6FAM-ATGATGTAGTCAGAGTCG	TATTTTGCAACCTTCTTG	Townsend et al. (2012)
Pau20^{2}	NED-AGTTGGGACTATTATCAC	ATAAATCAGTCCTGGAAG	Townsend et al. (2012)
Pau23^{2}	PET-CAGGGCATTTACAGATTTTCC	(MWG) CTCATCTTGCACACTGCTG	Townsend et al. (2012)
Pau26^{2}	6FAM-AAATTACTACAGTGTTACGGTGAAAA	(MWG) GGGACCACCAAGAACTTCAA	Townsend et al. (2012)
Pau66^{2}	NED-TGGGCCAGTTTATACCCTCT	(MWG) ATGAAAGGGTTCCATGATGC	Townsend et al. (2012)
Pau67^{j,2}	VIC-CCAGGAAAGGTGCTCAGAGT	(MWG)TGTCTGTGTTGGCCTGATCT	Townsend et al. (2012)
PC3^{j,2}	PET-GGTGTTTGTGAGCCGGGG	TGTTACAACCAAAGCGGTCATTTG	Dawson et al. (2000)
Pocc6^{2}	VIC-TCACCCTCAAAAACACACACA	ACTTCTCTCTGAAAAGGGGAGC	Bensch et al. (1997)
Ppi2^{k,l,m,n,}	PET-gtttcttCACAGACCATTCGAAGCAGA	GCTCCGATGGTGAATGAAGT	Martínez et al. (1999)
TG02^{a,1}	6FAM-TGTGTGTTGACAGTATTCTCTTGC	TTTAAACCTAATAAACGTCACACAGTC	Dawson et al. (2010)
TG11^{1}	VIC-ACAAACTAAGTACATCTATATCTGAAG	TAAATACAGGCAACATTGG	Dawson et al. (2010)
Tgu01^{i,o,1}	NED-TGCGGTCTGTATGGAAATAGTC	CTTGCAATACTCTCTGCCTCA	Forstmeier et al. (2007)
Tgu03^{c,m,2}	6FAM-TCTCTCTGCTAGGGATAAACAGTG	TGCTCCCTCCCTCCAGTAAC	Forstmeier et al. (2007)
TguEST09^{d,n,p,1}	PET-AACCCAACCAACAAAATTGG	CCAACTATCAGTTTTACAAGGCATAC	Dawson et al., unpublished

Appendix B

Details on the generalised linear model (GLM) on the effect of heterozygosity on the survival of offspring to adulthood

In order to test whether individuals with lower levels of HL (i.e., higher levels of heterozygosity) had a higher probability of surviving to one year of age, I fitted a generalised linear model (GLM) with a binary response variable (0 = did not survive to age one, 1 = survived to age one) with "individual HL" as a continuous predictor, "island" and "year of birth" as a categorical predictor, a binomial error distribution and the default logit link function. During step-wise model simplification, I deleted non-significant interactions, starting with the three-way interaction between the predicting variables (effect size = -6.189, 95% CI = -18.423 to 6.046, z-value = -0.991, p = 0.321), then the interaction between "individual HL" and "island" (effect size = 2.259, 95% CI = -3.529 to 8.047, z-value = 0.765, p = 0.444), and finally the factor "island" (effect size = -0.588, 95% CI = -1.338 to 0.161, z-value = -1.538, p = 0.124). The minimal model therefore retained only "individual HL", "year of birth", and the interaction between these two factors as significant predictors of probability of survivorship (see table B.1).

Individual HL had a significant negative effect on survival in that more heterozygous offspring (i.e., with lower levels of HL) had a higher probability of surviving to age one (p = 0.027; see table B.1). Survival of chicks born in 2009 was significantly lower compared to chicks born in 2008 (p = 0.026). The year 2009 was a particularly bad year, with above-average rainfall, storms, and associated variation in temperature; as a consequence, a high proportion of the nestlings in the monitored nests died due to hypothermia and starvation (SH, personal observation). The interaction between "individual HL" and "year of birth" was significantly different in the two years considered: while there was a strong and significant negative correlation between individual HL and survivorship in the year 2008 (effect size = -4.606, 95% CI = -8.683 to 0.529, p = 0.027), the interaction

between individual HL and survivorship was reversed in year 2009, with individuals with lower HL levels experiencing higher mortality. This relationship, however, was not significant (effect size = 1.449, 95% CI = -2.096 to 4.995, p = 0.423). These results support the hypothesis that the heterozygote excess prior to the translocation is due to higher survivorship among more heterozygote individuals.

Table B.1: Summary of output of the generalised linear model (GLM) of "year of birth" (2008/2009), "individual HL" on offspring survival, and the interaction of these two factors ("year*individual HL") after model simplification (n = 133).

	Estimate ($\pm$ s.e.)	z-value	p-value
Intercept	1.664 ± 0.893	1.863	0.062
Individual HL[1]	-4.606 ± 2.080	-2.214	0.027
Individual HL[2]	1.449 ± 1.809	0.801	0.423
Year of birth 2009	-2.6497 ± 1.187	-2.232	0.026
Individual HL*year of birth 2009	6.055 ± 2.757	2.197	0.028

[1]Effect of individual HL in reference to offspring born in 2008
[2]Effects of individual HL in reference to offspring born in 2009

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Chapter 5

Effects of reciprocal translocations on reproductive success, survival, and recruitment in two inbred South Island robin populations

Abstract: *I conducted reciprocal translocations between two severely bottlenecked and isolated populations of the South Island robin* Petroica australis *to investigate differences in key fitness measures such as breeding success, survival and recruitment between inbred individuals and crosses between the two populations (termed hybrids). I found a significant effect on overall levels of fitness, with hybrid birds experiencing increased levels of both survival and recruitment into the breeding population. Furthermore, sperm quality significantly increased in hybrid males compared with inbred males. The present study demonstrates that the exchange of individuals between two inbred bird populations has successfully increased fitness levels in the resulting hybrid offspring. The results of this experiment highlight the possible value of translocations as an effective tool for the 'genetic rescue' of endangered species lacking outbred donor populations.*

5.1 Introduction

The detrimental effects of isolation and small population size following a bottleneck event include the loss of genetic diversity and increased inbreeding (Frankham et al. 2010, Newman and Pilson 1997). A loss in genetic variation as a consequence of founder effects and random genetic drift in small populations may decrease fitness or hamper the evolutionary potential of populations by limiting their ability to respond to future environmental challenges, such as climatic extremes, pollutants, and diseases (Amos and Balmford 2001, England et al. 2003, Frankham 1995, Frankham et al. 2010, Nei et al. 1975). Similarly, the reduction in fitness of individuals born to closely related parents, termed 'inbreeding depression', can reduce the viability of endangered species (Crnokrak and Roff 1999, Hedrick and Kalinowski 2000, Keller and Waller 2002, Kristensen et al. 2010) and increase their risk of extinction (Frankham 2005). Key fitness measures, such as fertility and survival, are susceptible to inbreeding depression (Charlesworth and Charlesworth 1987, Charlesworth and Willis 2009). In fact, several studies have found a negative relationship between parental genetic similarity and survival in mammals (Ralls et al. 1979, Stockley et al. 1993), as well as between parental similarity and hatching success among birds (Bensch et al. 1994, Kempenaers et al. 1996, Spottiswoode and Møller 2004). The negative effects of inbreeding have also been shown to increase with the severity of the population bottleneck. For example, a comparative analysis across more than 50 bird species worldwide found that hatching failure was significantly negatively correlated to bottleneck size (Heber and Briskie 2010, see chapter 2).

The adverse effects of inbreeding can be mitigated and levels of genetic variability restored through the introduction of outbred individuals into bottlenecked populations ('genetic rescue'; e.g. Adams et al. 2011, Benson et al. 2011, Bouzat et al. 2009, Madsen et al. 2004, Vilá et al. 2002, Westemeier et al. 1998, see also Frankham et al. 2011, Hedrick and Fredrickson 2010, Weeks et al. 2011), but this approach depends on the availability of suitable outbred source populations. However, most endangered species survive only as a series of small, fragmented populations, with each likely subject to some loss of genetic variation and increased levels of inbreeding. Theoretically, crossing individuals from one inbred population with those of a second inbred population should mitigate the severity of inbreeding depression in the resulting hybrid offspring by masking deleterious recessive alleles in one population with alleles from the other population, and vice versa (Charlesworth and Charlesworth 1999, Edmands 2007, Lynch 1991, Whitlock et al. 2000). However, these theoretical models have not been tested in wild populations using a systematic and replicated approach

(but see Fredrickson et al. 2007, Wisely et al. 2008).

In the present study, I conducted experimental translocations between two insular, inbred populations of the South Island robin *Petroica australis* to investigate whether key fitness components such as breeding success, survival and recruitment, differ between hybrid and inbred control birds following the introduction of new individuals. Furthermore, I compared traits that could potentially affect fitness, such as sperm quality and various aspects of parental care, between inbred and hybrid individuals. My objective was to determine whether 'genetic rescue' could be implemented successfully to increase fitness levels of endangered species using only highly bottlenecked and inbred populations as donors.

5.2 Material and methods

5.2.1 Study populations

I used two isolated and bottlenecked populations of the South Island robin on Allports and Motuara islands in the Marlborough Sounds, New Zealand, for the experimental translocations. Both robin populations were founded with only five individuals each in 1973, and the founders of each population originated from different parts of New Zealand (Flack 1974, Armstrong 2000). Henceforth, I refer to the descendants of the five founders on each island as "inbred" individuals, even though they might not be the product of recent within-family matings. The founder individuals of each population were derived from different parts of New Zealand, with the population on Allports Island originating from a mainland site near Kaikoura, and the population on Motuara Island originating from Nukuwaiata Island, Marlborough Sounds (Ardern et al. 1997).

Both Allports and Motuara islands are located a minimum of 1.4 km from the nearest shoreline in the Queen Charlotte Sound. As robins are not capable of sustained flight (Boessenkool et al. 2007, Taylor et al. 2005), the two island populations are considered closed (i.e., genetically isolated) systems. At the time of the translocations, the populations had thus been isolated for nearly four decades ($\sim$10 generations). Despite being at carrying capacity, the populations were showing signs of inbreeding depression, such as reduced egg hatching success and problems with immune system function (Byrne 1999, Hale and Briskie 2007, Mackintosh and Briskie 2005). Although the islands differ in size (Allports Island: 16 ha, Motuara Island: 59 ha), the density of robins is similar on both islands (Allports: $\sim$60 adult individuals, $\sim$3.9 birds/ha; Motuara: $\sim$300 adult individuals,

$\sim$5.1 birds/ha; SH, personal observation).

In total, 31 female robins were exchanged between Allports and Motuara islands in 2008 and 2009 (see chapter 4). The number of individuals chosen for the experimental translocations was selected on the basis of leaving an appropriate number of non-manipulated individuals on each island to act as controls. Only females were translocated in order to minimise disturbance to territorial boundaries that the translocation of males would entail, and to ensure the formation of new "mixed" pairs upon release. The offspring of these mixed pairs will hereafter be referred to as "hybrid F1", and the offspring of hybrid F1 individuals as "hybrid F2". Individuals were considered "hybrid F2" if either one or both parents were hybrid F1 (however, only one hybrid F1 x hybrid F1 pair formed during the study period, wherefore the majority of "hybrid F2" individuals are backcrosses). As the sample size for the F2 generation was relatively small, and general patterns did not change when analysing all hybrids combined, I only present the combined results in the main text. Results for hybrid F1 and F2 separately can be found in appendices H–L, pp. 155–169.

5.2.2 Breeding success

Following the initial translocation in 2008, both islands were extensively searched for the translocated females, and all their nests located and monitored at regular intervals. Additionally, nests of all inbred control pairs were monitored on Allports Island, and, due to the relatively large population size, the nests of a sub-sample of inbred control pairs were monitored on Motuara Island. In 2008, I therefore monitored nests of mixed pairs and of inbred control pairs. As robins reach sexual maturity at age one (Heather and Robertson 2005), I was able to additionally monitor nests of 1- and 2-year-old inbred and hybrid F1 individuals in 2009 and 2010, and of 1-year-old hybrid F2 individuals in 2010. Nests were checked approximately every three days to quantify hatching, fledging, and overall breeding success. Nests that were abandoned before the incubation term had been completed were excluded from the analysis of hatching success if the eggs contained developing embryos: in those cases (n_{eggs} = 8) I could assume reasonably safely that the eggs failed to hatch because they were abandoned, as opposed to the nest being abandoned because the eggs failed to hatch. My estimate of hatching failure therefore reflects problems with fertility and embryo death.

5.2.3 Survival and recruitment

Throughout the breeding seasons of 2009 and 2010, a thorough search was repeated on both islands to determine the survival rate and breeding success of inbred and hybrid individuals. The survival and recruitment rate on Allports could be determined accurately as the smaller size and terrain of the island allowed it to be surveyed entirely. The survival and recruitment rates on Motuara, however, represent minimum estimates due to the larger size of the island, presence of inaccessible areas, and possibility of missing individuals even after repeatedly combing the island. I was, however, able to infer the survival and recruitment of some individuals on Motuara through allocating parentage to captured individuals using paternity analysis (see appendix C, p. 145). Furthermore, I calculated the probability of detection on each island to assess the accuracy of my survival estimates (see section 5.2.7).

Robins are sexually monomorphic until they reach sexual maturity (Heather and Robertson 2005). I therefore assigned the sex to 267 robins banded as nestlings and fledglings using molecular techniques in order to test whether there was a difference in survival to adulthood between the sexes (see appendix D, p. 147).

5.2.4 Sperm quality

The two most important determinants of male fertility are the number of motile and morphologically normal sperm (Birkhead and Pizzari 2002, Immler et al. 2010, Malo et al. 2005, Pizzari and Birkhead 2002). This is because only motile sperm can reach the site of fertilisation, and morphologically abnormal sperm are likely to suffer from reduced velocity or compromised direction of movement (midpiece and tail abnormalities; Saacke et al. 1994), or are unable to penetrate the ovum (acrosomal dysfunction; Drobnis and Overstreet 1992, Roldan et al. 1994). In passerine birds, the sperm midpiece is thought to consist of a single mitochondrial helix that is coiled around the flagellum (Humphreys 1972, Jamieson 2006, but see Birkhead et al. 2007) and provides the energy for movement (Cardullo and Baltz 1991). It is therefore possible that gross abnormalities or increased variation in the size of the midpiece (e.g. partial or complete aplasia, under- or oversized midpieces) restrict energy production, swimming speed, or the lifespan of spermatozoa (Gomendio and Roldan 1993, Roldan et al. 1998). Reduced midpiece length could also negatively affect fertilisation success or competitiveness, as it results in a decrease in the quantity or size of mitochondria

contained in each spermatozoon (Woolley and Beatty 1967), and hence in reduced power output and swimming velocity (Cardullo and Baltz 1991, Lüpold et al. 2009). A positive correlation between midpiece length and fertilisation success has indeed been found both *in vitro* (Sofikitis et al. 1994) and *in vivo* (Laskemoen et al. 2010). Midpiece length and abnormalities in midpiece structure have been found to be highly heritable in both mammals and birds (Birkhead et al. 2005, Woolley and Beatty 1967).

To test whether there is a difference in sperm quality between inbred and hybrid birds, I collected sperm samples from males in reproductive condition using the *gentle massage* technique (Wolfson 1952). Due to difficulties with measuring sperm motility in the field, I was only able to assess sperm abnormalities and morphometry. Samples were stored in 10% formalin until analysis in the laboratory. To estimate the proportion of abnormal sperm, 10 fields of view were randomly chosen per sample and sperm were counted and scored for abnormalities (mean number of sperm scored $\pm$ SD = 104 $\pm$ 77, range = 2 – 240, $n_{samples}$ = 33). Sperm morphology was categorised either as normal, or with structural abnormalities in the various sperm components (head, midpiece, and tail). In samples with very low sperm counts ($<$ 50 sperm, $n_{individuals}$ = 10), all detected sperm were scored for abnormality. Examples of the encountered abnormalities are illustrated in figure 5.3 on page 137.

Due to their importance in fertilisation success, I used sperm midpiece length (measured to the nearest 0.1 μm) and the within-male coefficient of variation of midpiece length (controlling for sperm size) as the morphometric traits of interest in this study. To obtain morphometric data, sperm were photographed using a Leitz Laborlux S microscope with a Spot Insight QE video camera at 250x magnification. Total length, head length, midpiece length, and tail length were measured using Leica IM50 v.4.0 software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). I calculated the number of sperm that would have to be measured in order for the sample to reflect within-male variation with 95% accuracy following Calhim et al. (2011). Based on the results of the accuracy estimates, which revealed high intra-male variation in sperm morphometry, a minimum of 15 sperm per male was measured whenever possible (see figure E.1 in appendix E, p. 149). All sperm measurements and abnormality screens were conducted by the same person (SH) and blind as to the identity of the bird. Table E.1 on page 150 summarises the relationship between the assessed sperm characteristics.

5.2.5 Unhatched eggs

Inbreeding depression is commonly expressed in the form of compromised fertility and embryogenesis (Gage et al. 2006, Keller and Waller 2002). It is important to distinguish between the causes of hatching failure (infertility or embryo death) in order to be able to assess the mechanisms by which inbreeding affects reproductive success. In the breeding seasons 2008–2010, I therefore collected eggs that failed to hatch two days after the last chick had hatched or upon desertion of the nest (n_{eggs} = 80 from n = 55 clutches). Eggs were cracked open upon collection and stored in 10% formalin until analysis at a later date. For eggs that did not show obvious signs of development (n_{eggs} = 34 from n = 26 clutches), all of the perivitelline layer (PVL) found in each sample was examined as described in Birkhead et al. (2008). In short, fertility was determined by staining the germinal disk (GD) and PVL using Hoechst 33342 dye (10 mg/ml solution in water; Molecular Probes, Eugene, Oregon, USA) and examining the GD for the presence of cell nuclei/embryonic tissue and/or the PVL for the presence of sperm using a Leica DMLB microscope at 250x magnification. In eggs in which GD degradation was too advanced to detect cell division, fertility was determined based on the presence of sperm on the PVL. Sufficient material was obtained from all but three eggs to determine their fertility status (n_{eggs} = 77 from n = 52 clutches). Eggs were classified as either (i) infertile (no cell division in the GD, no sperm on the PVL) or (ii) fertile but having suffered embryo mortality (presence of an embryo, cell division in the GD, and/or sperm on the PVL). Table F.1 in appendix F (p. 152) summarises the fertility status, sperm count, and presence of cell nuclei or embryonic tissue for eggs collected in the nests of 1- and 2-year-old inbred and hybrid birds.

5.2.6 Parental care

I estimated various aspects of incubation attentiveness and nestling provisioning using nest recordings in order to compare parental care between inbred and hybrid individuals. Nests were filmed for a period of six hours from first light using Sony digital video cameras that were installed on a tripod at 3 to 8 m distance from a nest. Individuals did not seem to be affected by the presence of a camera (SH, personal observation). Nests were typically found after egg laying had been completed, and it was therefore often not possible to determine the day of incubation at the time of nest filming. Incubation day could, however, be inferred in retrospect for all nests where at least one egg hatched. For those nests, day of incubation at nest-filming ranged between 2 and 18 (mean $\pm$ SD = 9.2 $\pm$ 4.3,

n = 80). As there was no evidence for a relationship between day of incubation and any of the assessed incubation measures (% of time spent on the nest, mean length of bouts on/off the nest, and number of visits per hour; see table G.1, p. 154, in appendix G), I used all incubation videos of 1- and 2-year-old inbred and hybrid females in my analysis, regardless of the day of incubation during nest-filming (n = 25).

I attempted to record food provisioning around pin-break stage, but due to logistical difficulties (i.e., simultaneous data collection on two islands, limited number of video cameras), nestling age during filming ranged from 6 to 20 days (mean $\pm$ SD = 11.3 $\pm$ 2.8, n = 58). Nonetheless, I found no evidence for a relationship between nestling age and food provisioning in this range of ages (see table G.2, p. 154, in appendix G), and therefore all food provisioning videos of 1- and 2-year-old inbred and hybrid birds were included in the analysis (n = 25). Nests were considered "hybrid" if one or both parents were hybrid, and "inbred" if both parents were inbred and at least one of them 1 or 2 years old. Feeding rates were calculated as the number of feeding visits by both parents per hour divided by the size of the brood. Chicks were weighed around pin-break stage and approximately one month after fledging to determine pre- and post-fledging body mass.

5.2.7 Data analyses

All analyses (except for the calculation of detectability, see section 5.2.7.2) were carried out within the R statistical environment (R Development Core Team 2011). Models with more than one fixed effect were simplified by stepwise deletion of the least significant terms until no further reduction of residual deviance (measured using the Akaike Information Criterion, AIC; Akaike 1973) was obtained. However, the predicting factor "group" (inbred vs. hybrid) was retained in all models regardless of its significance level, as this was the effect of interest. All mixed models were checked for overdispersion. P-values for models with Gaussian error distribution were obtained using the *pvals.fnc()* function in the "languageR" package (Baayen 2011). Both the parameter estimates and 95% CIs were unlinked for ease of interpretation.

5.2.7.1 Breeding success

To take into account multiple observations of the same individuals in my analysis of breeding success, I fitted generalised linear mixed-effects models (GLMMs) with a binomial error structure

and logit link (Armstrong and Cassey 2007) using functions implemented in the "lme4" package (Bates and Maechler 2009) of the R statistical environment (version 2.13.1, R Development Core Team 2011). The response variable was either the proportion of eggs that hatched, proportion of nestlings that fledged, or proportion of eggs that developed into a fledgling, and was coded in R as the number of successes and number of failures using the *cbind* function (see Crawley 2007). Due to an imbalanced experimental design, I used separate models to analyse differences in breeding success between nests of mixed pairs and inbred control pairs of unknown age (data existed for 2008–2010), and between nests of 1- and 2-year-old inbred and hybrid birds (data on breeding success of hybrid F1 individuals existed for 2009/2010, whereas data on breeding success of hybrid F2 individuals only existed for Allports Island in 2010; see appendix H, pp. 155–159). "Group" (mixed/inbred control nest, inbred/hybrid nest) was the only fixed categorical predictor in these models, as I was specifically interested in testing for differences in breeding success between members of these groups. In order to control for possible effects of island and year, these factors were included as random effects. I also included the identity of female and male parents as random effects, because many individuals laid multiple clutches per year and bred in multiple years.

5.2.7.2 Survival and recruitment

I tested whether there were differences in survival to one month after fledging, survival to one year after fledging, and recruitment between inbred and hybrid individuals by fitting GLMMs with a binary response (1 = alive/recruited, 0 = dead/not recruited), "group" and "sex" as categorical fixed predictors, and "island" and "year" as random effects. I also included "nest identity" as a random effect to control for non-independence of chicks within a nest. The same procedure was applied in my analysis for differences in time to recruitment between inbred and hybrid birds. However, in this case the response variable consisted of count data (number of years till recruitment), and the model was therefore fitted using a Poisson error structure and log link.

To determine the accuracy of my survival estimates, I estimated detectability separately for each island using a Bayesian approach within the software WinBUGS (Ntzoufras 2008). I modelled the probability (p_i) of each individual being identified in year i as a product of both its survival rate and its probability of being observed, given that it was alive that year (resighting probability). Recapture histories for the three study years of n = 90 individuals on Allports Island and n = 173 individuals on Motuara Island were used to estimate values of p_i and 95% credible intervals. A bird that was

observed in a given year was recorded as 1 in the recapture history for year *i*, and as 0 if it was not observed or dead. These gaps in each bird's individual recapture history were used to derive annual values of *p* (see also McCarthy 2007). A total of 100,000 samples after discarding the first 10,000 were used to produce an estimate of the detectability of individuals on each island.

5.2.7.3 Sperm quality

I tested for differences in the proportion of total abnormal sperm (head, midpiece and tail abnormalities combined), proportion of head abnormalities, proportion of midpiece abnormalities and proportion of tail abnormalities between inbred and hybrid males by fitting GLMMs with a binomial error structure and logit link. Mean midpiece length and individual coefficient of variation of sperm midpiece were continuous response variables, and were therefore analysed by fitting GLMMs with Gaussian error distribution and identity link. In all models related to sperm abnormalities or sperm morphometry, "group" was the only fixed effect, and "island" and "individual identity" were included as random factors to account for differences between the islands and individual effects of males that were sampled both at age one and age two. In two hybrid and two inbred sperm samples, less than 15 spermatozoa could be retrieved and measured. I therefore repeated all analyses related to sperm morphometry excluding samples with less than 15 sperm. As none of the results changed, I only present results including all samples.

5.2.7.4 Unhatched eggs

Of the 80 unhatched eggs collected during the study period, 12 eggs were collected in 7 hybrid nests, and 16 eggs in 11 nests of 1- or 2-year-old inbred individuals (see table F.1, p. 152). I carried out a Fisher's exact test to examine whether there was an association between the probability of an unhatched egg being truly infertile and the group the parents belonged to (an egg was assigned to the category "hybrid" if either one or both parents were hybrid).

5.2.7.5 Parental care

In order to compare measures related to incubation attentiveness, I fitted separate GLMMs with percent of time spent on the nest, mean length of on/off bouts and number of visits per hour as response variables. "Group" (inbred vs. hybrid) was the only fixed effect in these models, and

"year" and "island" were random effects. As in robins only the female incubates the eggs (Heather and Robertson 2005), female identity was also included as a random effect in all models relating to incubation to avoid pseudoreplication. Percent time spent on the nest was analysed with a binomial error structure and logit link. Mean length of time spent on and off the nest, and number of female visits/hour were fitted with a Gaussian error distribution and identity link. The models analysing differences in mean length of time spent on and off the nest were overdispersed (dispersion parameter $\hat{c}$ = 142.8 and 90.8, respectively), and were therefore re-fitted using Penalised Quasi-Likelihood within the *glmmPQL* function of the "MASS" package in R (Venables and Ripley 2002). I included brood size as a fixed effect in my analyses of the differences in the feeding rate per hour, per chick, between inbred and hybrid nests, to control for differences between nests with varying number of chicks. "Year", "island", and both male and female identity were included as random factors, as male and female robins share nestling provisioning (Heather and Robertson 2005).

5.2.7.6 Body mass

In addition to the factor "group", sex and capture time were included as fixed effects in my analyses of differences in pre-fledging, post-fledging, and adult body mass, due to expected differences between males and females, and an expected increase in weight associated with food intake during the course of the day. Capture month, year, island, and nest identity were included as random effects in a linear mixed model with a Gaussian error structure and identity link.

5.3 Results

5.3.1 Breeding success

I found no significant differences in hatching, fledging, or overall breeding success between nests of mixed pairs and nests of inbred control pairs (of unknown age). Similarly, measures of reproductive success were similar in the nests of 1- and 2-year-old inbred and hybrid individuals. However, all trends were in the predicted direction, with mixed pairs and hybrid pairs experiencing slightly higher levels of hatching, fledging, and overall breeding success (figure 5.1a-b on p. 135; see tables H.1–H.4, pp. 156–159, for exact values of the estimate and 95% CIs).

Hatching success (proportion of hatched eggs) calculated from the raw data was 76% in inbred

nests and 79% in hybrid nests. The GLMM produced much higher estimates of 96% and 99%, respectively (table H.4, p. 159). The reason for this discrepancy might be that whereas most birds had very high hatching success (100%), a few pairs tended to have particularly low hatching success. Since individual identity was treated as a random effect, less weight was given to individual observations from the same pair.

5.3.2 Survival and recruitment

Survival probability to one month post-fledging was significantly higher in hybrid individuals (mean of 91% survival, 95% CI = 77 – 96%) compared with inbred individuals (mean of 69% survival, 95% CI = 58 – 78%, p = 0.008; see figure 5.2 on p. 136; estimates for hybrid F1 and F2 individuals separately are presented in table I.1 on p. 162). Similarly, survival probability to one year was significantly higher in hybrid individuals: 76% of hybrids survived to age one (95% CI = 52 – 90%) compared to only 29% of inbred individuals (95% CI = 20 – 39%; p = 0.0001). Detection rates were very high on both islands with p_i = 98% (95% CI = 92 – 100%) on Allports and p_i = 92% (95% CI = 86 – 96%) on Motuara Island. Thus, I can be confident that my estimates of survival are reasonably accurate. Furthermore, 95% of hybrid individuals that were alive at age one or two were recruited into the breeding population (95% CI = 70 – 99%), compared to only 59% of inbred individuals (95% CI = 36 – 78%), which constituted a significant difference (p = 0.016; figure 5.2). As robins reach sexual maturity at age one (Heather and Robertson 2005), this means that more than 40% of inbred individuals that were alive at age one or two were either unable to establish a territory, or unable to find a mate. This was only the case for 5% of hybrid individuals. Time to recruitment was slightly shorter in hybrid individuals (1.11 years) compared to inbred individuals (1.23 years), but this difference was not significant (p = 0.74; table I.1, p. 162).

5.3.3 Sperm quality

There was a significant decrease in the proportion of total abnormal sperm in hybrid individuals (estimate = 13%, 95% CI = 3 – 44%) compared to inbred individuals (estimate = 46%, 95% CI = 22 – 72%; p = 0.039; figure 5.4 on p. 138). When comparing head, midpiece and tail abnormalities separately, the proportion of abnormal sperm was lower in hybrid individuals; however, this trend

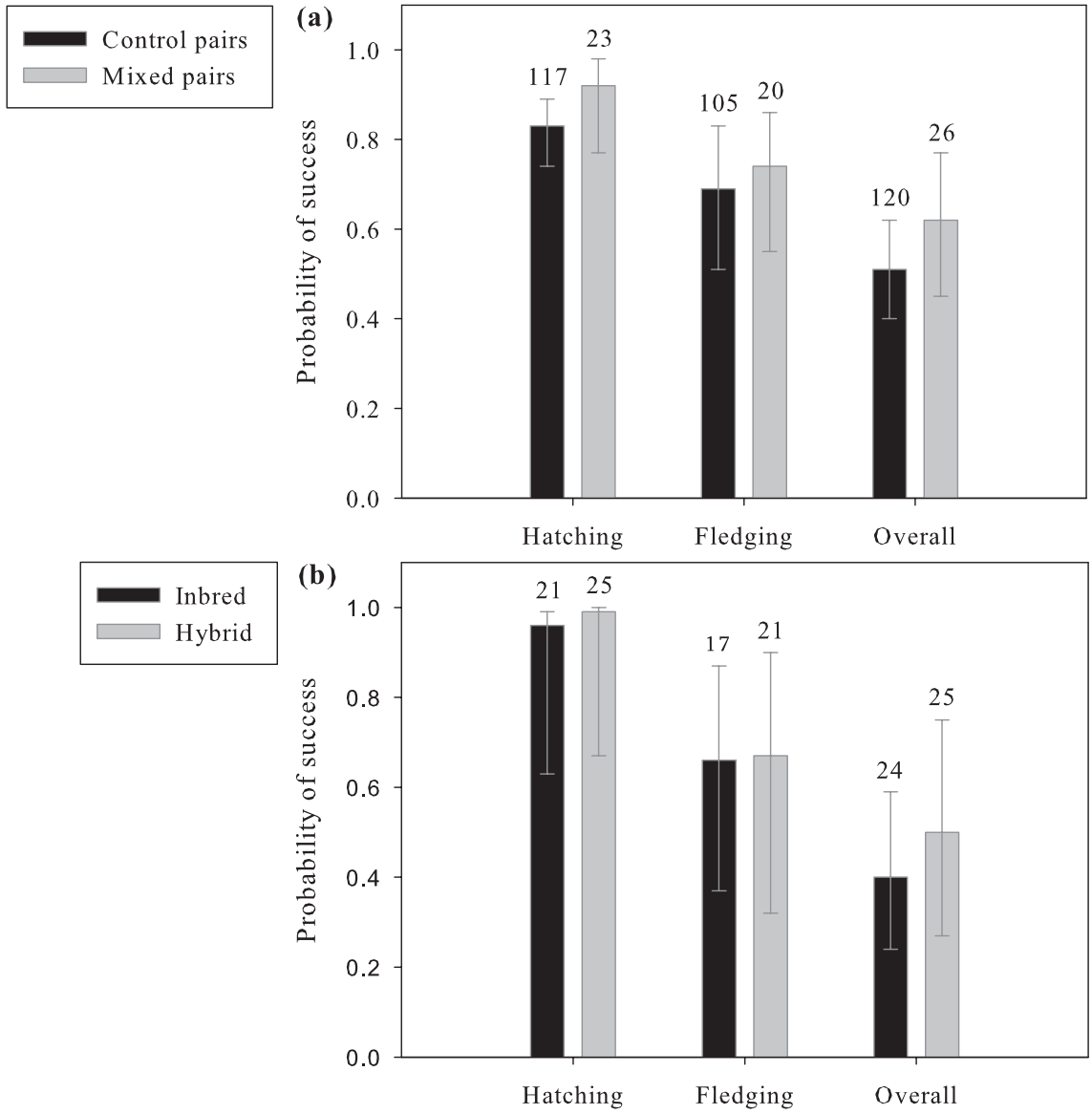

Figure 5.1: Comparison of hatching, fledging, and overall breeding success between control and treatment groups; error bars represent 95% confidence intervals; sample sizes (number of nests) are indicated above each bar. **a)** Inbred control pairs of unknown age and mixed pairs. **b)** 1- and 2-year-old inbred and hybrid pairs (a pair was classified as "inbred" if both parents were inbred and one or both were 1 or 2 years old, and as "hybrid" if one or both parents were hybrid, and by default 1 or 2 years old).

was not significant (see table J.1, p. 164). Hybrid males furthermore had significantly longer sperm midpieces (estimate = 94.8 μm, 95% CI = 92.9 – 96.8 μm) compared to inbred males (estimate = 92.5 μm, 95% CI = 90.8 – 94.2 μm; p = 0.029; table J.2, p. 165).

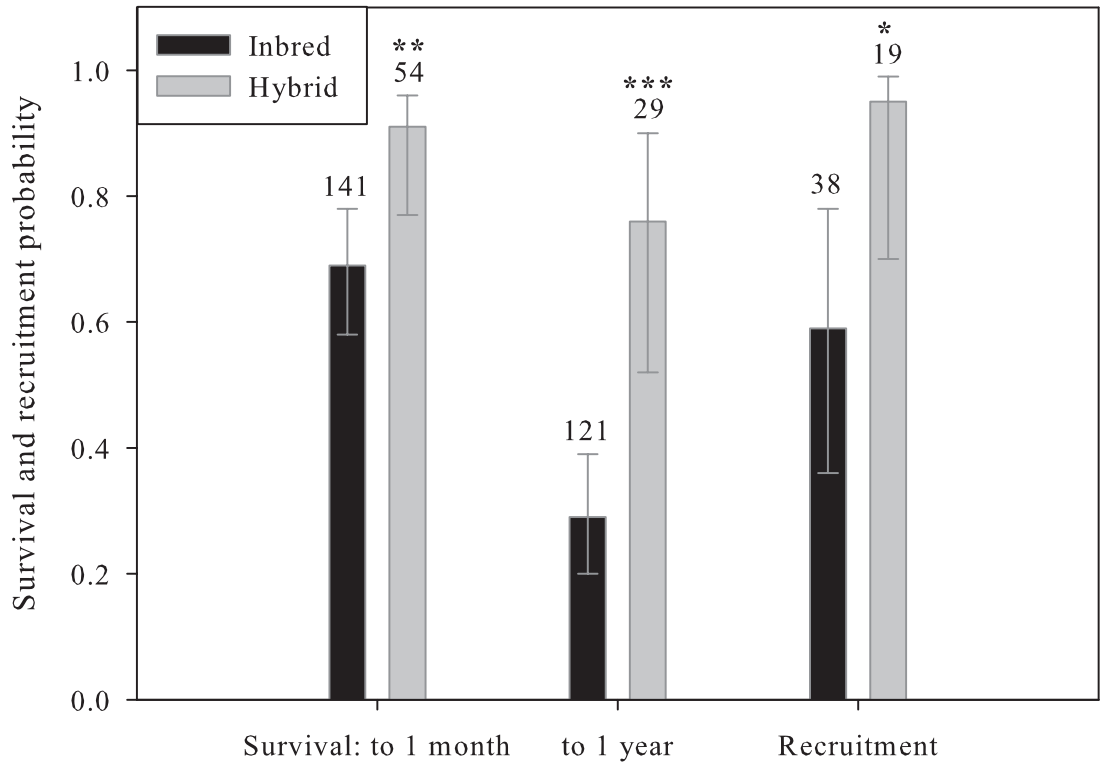

Figure 5.2: Comparison of survival to 1 month, survival to 1 year, and recruitment between inbred and hybrid individuals; error bars represent 95% confidence intervals; sample sizes (number of individuals) and significance are indicated above each bar (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

As the degree of coiling of the midpiece helix may vary between males, Birkhead et al. (2005) suggested calculating the straight midpiece length, which takes into account the number of complete helix gyres and the radius from the centre of the sperm flagellum to the centre of the midpiece helix (hereafter tail radius). As measuring the tail radius involves the use of transmission electron microscopy, I was unable to calculate straight midpiece length from my data. However, within-male mean midpiece length in my dataset was highly correlated with the mean number of helix gyres ($r = 0.66$, 95% CIs = 0.52 – 0.77; $p < 0.0001$; $n = 83$), indicating that differences in the unadjusted midpiece length between inbred and hybrid individuals are unlikely due to differences in the degree of coiling of the midpiece helix. I found no evidence for a difference in within-male variation (midpiece coefficient of variation; see table J.2, p. 165) between inbred and hybrid males.

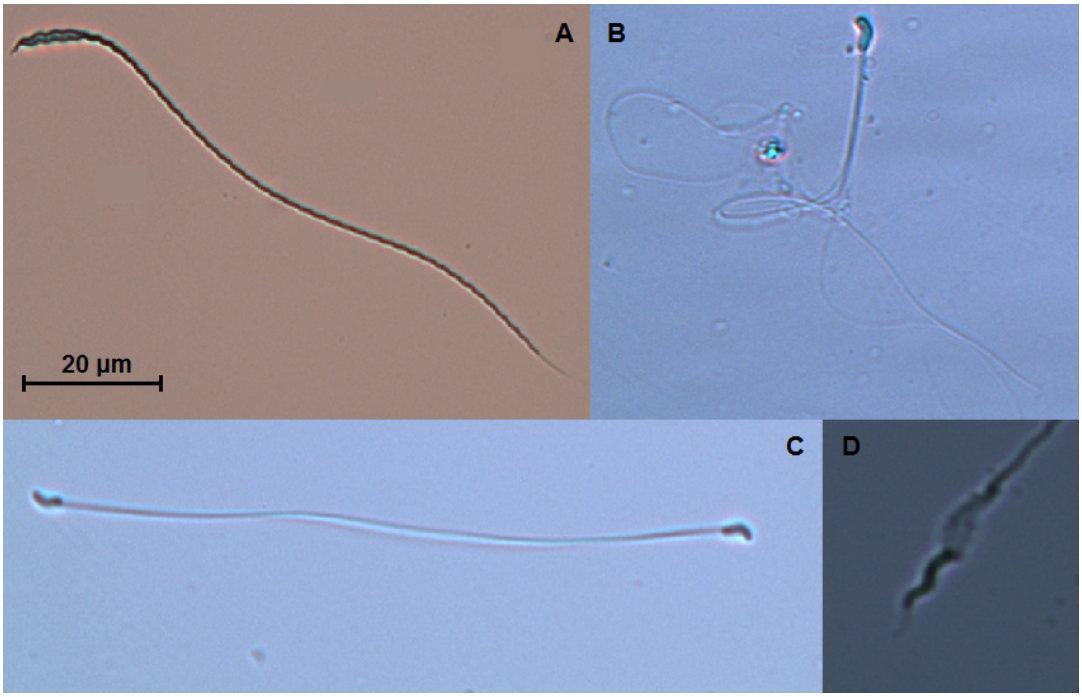

Figure 5.3: Images of South Island robin sperm viewed under a light microscope at 250x magnification: **(A)** normal sperm, **(B)** multiple tails and missing acrosome, **(C)** two-headed sperm, missing acrosomes and missing mitochondrion, **(D)** damaged head helix and acrosome.

5.3.4 Unhatched eggs

Only 10.4% of all unhatched eggs collected between 2008 and 2010 were truly infertile ($n_{infertile}$ = 8 from n = 6 clutches; includes eggs collected in nests of mixed pairs and inbred control individuals of unknown age), suggesting that embryo mortality—as opposed to true infertility caused by a lack of sperm reaching the ovum—was the major cause of hatching failure in these two populations. Eggs with as few as 23 sperm on the entire ovum were unambiguously confirmed to be fertile due to the presence of cell division on the germinal disk (see table F.1, p. 152). The proportion of truly infertile eggs was higher in inbred ($n_{infertile}$ = 4; 25%) than in hybrid nests ($n_{infertile}$ = 1; 8.3%), but my sample size was too small to detect statistically significant differences (odds ratio = 0.28, 95% CI = 0.005 to 3.483; p = 0.36).

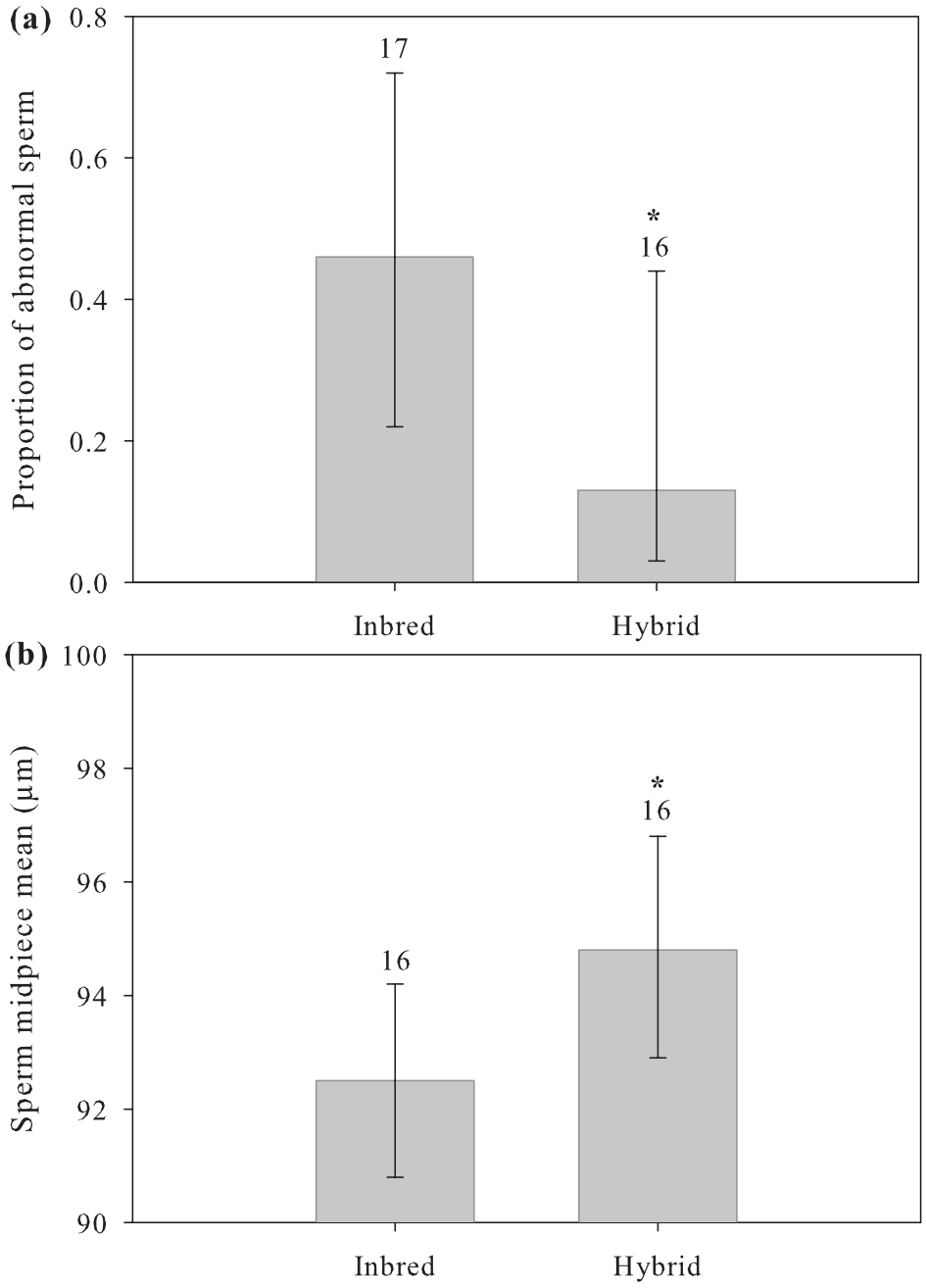

Figure 5.4: Comparison of sperm characteristics between 1- and 2-year-old inbred and hybrid males; error bars represent 95% confidence intervals; sample sizes (number of individuals) and significance are indicated above each bar (* $p < 0.05$). **a)** Proportion of total abnormal sperm. **b)** Mean sperm midpiece length (μm).

5.3.5 Parental care

None of the measures assessed to compare incubation behaviour between inbred and hybrid individuals differed significantly (see table K.1, p. 167). Brood size had a significant negative effect on

feeding rate in that chicks with one or more siblings were fed less frequently ($p < 0.01$). After controlling for brood size, there was no difference in feeding rate per chick, per hour, between inbred and hybrid nests (p = 0.39).

5.3.6 Body mass

Capture time and sex had a significant effect on pre-fledging body mass in that weight increased throughout the day (p = 0.001) and male nestlings were heavier than female nestlings (estimate$_{malenestlings}$ = 26.2 g, 95% CI = 25.4 – 27.1 g; estimate$_{femalenestlings}$ = 24.4 g, 95% CI = 19.3 – 29.6 g; p = 0.0001). After controlling for capture time and sex, there were no significant differences in body mass between inbred and hybrid nestlings (see appendix L on p. 169). There was no significant effect of capture time on post-fledging body mass (p = 0.45). After controlling for the effects of sex (estimate$_{malefledglings}$ = 40.2 g, 95% CI = 39.3 – 41.0 g; estimate$_{femalefledglings}$ = 37.0 g, 95% CI = 34.8 – 39.3 g; $p < 0.0001$), there was no difference in post-fledging body mass between inbred and hybrid birds. Neither capture time nor sex had a significant effect on body mass of 1-year-old robins (p = 0.33 and 0.11, respectively). There was no significant difference in adult body mass between inbred and hybrid individuals (see table L.1, p. 169).

5.4 Discussion

I have documented that the reciprocal translocation of individuals between two isolated and inbred robin populations can be used as an effective tool to increase overall levels of fitness: key fitness measures such as survival and recruitment showed a significant improvement in hybrid individuals compared to inbred ones. As opposed to previous studies that revealed that the translocation of outbred individuals into inbred populations caused an increase in reproductive success (Madsen et al. 1996, Westemeier et al. 1998), I found no clear evidence for such a difference in reproductive success between inbred and hybrid birds on a per breeding attempt basis (although it is worth noting that all trends were in the predicted direction, and it is possible that my results were affected by limited statistical power). Nonetheless, increased survival provides individuals with increased opportunities to reproduce, and can thereby potentially increase lifetime reproductive success. This finding alone carries important implications for the persistence of endangered species.

In birds, the female is the heterogametic sex (Pike and Petrie 2003). Deleterious recessive alleles

that are linked to the sex chromosomes can therefore not be masked in females, and I would expect inbred females to exhibit lower fitness than inbred males (Charlesworth and Charlesworth 1999, but see Frankham and Wilcken 2006). Survival was the only trait assessed that was directly comparable between males and females, but I found no effect of sex on the survival of inbred and hybrid birds. Sex differences in survival could, however, occur at earlier life stages from fertilisation till hatching. As I did not determine the sex of unhatched eggs, I was not able to address pre-hatching differential fitness between females and males. It is also possible that longevity of adults might vary between the sexes, but I could not measure this given the relative short duration of the study compared to the lifespan of the birds[1].

I found a significant difference in the frequency of sperm abnormalities, with the proportion of abnormal sperm being on average three times higher in inbred males compared to hybrid males. The increased proportion of abnormal sperm in inbred individuals is consistent with other studies that have found inbreeding or heterozygosity effects on ejaculate quality (Asa et al. 2007, Gage 2006, Gomendio et al. 2000, Roldan et al. 1998). Furthermore, a correlation between poor semen quality and reproductive success has been documented in several species (Gomendio et al. 2000, Malo et al. 2005). In my study, however, the difference found in the proportion of abnormal sperm between inbred and hybrid males did not seem to affect fertilisation or hatching success of eggs (see table F.1, p. 152, and tables H.2–H.4, pp. 157–159, in the appendix). It is possible that my sample size was not large enough to detect differences. The fact that I found that eggs with as few as 23 sperm on the entire ovum had been fertilised, however, suggests that very few sperm are needed to ensure fertilisation. Even if $> 50\%$ of the spermatozoa in an ejaculate are abnormal, there may still be sufficient functional sperm left to reach and fertilise the egg. Nonetheless, the sperm of hybrid individuals seems to be more competitive than sperm of inbred males, as indicated by their significantly longer midpieces. Since none of the 1- and 2-year-old inbred and hybrid males sired extra-pair young (see appendix C, p. 145), I was not able to assess the competitiveness of hybrid versus inbred sperm in this study.

In spite of the recognised benefits of translocations, harmful effects associated with the disruption of locally adapted gene complexes have also been documented as the result of translocations (Frankham et al. 2010, 2011, Edmands 2007, Huff et al. 2011, Tallmon et al. 2004). The effects of outbreeding depression are typically expressed in the F2 generation, when a recombination of the

[1] (SI robins have a mean life expectancy of three years, but can reach up to 16 years; Heather and Robertson 2005).

original parental gene arrangements occurs, thereby potentially affecting local adaptation (Edmands 1999, 2007, Huff et al. 2011, Marshall and Spalton 2000, but see McClelland and Naish 2007). I found no evidence for the occurrence of outbreeding depression as a result of the translocation, as hybrids of the F2 generation had trait values similar to those of the F1 generation (see table H.2, p. 157, and tables and I.1–L.1, pp. 162–169, in the appendix). However, sample size for the F2 generation was generally very small and therefore possibly not representative. Furthermore, effects of outbreeding depression could be delayed to the F3 generation (Marshall and Spalton 2000, Tallmon et al. 2004). It would therefore be necessary to assess the effects of the translocation after an extended period of time, when more generations of hybrids and larger sample sizes can be examined. As I crossed individuals of the same species with no fixed chromosomal differences, gene flow between the two populations had been present within the last 500 years, and both populations inhabit similar environments, the probability of outbreeding depression is minimal (Frankham et al. 2011). To avoid the potential problems of outbreeding depression, however, source populations that adaptively match the population of concern (e.g. adapted to similar environments) should be preferable for a translocation (Frankham et al. 2011, Hedrick and Fredrickson 2010, Weeks et al. 2011).

Although the reciprocal translocation was effective in increasing fitness levels, the potential recovery and long-term viability of a species is not solely dependent on genetic factors, such as the loss of genetic diversity and inbreeding depression. One of the main limiting factors is the availability of suitable habitat (Wolf et al. 1998, see also Bouzat et al. 2009). Even if translocations can be used to increase levels of fitness, these improvements will only be temporary if the focal population remains in its isolated state, as isolated populations will inevitably be affected by genetic drift and accumulate inbreeding over the long-term (Adams et al. 2011, Jamieson 2010). It is therefore essential that genetic interventions in form of reciprocal translocations be complemented with other management strategies aimed at the restoration or conservation of suitable habitat.

Appendices

Appendix C

Paternity assignment

In order to ensure that the individual birds were assigned to the correct group (hybrid F1, F2, or inbred) in the presence of extra-pair paternity (EPP), I carried out parentage analyses of all chicks hatched in monitored nests during 2008–2010 following the translocation, unless they disappeared before blood-sampling age. When nestlings were around 9 days old (pin-break), blood samples were collected via brachial venipuncture, and approximately 10-30 μl of blood was stored in 1 ml of Queens Lysis Buffer (0.01 M Tris-HCl, 0.01 M NaCl, 0.01 M Na-EDTA (pH 7.5), 1% (v/v) n-Lauroylsarcosine; pH 7.5; Seutin et al. 1991) at room temperature. If dead chicks could be found, a tissue or feather sample was taken and preserved in 95% ethanol or stored in an envelope, respectively. On the larger Motuara Island, where not all nests could be monitored, unbanded adults and their fledglings were additionally caught and sampled throughout the breeding seasons 2008–2010.

I thus collected blood samples from 658 individuals ($>$ 90% of adult individuals and their offspring in each island population) in three consecutive years. Maternity was determined for 203 individuals from field observations, either while the chicks were still in the nest, or after they had fledged but were still being fed by their parents. Later on, maternity was confirmed with parentage analysis. Parentage was assigned from genetic data for a total of 358 individuals of known age. All offspring and candidate parents were genotyped at 28 polymorphic microsatellite loci, and the genotypes were analysed using the maximum likelihood program CERVUS 3.0, set at 95% confidence (Kalinowski et al. 2007). A detailed description of the genotyping procedure is given in chapter 4 (appendix A on p. 107).

In 121 nests monitored between 2008 and 2010 on Allports and Motuara islands, 8.3% (n_{nests} = 10) contained at least one extra-pair offspring. In three cases of EPP, a 1- or 2-year-old inbred

male was the social father, and in one case, a hybrid F1 male was the social father. All other cases involved inbred individuals of unknown age. Neither inbred nor hybrid 1- and 2-year-old males sired any extra-pair offspring (all extra-pair offspring were sired by inbred males of unknown age).

Appendix D

Molecular sex determination

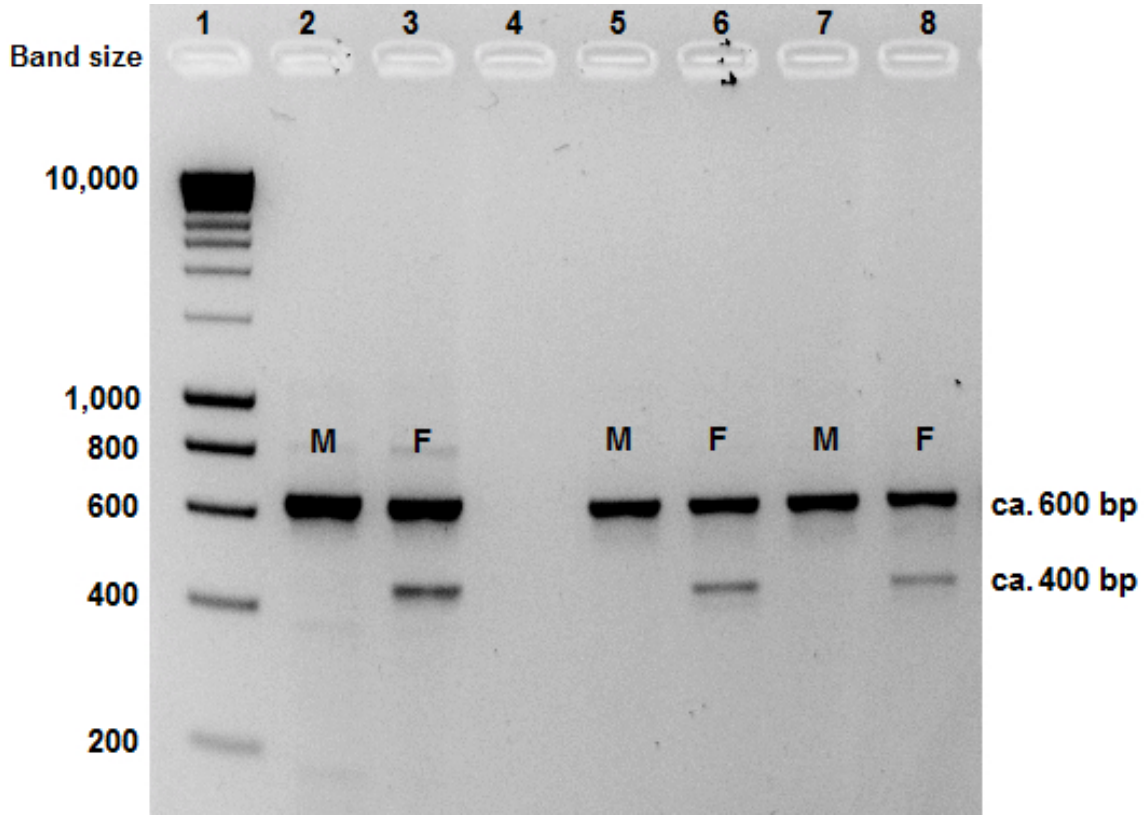

Figure D.1: Molecular sexing of robin nestlings and fledglings using PCR amplification of introns in the two homologous genes CHD-W and CHD-Z followed by 2% agarose electrophoresis. Column 1 contains hyperladder I, and columns 2 and 3 contain samples from adult birds of known sex; column 4 was left empty.

The sex of all inbred and hybrid robin nestlings and fledglings was determined by polymerase chain reaction (PCR) amplification of introns in two homologous genes (CHD-W and CHD-Z) using the primer pair 2550/2718 F/R for avian sex (Fridolfsson and Ellegren 1999). Amplification was carried out in a total volume of 20 μl containing 1 μl of primers, 10 μl of KAPA Blood PCR Mix B (2x), 7 μl molecular grade water, and 4 μl DNA template. Each reaction consisted of and initial denaturing cycle at $94\,^{\circ}\mathrm{C}$ for 2 min, 35 cycles of 30 s at $94\,^{\circ}\mathrm{C}$, 30 s at $50\,^{\circ}\mathrm{C}$, and 45 s at $72\,^{\circ}\mathrm{C}$, with a final 1 min extension step at $72\,^{\circ}\mathrm{C}$. PCR products were separated by electrophoresis at 120 V for 50 min on 2% agarose gels stained with SYBR SAFE DNA stain and visualised by UV-transillumination. Female sex was assigned if both the CHD-W and CHD-Z bands were present, and male sex was assigned if a single CHD-Z band was present. This technique was verified by testing it on adults of known sex (one male and one female per row in each gel; four different adults in total; see figure D.1). Of 54 individuals whose sex could be confirmed from morphological or behavioural characteristics as they were resighted at age one or two, 53 had been assigned the correct sex using the described molecular technique, yielding a consensus between field and molecular data of $\sim$98%.

Appendix E

Sperm abnormalities and morphometry

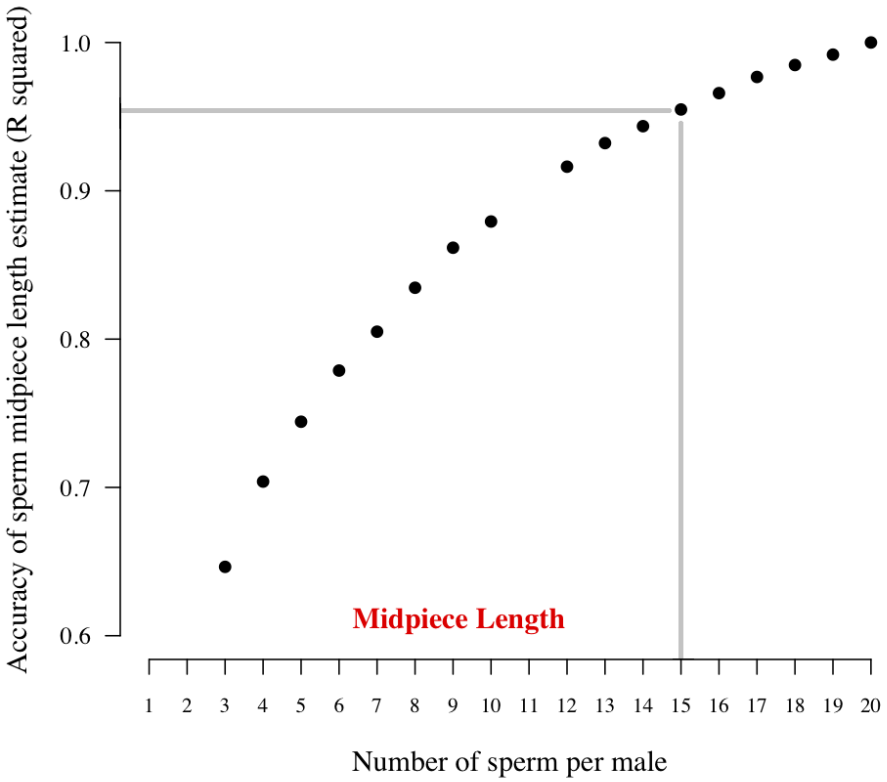

Figure E.1: Relationship between the accuracy of the sperm midpiece estimate and the number of sperm measured per male in South Island robins. The grey lines indicate that if 15 sperm are measured per male, the sample estimate is representative with 95% accuracy.

Table E.1: Pairwise Pearsons product-moment correlations for six sperm measurements (upper triangle) and the 95% confidence intervals (CI, lower triangle), n = 83. Statistically significant correlations and their CIs are in **bold**. CV = coefficient of variation.

Variable	Mean midpiece length	Midpiece CV	% head defects	% midpiece defects	% tail defects	% total defects
Mean midpiece length	–	**−0.507**	**−0.236**	−0.159	**−0.220**	**−0.373**
Midpiece CV	**−0.651 to −0.327**	–	0.022	**0.316**	**0.460**	**0.314**
% head defects	**−0.430 to −0.021**	−0.194 to 0.237	–	−0.081	**−0.240**	**0.580**
% midpiece defects	−0.363 to 0.058	**0.108 to 0.498**	−0.291 to 0.138	–	**0.546**	0.176
% tail defects	**−0.417 to −0.005**	**0.271 to 0.615**	**−0.434 to −0.026**	**0.374 to 0.681**	–	**0.401**
% total defects	**−0.545 to −0.171**	**0.105 to 0.496**	0.417 to 0.708	−0.041 to 0.377	**0.203 to 0.568**	–

Appendix F

Fertility status of unhatched eggs

Table F.1: Fertility status, sperm counts and presence of cell nuclei or embryonic tissue of unhatched eggs in nests of 1- or 2-year-old hybrid and inbred robins. The perivitteline layer (PVL) area (mm^2) denotes the size of the PVL fragment retrieved from each egg. The average PVL area of four intact yolks was 501.63 mm^2. GD = germinal disk; n/a = not available (no sperm count was conducted either because the egg contained an embryo, or because no PVL could be retrieved).

Group	Island	Nest	Result	Sperm count	PVL area (mm^2)	Cell nuclei/embryonic tissue
Hybrid F1	Motuara	1	Fertile	53	62.79	yes
Hybrid F1	Allports	2a	Fertile	54	173.32	yes
Hybrid F1	Allports	2a	Fertile	14	66.56	yes
Hybrid F1	Allports	2b	Fertile	n/a	n/a	yes
Hybrid F1	Allports	2b	Fertile	42	115.01	yes
Hybrid F1	Allports	3	Infertile	0	241.17	no
Hybrid F1	Motuara	4a	Fertile	137	356.53	yes
Hybrid F1	Motuara	4a	Fertile	49	138.22	yes
Hybrid F1	Motuara	4b	Fertile	144	176.56	GD not found
Hybrid F1	Motuara	4b	Fertile	88	111.33	GD not found
Hybrid F2	Allports	5	Fertile	302	458.94	yes
Hybrid F2	Allports	5	Fertile	258	549.24	yes
Inbred	Allports	6	Infertile	n/a	n/a	no (no yolk)
Inbred	Allports	7	Fertile	n/a	n/a	yes
Inbred	Allports	7	Fertile	n/a	n/a	yes
Inbred	Allports	8	Fertile	n/a	n/a	yes
Inbred	Allports	8	Fertile	n/a	n/a	yes
Inbred	Allports	9	Fertile	n/a	n/a	yes
Inbred	Allports	10	Fertile	n/a	n/a	yes
Inbred	Allports	10	Fertile	n/a	n/a	yes
Inbred	Allports	11	Infertile	0	223.78	no
Inbred	Allports	11	Infertile	0	142.33	no
Inbred	Motuara	12a	Fertile	23	583.02	yes
Inbred	Motuara	12a	Fertile	0	1.49	yes
Inbred	Motuara	12b	Infertile	0	251.29	GD not found
Inbred	Motuara	13	Fertile	2	22.47	yes
Inbred	Allports	14	Fertile	n/a	n/a	yes
Inbred	Allports	14	Fertile	n/a	n/a	yes

Appendix G

Incubation attentiveness and nestling provisioning

Table G.1: Pairwise Pearsons product-moment correlations for six incubation measures (upper triangle) and the 95% confidence intervals (CI, lower triangle), n = 80. Statistically significant correlations and their CIs are in **bold**.

Variable	Incubation attention	Mean ON time	Mean OFF time	Female visits/ hour	Incubation day	Clutch size
Incubation attention	–	0.147	**−0.694**	0.220	−0.030	0.115
Mean ON time	−0.075 to 0.356	–	**0.461**	**−0.854**	0.196	**0.254**
Mean OFF time	**−0.792 to −0.559**	**0.269 to 0.619**	–	**−0.678**	0.122	0.026
Female visits/hour	0.000 to 0.419	**−0.904 to −0.780**	**−0.782 to −0.540**	–	−0.124	−0.102
Incubation day	−0.248 to 0.191	−0.024 to 0.399	−0.101 to 0.332	−0.335 to 0.098	–	–
Clutch size	−0.107 to 0.327	**0.036 to 0.448**	−0.195 to 0.244	−0.315 to 0.120	–	–

Table G.2: Pairwise Pearsons product-moment correlations for five nestling provisioning measures (upper triangle) and the 95% confidence intervals (CI, lower triangle), n = 58 (except for maternal weight at pin-break stage, where n = 32). Statistically significant correlations and their CIs are in **bold**.

Variable	Total feeding rate/hour	Feeding rate/hour and chick	Maternal weight at pin-break	Nestling age	Brood size
Total feeding rate/hour	–	**0.550**	−0.013	0.078	**0.413**
Feeding rate/hour and chick	**0.340 to 0.708**	–	0.167	0.174	**−0.484**
Maternal weight at pin-break	−0.270 to 0.246	−0.095 to 0.408	–	−0.242	−0.163
Nestling age	−0.184 to 0.330	−0.088 to 0.414	−0.471 to 0.017	–	−0.171
Brood size	**0.173 to 0.607**	**−0.660 to −0.258**	−0.404 to 0.099	−0.411 to 0.091	–

Appendix H

Results of hatching success, fledging success, and overall breeding success analyses

Table H.1: Probability of hatching, fledging and overall breeding success of inbred control pairs (of unknown age; male and female from the same island) and mixed pairs (male and female from different islands). P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that the hybrid nests do not differ from the inbred nest intercept). CI = confidence interval.

Trait	Group	n_{nests}	n_{pairs}	Estimate	Lower 95% CI	Upper 95% CI	p-value
Hatching success	Inbred control pairs	117	61	0.83	0.74	0.89	
	Mixed pairs	23	16	0.92	0.77	0.98	0.17
Fledging success	Inbred control pairs	105	46	0.69	0.51	0.83	
	Mixed pairs	20	16	0.74	0.55	0.86	0.59
Overall breeding success	Inbred control pairs	120	62	0.51	0.40	0.62	
	Mixed pairs	26	18	0.62	0.45	0.77	0.21

Due to an imbalanced experimental design (data on breeding success of hybrid F2 individuals only existed for Allports Island in 2010), I used separate models to compare breeding success of 1- and 2-year-old inbred, hybrid F1, and hybrid F2 birds on Allports Island in 2010 (see table H.2), and to compare breeding success of 1- and 2-year-old inbred and hybrid F1 birds (data comprising both islands and both years; see table H.3). This model estimation involved numerical algorithm, and convergence was ensured by running the algorithm on a grid of starting values and choosing the one with the highest likelihood. I then repeated the analyses on breeding success for F1 and F2 hybrids combined (see table H.4).

Table H.2: Probability of hatching, fledging and overall breeding success of 1- and 2-year-old inbred, hybrid F1, and hybrid F2 individuals on Allports Island in 2010. P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that the hybrid nests do not differ from the inbred nest intercept). CI = confidence interval.

Trait	Group	n_{nests}	n_{pairs}	Estimate	Lower 95% CI	Upper 95% CI	p-value
Hatching success	Inbred	7	6	0.72	0.07	0.99	
	Hybrid F1	10	8	0.99	0.29	0.99	0.20
	Hybrid F2	4	4	0.97	0.09	0.99	0.37
Fledging success	Inbred	4	4	0.93	0.16	0.99	
	Hybrid F1	9	8	0.92	0.08	0.99	0.96
	Hybrid F2	3	3	0.50	<0.01	0.99	0.38
Overall breeding success	Inbred	7	6	0.32	0.09	0.69	
	Hybrid F1	10	8	0.62	0.19	0.92	0.22
	Hybrid F2	4	4	0.36	0.05	0.86	0.88

Table H.3: Probability of hatching, fledging and overall breeding success of 1- and 2-year-old inbred and hybrid F1 individuals across all study years and both islands. P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that the hybrid nests do not differ from the inbred nest intercept). CI = confidence interval.

Trait	Group	n_{nests}	n_{pairs}	Estimate	Lower 95% CI	Upper 95% CI	p-value
Hatching success	Inbred	21	16	0.97	0.68	1.00	
	Hybrid F1	21	14	0.99	0.58	1.00	0.64
Fledging success	Inbred	17	14	0.65	0.34	0.87	
	Hybrid F1	18	13	0.72	0.36	0.92	0.69
Overall breeding success	Inbred	24	17	0.40	0.24	0.59	
	Hybrid F1	21	14	0.54	0.29	0.78	0.32

Table H.4: Probability of hatching, fledging and overall breeding success of 1- and 2-year-old inbred and hybrid individuals (hybrid F1 and F2 combined) across all study years and both islands. P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that the hybrid nests do not differ from the inbred nest intercept). CI = confidence interval.

Trait	Group	n_{nests}	n_{pairs}	Estimate	Lower 95% CI	Upper 95% CI	p-value
Hatching success	Inbred	21	16	0.96	0.63	0.99	
	Hybrids combined	25	18	0.99	0.67	1.00	0.53
Fledging success	Inbred	17	14	0.66	0.37	0.87	
	Hybrids combined	21	16	0.67	0.32	0.90	0.98
Overall breeding success	Inbred	24	17	0.40	0.24	0.59	
	Hybrids combined	25	18	0.50	0.27	0.75	0.43

Appendix I

Results of analyses on survival to one month, survival to one year, recruitment, and time to recruitment

Table I.1: Probability of surviving to 1 month, surviving to 1 year, and being recruited into the breeding population of inbred, hybrid F1, and hybrid F2 individuals. P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that hybrid individuals do not differ from the inbred intercept). Significant p-values are highlighted in **bold**. CI = confidence interval.

Trait	Group	$n_{individuals}$	Estimate	Lower 95% CI	Upper 95% CI	p-value
Survival to 1 month (%)	Inbred	141	0.69	0.58	0.78	
	Hybrid F1	30	0.85	0.63	0.95	0.122
	Hybrid F2	21	0.97	0.70	1.00	**0.046**
	Hybrids combined[1]	54	0.91	0.77	0.96	**0.008**
Survival to 1 year (%)	Inbred	121	0.29	0.20	0.39	
	Hybrid F1	24	0.74	0.48	0.90	**0.0006**
	Hybrid F2	5	0.84	0.28	0.99	**0.054**
	Hybrids combined	29	0.76	0.52	0.90	**0.0001**
Recruitment (%)	Inbred	38	0.59	0.36	0.78	
	Hybrid F1	15	0.93	0.62	0.99	**0.037**
	Hybrid F2	4	1.00	<0.01	1.00	0.995
	Hybrids combined	19	0.95	0.70	0.99	**0.016**
Time to recruitment (years)	Inbred	22	1.23	0.84	1.79	
	Hybrid F1	14	1.14	0.62	2.12	0.821
	Hybrid F2	4	1.00	0.35	2.86	0.702
	Hybrids combined	18	1.11	0.53	1.69	0.736

[1]total hybrid count includes 3 hybrid F3 individuals (offspring of at least one hybrid F2 parent) fledged in 2010

Appendix J

Results of sperm analyses

Table J.1: Sperm abnormalities in 1- and 2-year-old inbred, hybrid F1, and hybrid F2 South Island robin males on Allports and Motuara islands. P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that the proportion of sperm abnormalities in hybrids does not differ from the inbred male intercept). Significant p-values are highlighted in **bold**. CI = confidence interval.

Trait	Group	$n_{individuals}$	Estimate	Lower 95% CI	Upper 95% CI	p-value
Total abnormalities	Inbred	17	0.46	0.22	0.72	
	Hybrid F1	14	0.15	0.03	0.48	0.064
	Hybrid F2	2	0.08	0.00	0.68	0.164
	Hybrids combined	16	0.13	0.03	0.44	**0.039**
Head abnormalities	Inbred	17	0.28	0.10	0.58	
	Hybrid F1	14	0.08	0.01	0.37	0.115
	Hybrid F2	2	0.04	0.00	0.65	0.254
	Hybrids combined	16	0.07	0.01	0.33	0.080
Midpiece abnormalities	Inbred	17	0.009	0.003	0.031	
	Hybrid F1	14	0.007	0.001	0.039	0.658
	Hybrid F2	2	0.003	0.000	0.105	0.542
	Hybrids combined	16	0.006	0.001	0.033	0.564
Tail abnormalities	Inbred	17	0.018	0.007	0.043	
	Hybrid F1	14	0.006	0.001	0.025	0.127
	Hybrid F2	2	0.016	0.001	0.169	0.917
	Hybrids combined	16	0.007	0.002	0.027	0.178

Table J.2: Measures of sperm morphometry in 1- and 2-year-old inbred, hybrid F1, and hybrid F2 males on Allports and Motuara islands. P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that the sperm midpiece length in hybrids and its within-male coefficient of variation does not differ from the inbred male intercept). Significant p-values are highlighted in **bold**. CI = confidence interval; CV = coefficient of variation.

Trait	Group	n	Estimate	Lower 95% CI	Upper 95% CI	p-value
Mean midpiece length (μm)	Inbred	16	92.5	90.8	94.2	
	Hybrid F1	14	94.9	92.8	97.0	**0.035**
	Hybrid F2	2	94.5	90.4	98.6	0.342
	Hybrids combined	16	94.8	92.9	96.8	**0.029**
Midpiece CV	Inbred	16	2.2	1.8	2.7	
	Hybrid F1	14	1.9	1.4	2.5	0.242
	Hybrid F2	2	2.3	1.2	3.4	0.891
	Hybrids combined	16	2.0	1.5	2.5	0.305

Appendix K

Results of analyses related to parental care

Table K.1: Comparison of measures related to parental care (1-4: incubation; 5: nestling provisioning) between 1- and 2-year-old inbred, hybrid F1, and hybrid F2 robins. P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that hybrid individuals do not differ from the inbred intercept.). SD = standard deviation.

	Trait	Group	Mean	Range	SD	n	p-value
(1)	Incubation attention (%)	Inbred	70	18 – 85	16	15	
		Hybrid F1	77	69 – 92	7	9	0.72
		Hybrid F2	83	n/a	n/a	1	0.79
		Hybrids combined	77	69 – 92	7	10	0.69
(2)	Mean ON time (s)	Inbred	561	373 – 872	159	15	
		Hybrid F1	613	307 – 895	214	9	0.52
		Hybrid F2	690	n/a	n/a	1	0.50
		Hybrids combined	620	307 – 805	202	10	0.43
(3)	Mean OFF time (s)	Inbred	221	85 – 423	120	15	
		Hybrid F1	181	57 – 345	84	9	0.41
		Hybrid F2	153	n/a	n/a	1	0.56
		Hybrids combined	179	57 – 345	80	10	0.35
(4)	Female visits/hour	Inbred	4.9	1.5 – 7. 8	1.7	15	
		Hybrid F1	5.1	3.3 – 8.9	1.8	9	0.82
		Hybrid F2	3.9	n/a	n/a	1	0.80
		Hybrids combined	5.0	3.3 – 8.9	1.8	10	0.77
(5)	Feeding rate/hour/chick	Inbred	6.1	2.0 – 10.7	2.9	10	
		Hybrid F1	5.0	2.7 – 8.1	1.4	12	0.48
		Hybrid F2	4.5	3-6 – 5.5	1.0	3	0.45
		Hybrids combined	4.9	2.7 – 8.1	1.3	15	0.39

Appendix L

Results of analyses related to differences in body mass

Table L.1: Pre-fledging, post-fledging, and adult weights (at age one) of inbred, hybrid F1, and hybrid F2 South Island robins after controlling for the effects of capture time and sex. P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that hybrid individuals do not differ from the inbred intercept). CI = confidence interval.

Trait	Group	n	Estimate	Lower 95% CI	Upper 95% CI	p-value
Pre-fledging weight (g)	Inbred	105	24.7	19.5	29.9	
	Hybrid F1	30	23.6	21.2	26.0	0.36
	Hybrid F2	20	24.9	22.4	27.3	0.89
	Hybrids combined[1]	54	24.7	22.9	26.6	0.44
Post-fledging weight (g)	Inbred	135	37.0	34.8	39.3	
	Hybrid F1	20	36.5	34.9	38.2	0.54
	Hybrid F2	16	37.0	36.5	37.6	1.00
	Hybrids combined	36	36.8	35.6	38.0	0.67
Adult weight (g)	Inbred	35	38.1	35.0	41.1	
	Hybrid F1	17	40.0	37.9	42.1	0.08
	Hybrid F2	4	38.2	34.9	41.6	0.93
	Hybrids combined	21	39.7	37.9	41.6	0.13

[1]total hybrid count includes 4 hybrid F3 nestlings (offspring of at least one hybrid F2 parent) weighed in 2010

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Chapter 6

Effects of reciprocal translocations on immunocompetence and pathogen loads in two inbred South Island robin populations

Abstract: *I investigated the effects of reciprocal translocations between two severely bottlenecked populations of the South Island robin* Petroica australis *on two components of the avian immune system and pathogen loads. The objective was to determine whether 'genetic rescue' using only highly bottlenecked populations as donors can be implemented successfully to decrease the susceptibility of hybrid individuals (crosses between the two populations) to pathogens. I found a significant increase in one aspect of cell-mediated immunity as indicated by an eightfold stronger immune response in hybrids to the mitogen phytohaemagglutinin (PHA). In contrast, total estimated and differential leucocyte counts did not differ between inbred and hybrid individuals. Despite the apparent increase in cell-mediated immunocompetence in hybrids, I found no difference in the prevalence of* Campylobacter *infection or ectoparasite loads between inbred and hybrid individuals. I conclude that the exchange of individuals between two inbred bird populations successfully increased levels of one component of cell-mediated immunity in the resulting hybrid offspring. The results of this experiment highlight the possible value of translocations between different inbred populations of endangered species as a tool to increase population viability.*

6.1 Introduction

The probability of an individual becoming infected with a pathogen or infested by a parasite depends both on environmental factors influencing exposure (Rogers and Randolph 2006), and on intrinsic factors such as age, reproductive effort, immunocompetence and ultimately the genetic make-up of the host (Kaslow et al. 2008, Norris et al. 1994, van Oers et al. 2010, Palacios et al. 2007, Sol et al. 2003, Stjernman et al. 2004). Small and isolated populations are particularly vulnerable to novel challenges such as pathogens and parasites (Altizer et al. 2003, Amos and Balmford 2001) due to the loss of genetic diversity and increased levels of inbreeding (Frankham et al. 2010, Keller and Waller 2002), both of which can result in compromised immune systems (Hale and Briskie 2007*b*, Hawley et al. 2005, O'Brien and Evermann 1988, Reid et al. 2003, 2005, Whiteman et al. 2006). During a bottleneck event, for example, alleles that influence resistance may be lost (e.g. Hedrick et al. 1999) and the frequency of deleterious mutations may increase (Lynch et al. 1995). An association between low levels of genetic variation and disease outbreaks has indeed been found in several wild populations of endangered species (e.g. O'Brien et al. 1985, Roelke et al. 1993, Thorne and Williams 1988). Subsequent inbreeding and the concomitant increase in homozygosity can further exacerbate susceptibility to infection (O'Brien and Evermann 1988, Reid et al. 2003). Diseases and parasites are therefore of particular concern in endangered species that have undergone a population bottleneck (Frankham et al. 1999). In fact, previous studies on endangered bird species that went through severe bottlenecks have shown that these birds are immunocompromised and therefore potentially more susceptible to novel pathogens (Hale and Briskie 2007*b*, Tompkins et al. 2006). Because infection with some diseases or high parasite loads can compromise an individual's fitness (e.g. Asghar et al. 2011, Hudson 1986, Martínez-de la Puente et al. 2011, Oppliger et al. 1993, but see Kilpatrick 2006) and even lead to death (Martínez-de la Puente et al. 2010), they have the potential to increase the risk of extinction of endangered species.

Intra-specific hybridisation, that is, offspring produced through the deliberate translocation of individuals between different populations of the same species, may improve population viability by enriching depauperate gene pools (Hedrick and Fredrickson 2010, Weeks et al. 2011). In fact, the translocation of outbred individuals into inbred populations has been shown to increase fitness by mitigating the negative effects of inbreeding and restoring genetic variation (e.g. Madsen et al. 1999, Westemeier et al. 1998). The hybridisation of related species (inter-specific hybridisation) has shown similar effects on the viability of at least one endangered species (Tompkins et al. 2006).

This, however, raises concerns about the genetic swamping of populations (Allendorf et al. 2001, Frankham et al. 2010) and the inherent risks of outbreeding depression (Frankham et al. 2011, Goldberg et al. 2005, Huff et al. 2011), which could work contrary to the desired effects by breaking up either locally adapted (Hendry et al. 2007), or co-adapted gene complexes in the immune system, such as the major histocompatibility complex (MHC; Reid et al. 2003).

An increasing number of endangered species survive only as a series of small, fragmented populations, with each likely subject to some loss of genetic variation and elevated levels of inbreeding. The objective of the present study was therefore to determine whether—in the absence of outbred donor populations of the same species—'genetic rescue' using only highly bottlenecked and inbred populations as donors could be implemented successfully to decrease the susceptibility of hybrid individuals to pathogens. To test this, I conducted experimental translocations between two isolated and inbred populations of the South Island robin *Petroica australis* and investigated whether hybrids (crosses between the two populations) and coeval inbred control birds differed in two components of the avian immune system, acquired and innate immunity (Norris and Evans 2000). First, I tested their ability to mount an immune response by artificially challenging the non-specific cell-mediated immune response using the phytohaemagglutinin assay (PHA; Smits et al. 1999). Acquired cell-mediated immunity is crucial to controlling a range of pathogens and parasites, including fungi, viruses, intra- and extra-cellular parasites, and ectoparasites (Male et al. 1998, Wakelin 1996). As a measure of innate immune function, which is considered to be the primary means of controlling bacterial infections (Campbell and Ellis 2007, Male et al. 1998), I analysed peripheral blood smears for estimated total and differential white blood cell counts (reviewed in Davis et al. 2008). Finally, I investigated potential differences in pathogen loads between inbred and hybrid individuals by collecting ectoparasites, and screening faecal samples for infection with species of *Salmonella* and *Campylobacter*.

6.2 Material and methods

6.2.1 Field work and study populations

The study was carried out on Allports and Motuara islands in the Marlborough Sounds, New Zealand, between 2008 and 2010. The South Island robin populations on both islands were founded with only five individuals each in 1973, have been isolated since and are showing signs of inbreed-

ing depression such as reduced egg hatching success and problems with immune system function (Hale and Briskie 2007*b*, Mackintosh and Briskie 2005). I refer to the descendants of the five original founders on each island as "inbred" individuals, even though they might not be the product of recent within-family matings. In 2008 and 2009, a total of 31 female robins were exchanged between Allports and Motuara islands (see chapter 4). The number of individuals chosen was selected on the basis of leaving a sufficient number of non-manipulated individuals on each island to act as controls. Only females were translocated in order to minimise disturbance to territorial boundaries that would occur if males were removed, and to ensure the formation of new "mixed" pairs. Newly formed pairs (translocated female with a male native to the island) and control pairs (both male and female from the same island) were followed and their nests monitored. The offspring of the mixed pairs (crosses between the two populations) will hereafter be referred to as "hybrids".

Hybrid and inbred control individuals were banded as nestlings and given a unique combination of one aluminium and three colour rings for identification in the field. A blood sample of 10–30 μl was collected from all nestlings by brachial venipuncture and stored in Queen's lysis buffer (0.01 M Tris-HCl, 0.01 M NaCl, 0.01 M Na-EDTA (pH 7.5), 1% (v/v) n-Lauroylsarcosine; pH 7.5; Seutin et al. 1991) until DNA extraction at a later date. I carried out paternity analyses to ensure the correct allocation of parentage in the presence of extra-pair paternity (and hence the assignment of individuals to the correct group, inbred or hybrid). A detailed description of the paternity analysis is given in chapter 5 (appendix C, p. 145). Surviving inbred and hybrid individuals were recaptured at one year of age and subjected to a number of tests and sampling procedures as detailed below in order to assess their immunocompetence and pathogen loads.

6.2.2 Phytohaemagglutinin assay

In March 2010, a total of 19 1-year-old South Island robins including 10 inbred and 9 hybrid birds were caught and their immune systems challenged using the PHA immune test (Smits et al. 1999). This assay consists of subcutaneously injecting an immunostimulant (the kidney bean lectin phytohaemagglutinin) into an individual's wing web (patagium) and measuring the inflammatory response in the form of the extent of the swelling at the injection site after a standardised interval, usually 24 h (Smits et al. 1999, Tella et al. 2002, but see Møller and Cassey 2004, Navarro et al. 2003). In response to PHA, T lymphocytes proliferate and stimulate the activation of macrophages, heterophils, B lymphocytes, and basophils (Stadecker et al. 1977, Tella et al. 2002). The dense infil-

tration of leucocytes in the postcapillary venules causes inflammation at the injection site (Stadecker et al. 1977) that can then be used as a general index of one aspect of cell-mediated immunity (Tella et al. 2002).

Prior to capture, the birds were fed several mealworms to ensure that they would not dehydrate during the holding period. A fresh PHA suspension was made each day by mixing 5 mg of PHA-P (Sigma, USA; Lot # L-8754) with 1 ml of pyrogen-free phosphate buffered saline (PBS) in a sterile 1.5 ml Eppendorf vial to ensure that the solution was not contaminated (5 mg is the amount needed for 20 birds). Patagium thickness was measured three times immediately prior to injection using a digital micrometer (Mitutoyo, 395-371, Tokyo, Japan). The micrometer was set back to zero and the wing closed in between measurements to ensure independence of measurements. The patagium was then sterilised with a cotton swab dipped in ethanol. A 50 μl aliquot of the 5 mg ml^{-1} PHA suspension was drawn into a sterile $27\frac{1}{2}$ gauge syringe. After ensuring there were no air bubbles in the suspension it was injected subcutaneously into the left patagium of each bird. The birds were then kept overnight for a period of 14 hours in individual holding cages fitted with a perch, and with *ad libitum* access to mealworms and water. Due to the required confinement of individuals over the experimental period, I chose to conduct the immune challenge in the post-breeding season, as adults could be retained in captivity without affecting breeding activities. Furthermore, I chose the 14 h overnight holding period in order to minimise the risk of interfering with territory loss caused by the prolonged absence of the territory holder, and taking into account the daylight hours at that time of the year. Navarro et al. (2003) and Møller and Cassey (2004) found no significant increase in the response to PHA after a period of 6 h, hence justifying the modification of Smits et al.'s (1999) original protocol by reducing the holding time and thereby the stress imposed on the birds (Hale and Briskie 2007*a*, but see Smits et al. 1999). After 14 hours, the measurements of patagium thickness at the injection site were repeated as performed prior to injection, and the birds were subsequently released into their respective territories. The cell-mediated immune response was calculated as the difference between mean pre- and post-injection measurements of patagium thickness. All injections and measurements were conducted by the same person (SH) to ensure consistency of the sampling method. To warrant as little measurement bias as possible, the experiment was conducted in a semi-blind fashion in that the birds were handled by an assistant and passed to me with a piece of cloth covering the colour identification bands and hence concealing the status of the bird (inbred or hybrid).

6.2.3 Blood smears

In the Southern Hemisphere spring (late August–early November) of 2009 and 2010, blood was collected from 54 individuals (18 hybrid and 36 inbred control birds) via brachial venipuncture. Blood smears were prepared from a drop of blood thinly smeared onto a glass slide. The blood smears were immediately air-dried, and at a later date fixed by immersion in 100% methanol. After fixation, smears were stained with May-Grünwald/Giemsa stain (Raymond A. Lamb, London, UK; Lucas and Jamroz 1961). Subsequently, the slides were mounted using Eukitt mounting medium (EMS, Hatfield, USA) and no. 0 cover slips.

Due to technical difficulties of blood smear preparation and storage in the field, and problems with the staining technique, 32 of the prepared smears were not suitable for interpretation. The remaining 22 smears (15 inbred and 7 hybrid samples) were included in the analysis. For each slide, between 10 and 41 fields of view in the monolayer (single layer of cells) were analysed per smear to assess: (1) total estimated white blood cell (WBC) count and (2) differential WBC count. All WBC counts were conducted by the same person (Eloise Jillings) without knowledge of the identity of the bird.

The total estimated WBC count ($x10^9$/l) was calculated from the average number of leucocytes per high power field. Avian blood is comprised of five types of WBC (aka leucocytes), namely lymphocytes, heterophils, monocytes, eosinophils, and basophils (Campbell and Ellis 2007, Davis et al. 2008). The differential WBC count was obtained by screening the blood smear under oil immersion at 1,000x magnification and determining the relative frequency of each leucocyte type for a total of at least 100 leucocytes. From the WBC differential, the heterophil to lymphocyte ratio (hereafter H:L ratio) was calculated.

6.2.4 *Salmonella* and *Campylobacter* screen

In order to collect faecal samples for the purpose of screening individuals for infection with *Salmonella* and *Campylobacter*, the holding cages used during the PHA immune challenge were lined with baking paper. All faecal matter excreted during the 14 h holding period could thereby be collected. Faecal samples were stored in 95% ethanol, and DNA subsequently extracted using the UltraClean® Fecal DNA Isolation Kit (MoBio, USA) and then purified using the DNA Clean & Concentrator™-5 Kit (Zymo Research, USA).

I tested for *Salmonella* infection following the protocol of Pathmanathan et al. (2003). The primers used in the polymerase chain reaction (PCR) to obtain a 784 bp product were HilA2 Forward 5'-CTG CCG CAG TGT TAA CCA TA-3' and HilA2 Reverse 5'-CTG TCG CCT TAA TCG CAT GT-3', targeting the hilA gene of *Salmonella typhimurium*. Amplification was carried out in a total volume of 20 μl containing 1 μl of primers, 10 μl of KAPA Blood PCR Mix B (2x; Kapa Bioscience, USA), 7 μl molecular grade water, and 4 μl DNA template. For each row on the gel, one negative control containing the same reaction mixture except the DNA and one positive control were included.

Amplification was performed using a program of initial denaturation of 4 min at $94\,^{\circ}\mathrm{C}$, followed by 30 cycles of denaturation at $94\,^{\circ}\mathrm{C}$ for 30 s, annealing at $65\,^{\circ}\mathrm{C}$ for 30 s, and extension at $72\,^{\circ}\mathrm{C}$ for 1 min. Finally, an additional extension was achieved for 5 min at $72\,^{\circ}\mathrm{C}$. The PCR product was electrophoresed on a 1% agarose gel stained with CYBR SAFE DNA stain for 30 min at 120 V, and visualised and photographed under UV illumination using a GelDoc imaging system using GeneSnap software vs. 6.05 (Syngene, Synoptics Ltd.).

The primers used for the *Campylobacter* screen were Therm 1M Forward 5'-AAA TTG GTT AAT ATT CCA ATA CCA ACA TTA G-3' and Therm 2M Reverse 5'-GGT TTA CGG TAC GGG CAA CAT TAG-3' for the detection of thermotolerant *Campylobacter* species, LpxA Forward 5'-CCG AGC TTA AAG CTA TGA TAG TGG AT-3' and LpxA Reverse 5'-TCT ACT ACA ACA TCG TCA CCA AGT TGT-3' for the detection of *C. jejuni*, and CeuE Forward 5'-CAT GCC CTA AGA CTT AAC GAT AAA GTT-3' and CeuE Reverse 5'-GAT TCT AAG CCA TTG CCA CTT GCT AG-3' for the detection of *C. coli*. PCR amplifications were performed in a total volume of 22 μl using 10 μl KAPA Blood PCR Mix B (2x; Kapa Bioscience, USA), 7 μl molecular grade water, 1 μl of primer mix (containing LpxA forward and reverse, Therm 1M forward and Therm 2M reverse, CeuE forward and reverse) and 4 μl of DNA template.

Thermal cycling conditions for the Eppendorf Mastercycler epGradient S (Eppendorf, Hamburg, Germany) were an initial denaturing cycle at $94\,^{\circ}\mathrm{C}$ for 3 min, followed by 40 cycles of denaturation at $94\,^{\circ}\mathrm{C}$ for 30 s, $60\,^{\circ}\mathrm{C}$ annealing for 30 s, $72\,^{\circ}\mathrm{C}$ extension for 1 min, with a final 5 min extension step at $72\,^{\circ}\mathrm{C}$. The PCR products were visualised by electrophoresis on a 1.5% agarose gel for 40 min at 120 V (Savill et al. 2001). For each row on the gel, two positive controls (1 μl of either *C. jejuni* or *C. coli*, 30 ng of DNA each) and two negative controls (replacing the DNA template in the reaction mixture with 4 μl of molecular biology grade water) were included. The bands were

captured and processed in a Chemi-Genius Bio Imaging System (Syngene, Cambridge, UK) and analysed using GeneSnap software vs. 6.05 (Syngene, Synoptics Ltd.).

6.2.5 Ectoparasite loads

Ectoparasites were collected by dust-ruffling the birds with flea powder (Vitapet Dog Flea Powder, 19.5 g/kg Permethrin, Vitapet Corporation, Lower Hutt, NZ; Walther and Clayton 1997). A total of 10 inbred control and 9 hybrid birds were sampled in March 2010. Upon capture, each bird was placed in a paper bag lining a cotton holding bag, and held until ready to be processed. The number of hippoboscid flies (*Ornithomya* spp. [Latreille, 1802] and *Ornithoica* spp. [Rondani, 1878]) flying off each bird was counted.

On removal from the bag, the bird was held over a large funnel with a 35 ml vial attached to its mouthpiece with adhesive tape. The bird was then dusted with sufficient flea powder to cover the plumage, whilst being held over the funnel. The flea powder was thoroughly distributed throughout the bird's feathers by hand (wearing latex gloves), and the plumage 'ruffled' for a period of 2 min to dislodge the parasites. The bird was then returned to the paper bag for a period of 3 min to allow the insecticide to take effect, and after 3 min the ruffling process was repeated for another 2 min before releasing the bird. The content of the paper bag was emptied into the funnel, and the funnel contents were flushed into the vial using 70% ethanol. To avoid contamination, a new paper bag was used for each bird, and the funnel and latex gloves were swabbed with alcohol. All birds were dust-ruffled by the same person (SH) to ensure consistency of the sampling method. The samples were sent to the Museum of New Zealand Te Papa Tongarewa, Wellington, for identification of the ectoparasites to genus or species level.

6.2.6 Statistical analyses

All analyses were carried out within the R statistical environment (ver. 2.13.2, R Development Core Team 2011), and data were analysed using $\alpha = 0.05$ as the significance level.

6.2.6.1 PHA immune test

The results of the PHA immune challenge were analysed using a one-way ANOVA. My model included the swelling (in mm) as a response variable, and "group" (inbred vs. hybrid) as predicting

variable. Due to my small sample size, I could not test for the effect of sex, island, and body mass. However, my sample was relatively well balanced in that each group contained a similar number of individuals from each sex and island (see table 6.1), and the individuals in each group were evenly distributed over different weight classes (inbred: mean ± SD = 38.4 g ± 3.1 g, range = 34.8 g – 44.8 g; hybrid: mean ± SD = 40.2 g ± 2.6 g, range = 36.3 g – 43.8 g), suggesting that any observed pattern was unlikely to be caused by a skewed sample. The effect size and 95% confidence interval (CI) were computed using the *Tukey HSD* function. I calculated the intra-class correlation coefficient (r_i) as a measure of within-individual repeatability of the three patagium measurements (pre- and post-injection) using the function implemented in the "ICC" package (Lessells and Boag 1987) of the R statistical environment (R Development Core Team 2011).

Table 6.1: Number of South Island robins sampled for each variable (sex, island and year) within each group (treatment: hybrid, control: inbred) per test (PHA immune challenge, WBC counts, and ectoparasite loads). P_{sex} indicates the p-value when sex was tested as the only predicting variable. PHA = phytohaemagglutinin; WBC = white blood cells.

Test	Variable	Level	Hybrid	Inbred	p_{sex}
PHA immune challenge	Sex	Female	3	5	0.84
		Male	6	5	
	Island	Motuara	6	5	
		Allports	3	5	
WBC counts	Sex	Female	1	8	> 0.131[1]
		Male	6	7	
	Island	Motuara	5	9	
		Allports	2	6	
	Year	2009	4	5	
		2010	3	10	
Ectoparasite load	Sex	Female	3	5	0.11
		Male	6	5	
	Island	Motuara	6	5	
		Allports	3	5	

[1] $p > 0.13$ when testing the effect of sex as only predicting variable on absolute numbers of heterophils, lymphocytes, monocytes, eosinophils, or basophils, and the H:L ratio. However, there was a significant effect of sex on total WBC counts (p = 0.001). Due to the larger sample size for males, I therefore tested the effect of group (inbred/hybrid) on males only. The group effect remained non-significant (p = 0.58).

6.2.6.2 Blood smears

I fitted generalised linear mixed-effects models (GLMMs) with a Gaussian error structure using functions implemented in the "lme4" package (Bates and Maechler 2009) in R to analyse differences in haematological parameters between inbred and hybrid individuals. I used separate models to investigate differences in the total estimated WBC count, absolute numbers of heterophils, lymphocytes, monocytes, eosinophils, or basophils, and the H:L ratio. Because of my small sample size, "group" (inbred/hybrid) was the only fixed categorical predictor in these models. Due to the presence of siblings in my dataset, I also included "nest ID" as random effect to control for non-independence of individuals raised in the same nest. As the sample within the hybrid group was strongly skewed towards males (see table 6.1, p. 187), I separately tested for the effect of sex when included as only predicting variable in the analysis. P-values were obtained using the *pvals.fnc()* function in the languageR package (Baayen 2011). All models were checked for overdispersion. Models with a dispersion parameter of $\hat{c} > 2$ were re-fitted using Penalised Quasi-Likelihood within the *glmmPQL* function of the "MASS" package in R (Venables and Ripley 2002).

6.2.6.3 *Salmonella* and *Campylobacter* screen

None of the tested birds were infected with *Salmonella* species (all results were validated through the use of a positive control). I carried out a Fisher's exact test to examine whether there was an association between the probability of infection with *Campylobacter* sp. and the group the individual belonged to.

6.2.6.4 Ectoparasite loads

Differences in ectoparasite loads were analysed using multivariate analysis of variance (MANOVA) by combining parasite loads of the various species encountered on each bird in a multivariate response variable. As data on the relative proportion of mite species per sample were not available, I used the combined number of individuals for all mite species (except for the nest mites) in my analysis. Because of my small sample size, the MANOVA was then fitted with "group" (inbred/hybrid) as the only fixed categorical predictor. However, individuals from each sex and island were fairly evenly represented in my sample (table 6.1, p. 187). I then analysed whether there were any differences in the number of ectoparasite species encountered on inbred and hybrid individuals using a

one-way ANOVA. The model included only “group” as fixed predictor.

6.3 Results

6.3.1 PHA immune test

Within-individual repeatability (r_i) of the patagium measurements was 0.87 (95% CI = 0.81 to 0.94), and thus well within the range found in 35 ecological studies published between 1997 and 2001 where the PHA assay was used (range: 0.70 – 0.99; see Smits et al. 2001). I found a significant difference in the extent of the swelling of the patagium between inbred (mean $\pm$ s.e.: 0.012 $\pm$ 0.03 mm, n = 10) and hybrid individuals (mean $\pm$ s.e.: 0.099 $\pm$ 0.02 mm, n = 9; p = 0.006). Hybrid individuals consequently had a significantly stronger immune response compared to inbred individuals (effect size = -0.09, 95% CI = -0.15 to -0.03; figure 6.1).

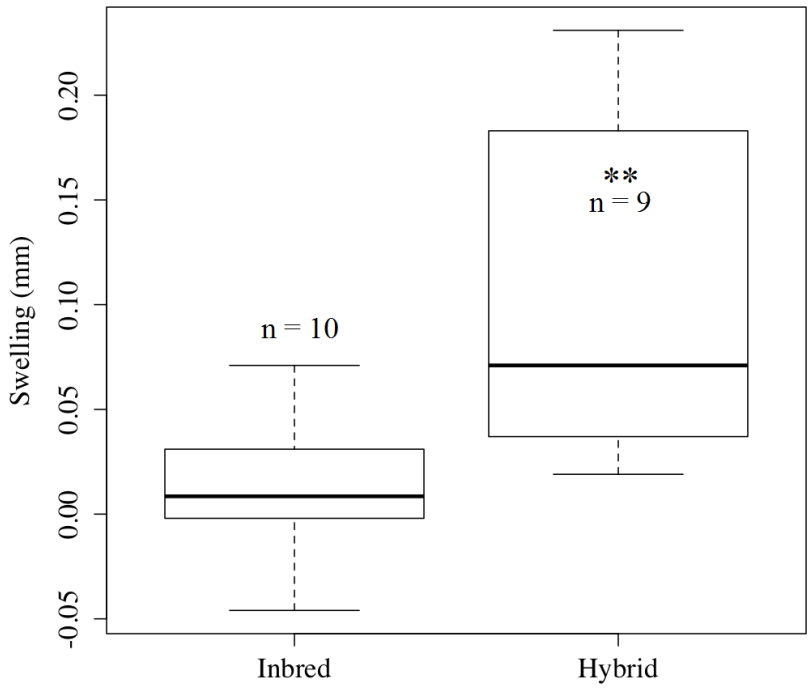

Figure 6.1: Comparison of the swelling of the wing web (mm) between 1-year-old inbred control and hybrid South Island robins following artificial immune challenge with phytohaemagglutinin (PHA); the lower and upper bars represent minimum and maximum values, respectively; the bottom and top of each box show the 25th and 75th percentiles, respectively; the thick black line indicates the median value; sample sizes (number of individuals) and significance are indicated above/within the box plots (** $p < 0.01$).

6.3.2 Blood smears

There was no significant difference between the total WBC count, differential WBC counts, or H:L ratio between 1-year-old inbred and hybrid individuals (table 6.2). Sex had no significant effect on absolute numbers of heterophils, lymphocytes, monocytes, eosinophils, basophils, or the H:L ratio (all $p > 0.13$). However, sex had a significant effect on total WBC counts ($p = 0.001$). Due to the comparatively larger sample size, I therefore tested the effect of "group" (inbred/hybrid) on males only. The group effect remained non-significant ($p = 0.58$) when including only males in the analysis.

Table 6.2: Haematological parameters estimated from blood smears of 1-year-old inbred (n = 15) and hybrid (n = 7) South Island robins. The measurement unit is given as the absolute value * 10^9/L (except for the H:L ratio). P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that the hybrid individuals do not differ from the inbred intercept). CI = confidence interval; WBC = white blood cell; H:L = heterophil/lymphocyte ratio.

Haematological parameter	Group	Estimate	Lower 95% CI	Upper 95% CI	p-value
Total estimated WBC count	Inbred	16.70	14.23	19.17	
	Hybrid	16.93	12.43	21.43	0.92
Heterophil count	Inbred	1.90	1.00	2.80	
	Hybrid	2.69	1.06	4.32	0.35
Lymphocyte count	Inbred	10.88	9.02	12.73	
	Hybrid	10.31	6.96	13.65	0.74
Monocyte count	Inbred	1.58	1.09	2.07	
	Hybrid	1.26	0.39	2.13	0.49
Eosinophil count	Inbred	1.70	1.19	2.20	
	Hybrid	2.05	1.16	2.94	0.45
Basophil count	Inbred	0.54	0.31	0.76	
	Hybrid	0.59	0.18	0.99	0.81
H:L ratio	Inbred	0.25	0.10	0.41	
	Hybrid	0.27	0.00	0.54	0.90

6.3.3 *Campylobacter* screen

Two of 9 hybrid individuals (22.2%), and two of 10 inbred individuals (20.0%) were infected with species of *Campylobacter*. Three of those birds were infected with *C. lari/upsaliensis*, while one of the hybrid individuals was infected with *C. jejuni*. I found no evidence for an association between the probability of infection with *Campylobacter* sp. and the group the individual belonged to (odds ratio = 1.13, 95% CI = 0.07 to 19.69; p = 1.0).

6.3.4 Ectoparasite loads

Using my sampling technique, I was able to collect seven different ectoparasite species from the plumage of my study birds. These included two species of hippoboscid flies (*Ornithomya* sp. [Latreille, 1802] and *Ornithoica* sp. [Rondani, 1878], Family Hippoboscidae), one species of chewing louse (*Menacanthus eurysternus* [Burmeister, 1838], Family Menoponidae), two species of feather mite (*Pedanodectes* sp. [Park and Atyeo, 1971], Family Proctophyllodidae, and the quill mite *Paralges* sp. [Mégnin and Trouessart, 1884], Family Dermoglyphidae), one species of free-living, stored-products mite (*Tyroborus lini* [Oudemans, 1924], Family Acaridae), and one species of blood-sucking nest mite (*Ornithonyssus bursa* [Berlese, 1888], Family Macronyssidae). Voucher specimens of the above species have been deposited at the Museum of New Zealand Te Papa Tongarewa, Wellington, New Zealand.

Table 6.3: Mean, standard deviation (SD), and range of ectoparasite counts in inbred (n = 10) and hybrid (n = 9) South Island robins on Allports and Motuara islands in March 2010. P-values test a null hypothesis that the parameter estimate does not differ significantly from zero (i.e., that hybrid individuals do not differ from the inbred intercept in their respective ectoparasite load).

	Inbred			Hybrid			
Species	Mean	SD	Range	Mean	SD	Range	p-value
Ornithomya sp.	2.2	0.9	1 - 4	1.6	1.0	0 - 3	0.16
Ornithoica sp.	1.3	2.2	0 - 7	0.2	0.4	0 - 1	0.17
Menacanthus eurysternus	1.3	4.1	0 - 13	0.0	0.0	0	0.36
Mites[1]	27.5	24.7	1 - 64	22.7	25.0	0 - 70	0.68
Ornithonyssus bursa	0.6	1.1	0 - 3	0.9	1.7	0 - 5	0.66

[1]includes *Pedanodectes* sp., *Paralges* sp., and *Tyroborus lini*

The multivariate analysis revealed no difference in ectoparasite loads between inbred and hybrid individuals ($p > 0.16$ for all ectoparasite species; table 6.3). On average, 2.3 ectoparasite species were encountered on hybrid individuals, compared with 2.9 species on inbred individuals. However, this difference was not significant ($p = 0.14$).

6.4 Discussion

As a result of the reciprocal translocation between two isolated and inbred populations of the South Island robin *Petroica australis*, I found a significant increase in one aspect of cell-mediated immunity as indicated by the eightfold stronger immune response to a novel mitogen in hybrids between the two populations. This finding is consistent with the reversal of an expected decline in host immunity with inbreeding (O'Brien and Evermann 1988). A negative correlation between PHA response and inbreeding coefficient has indeed been found in an inbred population of song sparrows *Melospiza melodia* on Mandarte Island, British Columbia, Canada (Reid et al. 2003). The relationship between the degree of inbreeding and aspects of immune system strength is thought to be mediated through reduced genetic diversity at MHC loci in inbred individuals (Potts and Wakeland 1990, Carrington et al. 1999). Genotyping at 28 microsatellite loci revealed that hybrid individuals in my study had significantly increased levels of both heterozygosity and allelic richness (see chapter 4), potentially reflecting increased genetic diversity at MHC loci. This in turn could explain the stronger response to a novel antigen in the genetically more diverse hybrids (see also Owen et al. 2008, 2009, Westerdahl 2007).

It has been shown previously that the strength of responsiveness to the PHA immune challenge is a reliable predictor of both survival (Møller and Saino 2004) and reintroduction success (Møller and Cassey 2004) across a number of bird species, indicating that increased levels of this measure of cell-mediated immunity may indeed reflect general resilience and hence population viability (González et al. 1999). It is unlikely that environmental differences, such as temporally and spatially localised and current infections, were underlying the differences observed between inbred and hybrid individuals in this study, as all individuals were caught within one week on two adjacent islands (Allports and Motuara islands in the Queen Charlotte Sound, Marlborough Sounds). Moderate changes in immunological parameters may have no effect on disease resistance (Keil et al. 2001). However, since I measured a more than eightfold increase in immune response in hybrid in-

dividuals compared with inbred ones, I am inclined to assume that this is an indication of increased immune fitness.

It has been suggested that the compromising effects of population bottlenecks on immune system function may only be of transient nature (Tompkins 2007). However, a study conducted on the South Island robin population on Motuara Island in 2005—32 years (8 generations) after the bottleneck event—found that a significant reduction in immune system response persisted in the bottlenecked population when compared to the source population (Hale and Briskie 2007*b*). My study, conducted five years after Hale and Briskie's (2007*b*) study (thus $\sim$9 generations following the bottleneck event), confirms the continued persistence of reduced immune system function in inbred individuals. In fact, the immune response of inbred individuals measured in autumn 2010 was a fraction of that measured in the same season in 2005 (see figure 3 in Hale and Briskie 2007*b*), possibly indicating a further reduction in immune responsiveness with time (although the time difference between the two studies is only approximately one robin generation). It is worth noting that, even though hybrid individuals in my study exhibited an eight times stronger immune response compared with inbred individuals, the absolute swelling of the wing web was only a fraction of that found by Hale and Briskie (2007*b*). As I only used 1-year-old individuals in my study, it is possible that age was a confounding factor in this case. The fact that Hale and Briskie (2007*b*) measured immune responsiveness after 6 h, whereas I used a period of 14 h, further complicates the direct comparison of the results between the two studies, although the general trend shows a similar pattern in that in both studies, relatively more inbred individuals had a weaker immune response.

I found no differences in total estimated WBC count, differential WBC counts, or H:L ratio between inbred and hybrid individuals. The H:L ratio was used as a measure of stress response to compare between inbred and hybrid birds, as it has been shown to be influenced by diseases and infections (Davis et al. 2008) and to be related to survival probability (Kilgas et al. 2006, Møller and Saino 2004), whilst not being immediately affected by routine handling time of the birds (Davis 2005, Campbell and Ellis 2007). It is important to note that 'normal' reference parameters for the leucocyte profile of South Island robins have not been published, and I am therefore currently unable to determine if the leucocyte differentials obtained from my blood smears differ from what normally occurs in this species.

Evidence for a trade-off between investments in different immune system components has been reported in several studies (Gross et al. 1980, Lindström et al. 2004, Mallon et al. 2003). Cheng

et al. (1991, cited in Tella et al. 2002) found negative correlations between the strength of the cell-mediated immune response and other measures of immune system function, suggesting that the complex reactions implicated in the defence against pathogens might not be adequately assessed by one single immune assay (Tella et al. 2002). If selection pressure generated by prevailing pathogens can influence investment in one component of the immune system to the detriment of other components, single measures of immune system strength should not be interpreted as demonstrating overall resilience to pathogens (Boa-Amponsem et al. 1999, Goüy de Bellocq et al. 2007, Norris and Evans 2000, Westneat and Birkhead 1998, Zuk and Johnsen 1998, Zuk and Stoehr 2002). At the same time, a non-significant result when assessing only a single immune system component should be interpreted cautiously, as it does not necessarily mean that no change in disease resistance occurred (Norris and Evans 2000). Significant changes in any component of the immune system, such as the marked difference between inbred and hybrid individuals in cell-mediated immune response found in this study, should, however, be a reliable indicator for the effect of a treatment (Norris and Evans 2000).

Relatively more inbred individuals are expected to be more susceptible to infection when exposed to relevant parasites (Male et al. 1998). However, I found no difference in pathogen loads (measured in terms of *Salmonella* and *Campylobacter* infections and ectoparasite loads) between inbred and hybrid individuals, although sample sizes were small and differences would have to be large to be detected. I classified the two species of feather mites (*Pedanodectes* sp. and *Paralges* sp.) found in this study as ectoparasites, although their effect on host viability is potentially negligible. In fact, only some species of avian mites, such as astigmatid mites (suborder Astigmata, order Acariformes) are true parasites (Proctor and Owens 2000). There is evidence that feather mites live as commensals on their hosts, thus presenting neither costs nor benefits to the host (Blanco et al. 2001, Proctor and Owens 2000). Nevertheless, some studies found a link between high mite loads and a reduction of body condition or plumage coloration in birds (Colloff et al. 1997, Figuerola et al. 2003, Harper 1999, Thompson et al. 1997). This suggests that mites could potentially have a detrimental effect on their hosts when present in high numbers, that high feather mite loads are associated with the occurrence of other more virulent pathogens (Lindström et al. 2004), or that the mites themselves might be infected with hyperparasites (e.g. Skoracki et al. 2006). The mite numbers dislocated through my sampling technique were generally very low (mean $\pm$ SD = 25 $\pm$ 24, range: 0 – 70; see also table 6.3 on p. 191), and it is therefore unlikely that they would have a large negative impact on their hosts. Notwithstanding, my estimates of feather mite load might not

represent true mite load: in order to dislocate every feather mite one would have to destructively sample the whole bird and its feathers, as quill mites in particular reside inside the shaft of the feather (Dabert and Mironov 1999).

Ornithonyssine mites, such as the species of nest mite I identified in this study (*Ornithonyssus bursa*), on the other hand, are truly parasitic as they feed on the host's blood and can therefore be vectors for diseases (Proctor and Owens 2000). However, as they do not stay on the host for prolonged periods of time, but only feed for a brief while and then return to the nest to digest their meal, their numbers on a bird are not indicative of overall parasite load (A. C. G. Heath, pers. comm.). To get a rough assessment of nest mite numbers, I would have had to destructively sample nests. It is also possible that the lack of a difference in parasite load between inbred and hybrid individuals is caused by the small number of birds sampled, thus reflecting insufficient statistical power. A further possibility could be a potential build-up of parasite loads over time, so that larger differences might only become apparent as birds age.

Infections with certain pathogens can reduce host survival (Martínez-de la Puente et al. 2010, Sol et al. 2003). A number of studies have found evidence for a positive correlation between immunocompetence and survival (e.g. Christe et al. 1998, Nordling et al. 1998, Saino et al. 1997, but see Sheldon and Verhulst 1996), and general host fitness (Moret and Schmid-Hempel 2000, Schmid-Hempel 2003), and a negative correlation between immunocompetence and pathogen load (Christe et al. 2000, González et al. 1999, Navarro et al. 2003). My findings of reduced survival (see chapter 5) and a weaker ability to mount an immune defence in inbred individuals potentially represent one mechanism of how inbreeding depression might affect viability, even if there was no apparent association of infection with the parasites measured in this study and immune system strength.

Any potential benefits resulting from the translocation of individuals into inbred populations to the immunocompetence of the resulting hybrid offspring, however, need to be weighed against the risk of introducing new pathogens into the recipient population (Cleaveland et al. 2002, Cunningham 1996, Griffith et al. 1993, Woodford 1993). Many translocation schemes, both in New Zealand and around the world, do not consider diseases and few involve thorough health checks of individuals by experienced biologists or veterinarians (Griffith et al. 1993, but see Hedrick and Fredrickson 2010). Prevention of the introduction of disease, however, is of utmost importance and will require thorough disease screening prior to any movement of wildlife.

In conclusion, although the marked difference between inbred and hybrid individuals in one as-

pect of avian immunity (cell-mediated immune response) might not reflect overall superiority of the immune system *per se*, it could be interpreted as a change in resistance to at least some pathogens. It is possible that the lack of difference in disease and parasite loads between inbred and hybrid individuals in this study is partially due to the fact that I did not measure the pathogen to which individual resistance changed, as opposed to there not being a change in resistance. Another possibility is that the reduced cell-mediated immune response in inbred individuals could be overcome by other compensatory mechanisms of the complex avian immune system. My results are thus at least partially in concordance with other studies that have suggested that immigration may increase an inbred population's ability to respond to a novel immune challenge (Reid et al. 2003), and reaffirm the possible value of translocations between different inbred populations of endangered species as a tool to mitigate the negative effects of inbreeding (Weeks et al. 2011).

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Chapter 7

General Discussion

Many wild animal and plant species around the world have gone through, or are currently going through, population bottlenecks. This raises concerns about the effects of increased levels of inbreeding and the loss of genetic variation on population viability and extinction probability (Brook et al. 2005, Frankham et al. 2010, Keller and Waller 2002, Moritz 1999, O'Grady et al. 2006). Although the relevance of inbreeding depression to wild populations has long been debated (Caro and Laurenson 1994, Caughley 1994, Pusey and Wolf 1996), ample evidence for its contribution to extinction risk has accumulated over the past decades (Frankham 2003, 2005, Keller and Waller 2002, and references therein), and its detrimental effects are no longer disputed. Despite earlier suggestions that bottlenecks might even prove beneficial through "purging" of deleterious alleles (see Byers and Waller 1999, and references therein), whatever advantage this may bring in some situations seems to be countered by even greater negative effects of inbreeding and loss of genetic diversity (Frankham et al. 2001, Jamieson et al. 2006, Keller and Waller 2002).

In the first data chapter of my thesis (chapter 2), I investigated whether the negative effects of inbreeding increased with the severity of the bottleneck through which a species or a population passed. For this purpose, I conducted a comparative analysis across 51 bird species worldwide, using egg hatching failure rates as the fitness measure of interest, as increases in the levels of hatching failure are a common result of inbreeding in birds (Bensch et al. 1994, Spottiswoode and Møller 2004). The main conclusion of this work is that there is a strong and significant inverse relationship between population bottleneck size and level of hatching failure among endangered bird species. In other words, those species that have passed through severe bottlenecks show levels of hatching fail-

ure several times higher than that seen in less endangered species. This pattern was evident across both continental and island species of birds worldwide and remained significant when controlling for potentially confounding variables such as body mass, clutch size, time since bottleneck, latitude and phylogeny. Furthermore, the results of my analysis allowed estimating the minimum population size needed to avoid increased hatching failure in birds (roughly about 100–150 individuals). In addition to confirming that the negative effects of inbreeding increase with population bottleneck size, the results of this chapter also provide added impetus for conservation biologists to ensure endangered species do not pass through severe bottlenecks. Whether other fitness traits are affected in a similar fashion remains to be explored and it may turn out that these may be even more sensitive to small bottleneck size (i.e., other traits may be affected by bottleneck sizes of a higher magnitude).

Many species and populations, however, have already declined below this limit of 100–150 individuals, and urgent action is therefore required to ensure their long-term viability. The remainder of my thesis therefore focused on the experimental use of translocations as a technique to mitigate the negative effects of inbreeding and restore levels of genetic variation once a species or population has passed through a bottleneck. In the past, two approaches have been used with some success: (1) the introduction of outbred individuals into inbred populations, and (2) the augmentation of inbred populations through the release of captive-reared individuals. However, both approaches have severe limitations. Firstly, in many threatened species, there are no outbred populations left to use as a source for introducing new individuals into inbred populations. For example, the black stilt *Himantopus novaezelandiae* survived as only a single population that passed through a bottleneck of just 23 birds and there are no other surviving populations that can be used as a source for donor birds (Reed and Murray 1993). Secondly, captive populations may not be available, and if they are, captive individuals may also be inbred and perhaps less likely to survive in free-living conditions. As many endangered species only survive as a series of small and inbred populations and do not have outbred individuals available for translocation into inbred populations, I was interested to test the value of using highly bottlenecked populations as donors. A few studies of wild populations have found positive effects of augmenting inbred populations with inbred individuals (Fredrickson et al. 2007, Wisely et al. 2008). Nonetheless, these studies involve mixing wild and captive individuals and differences in environmental conditions, and lack replicate crosses. In order to test the general effectiveness of using inbred individuals as donors, I therefore conducted experimental crossings between two inbred populations using a replicated approach (in the laboratory) and a systematic approach (in the wild).

In the laboratory experiment, I crossed individuals within and between two artificially inbred strains of the fruit fly *Drosophila melanogaster* and investigated changes in the viability of the resulting within- and between-strain hybrids compared to inbred control individuals (chapter 3). I found that crossing individuals between the two bottlenecked strains reversed the effects of inbreeding and led to increases in overall breeding success and survival that persisted into the second generation of hybrid offspring. In contrast, crosses within each strain (but between different replicate lines) resulted in only slight improvements in some fitness components, and this positive trend was reversed in the second generation. This experiment confirmed the potential value of translocations between different inbred populations of endangered species as a tool to mitigate the negative effects of inbreeding, but this benefit may depend upon the origin of the populations. If genetic diversity contributes to such a rescue effect, this study suggests that even greater value should be placed on inbred populations that could act as donors to other inbred populations of differing origin.

As the impact of hybridisation on fitness also depends on environmental effects, I repeated the above experiment by conducting reciprocal translocations between two severely bottlenecked and isolated, wild populations of the South Island robin *Petroica australis*. I found significant differences in mean levels of homozygosity by locus (HL) between inbred and hybrid birds on both islands, with hybrids (crosses between the two populations) exhibiting higher levels of heterozygosity (chapter 4). Similarly, average expected heterozygosity, allelic richness and the frequency of rare alleles all increased significantly in both populations within the first year after the translocation. The significant increase in genetic diversity was accompanied by increases in overall levels of fitness. Hybrid birds experienced increased levels of both survival and recruitment into the breeding population, and sperm quality improved significantly in hybrid males compared with inbred males (chapter 5). Finally, the eightfold stronger immune response to the mitogen phytohaemagglutinin (PHA) in hybrid individuals indicated a significant increase in one aspect of cell-mediated immunity (chapter 6). Although I found no clear evidence of an effect of the translocation on breeding success, measures of parental care, or body mass, the experimental translocation demonstrated the value of using previously bottlenecked populations as donors to alleviate at least some of the other fitness measures of inbreeding depression.

Both field and laboratory experiments yielded similar results and confirmed the value of inbred populations as donors for genetic rescue programs. As already found in the *Drosophila* experiment, the most important fitness improvement in hybrid robins was survival probability. This is in agree-

ment with a number of other studies that found that fitness differences between inbred and outbred populations are primarily due to survivorship differences (e.g. Molina-Freaner and Jain 1993, Pimm et al. 2006). Even though I found no clear evidence for a difference in reproductive success between inbred and hybrid birds on a per breeding attempt basis, increased survival can potentially increase lifetime reproductive success by providing individuals with increased opportunities to reproduce.

The observed decrease in heterozygosity in the first three generations of hybrid robins (chapter 4) did not seem to be associated with a corresponding decrease in fitness traits, as indicated by similar trait values in hybrid F2 individuals compared to first-generation hybrids (chapter 5). Nevertheless, it would be valuable to monitor these two robin populations over the long-term to assess the effects of the translocation at regular intervals many generations down the path. A few studies found that the benefits of gene flow into an inbred population were only short-lived (Adams et al. 2011, Bensch et al. 2006, Hagenblad et al. 2009), leading to the conclusion that a singular reciprocal translocation between inbred populations cannot be used to halt the erosion of genetic diversity. This is especially true in spatially confined populations, such as island populations (see also Adams et al. 2011). Similar to populations that are maintained at a large size, populations that experience growth are less likely to lose genetic diversity than small populations, as the effects of genetic drift and inbreeding are less pronounced (Gilpin and Soulé 1986). The benefits of genetic rescue might therefore be more long-lived in populations that live in environmental conditions that permit population expansion (e.g. Bouzat et al. 2009, Johnson et al. 2010, Madsen et al. 2004). A spatially confined population, however, has no possibility to expand beyond carrying capacity, and will thus inevitably lose genetic variation and accumulate inbreeding (Jamieson 2010). This is of particular importance for translocation schemes that involve translocations of endangered species to islands where introduced predators have been eradicated, as it is often the case in New Zealand (Armstrong and McLean 1995). Nonetheless, even if the benefit of outcrossing and heterosis declines over the generations, the temporary increase in fitness could prove crucial for small populations in the longer term by reducing extinction probability from demographic stochasticity. My findings suggest that reciprocal translocations between inbred populations can certainly be used to decelerate the process of genetic erosion, and could thus be a valuable short-term measure to prevent a species from becoming extinct (see also Frankham et al. 2011, Hedrick and Fredrickson 2010, Weeks et al. 2011). According to this view, the periodical introduction of new genetic stock should be part of a wider management scheme of island populations including other measures such as habitat restoration and predator control. Additionally, long-term population viability requires

comprehensive genetic restoration, not just the temporary benefits of genetic rescue (Bouzat et al. 2009, Hedrick and Fredrickson 2010, Weeks et al. 2011).

The main concern regarding the augmentation of inbred populations (whether using the traditional approach of sourcing individuals from outbred populations, or using inbred donors as in this thesis) is the potential for outbreeding depression to occur (Goldberg et al. 2005, Templeton 1986), and harmful effects associated with the disruption of locally adapted gene complexes have indeed been documented as the result of translocations (Edmands 2007, Frankham et al. 2010, 2011, Tallmon et al. 2004). However, in many cases the benefits of a translocation are likely to outweigh any negative effects of outbreeding (e.g. Willi et al. 2007). Guidelines are now available to predict the likelihood of outbreeding depression as a result of a translocation, which involves determining whether the species or populations used for the exchange of individuals have fixed chromosomal differences, have been isolated for more than 500 years, and inhabit different environments (Frankham et al. 2011). To avoid the potential problems of outbreeding depression, source populations that adaptively match the population of concern (e.g. adapted to similar environments) should be preferable for a translocation (Frankham et al. 2011, Hedrick and Fredrickson 2010, Weeks et al. 2011). The implementation of genetic rescue management should evidently only be undertaken if it is clear that the benefits outweigh the risks.

Another concern regarding the use of translocations as a tool in conservation management is the danger of introducing diseases (Cleaveland et al. 2002, Cunningham 1996, Griffith et al. 1993, Hogg et al. 2006, Woodford 1993). Small populations of threatened species are commonly the targets of such translocations, and, due to reduced genetic diversity and increased levels of inbreeding, it is precisely those populations that are most vulnerable to novel pathogens. There is a number of extinctions or near extinctions of endangered species that have been attributed to disease outbreaks, such as facial tumour disease in Tasmanian devils *Sarcophilus harrisii* (McCallum et al. 2009), Ebola virus in western gorilla *Gorilla gorilla* (Leroy et al. 2004), avian malaria and birdpox in Hawaiian land birds (van Riper et al. 1986, 2002), canine distemper virus in the black footed ferret *Mustela nigripes* (Thorne and Williams 1988), and rabies in African wild dogs *Lycaon pictus* (Vial et al. 2006) and Ethiopian wolves *Canis simensis* (Randall et al. 2006). Many translocation schemes, both in New Zealand and around the world, do not consider diseases and few involve thorough health checks of individuals by experienced biologists or veterinarians (Griffith et al. 1993, but see Hedrick and Fredrickson 2010, Trinkel et al. 2008). Prevention of the introduction of disease, however, is of

utmost importance and will require thorough disease screening prior to any movement of wildlife. This raises new practical issues in the capture and handling of animals for translocation as time may be needed for tests to be conducted and for animals to be held in quarantine before release—procedures that are often at odds with protocols to capture and release animals quickly to reduce stress.

The management of endangered species that only survive as a single, inbred population, such as the kakapo *Strigops habroptilus* and the black stilt *Himantopus novaezelandiae*, poses yet another challenge, as in these cases there are neither inbred nor outbred individuals of differing origin available for translocation. The hybridisation with a closely related species or subspecies could be a measure of last resort, and might prove successful in increasing population viability (e.g. Benson et al. 2011, Tompkins et al. 2006). However, this option again bears the risks of inducing outbreeding depression in the hybridised offspring, and controlled trials before any implementation in the wild would be necessary on a case-by-case basis, an option that might hardly be feasible in long-lived species. For some species, such as the kakapo, it is unlikely any other species could be used given its uniqueness and phylogenetic distance from other related species. For such species, the best strategy is to try to prevent bottlenecks and inbreeding in the first place.

In conclusion, although I was able to demonstrate that reciprocal translocations between different inbred populations can be an effective tool to increase fitness levels, the potential recovery and long-term viability of a species is not solely dependent on genetic factors, such as the loss of genetic diversity and inbreeding depression. One of the main limiting factors is the availability of suitable habitat (Wolf et al. 1998). Even if translocations can be used to increase levels of fitness, these improvements will only be temporary if the focal population remains in its isolated state, as isolated populations will inevitably be affected by genetic drift and accumulate inbreeding over the long-term (Adams et al. 2011, Bensch et al. 2006, Hagenblad et al. 2009, Jamieson 2010). It is essential to realise that genetic interventions in form of reciprocal translocations need to be complemented with other management strategies aimed at the restoration or conservation of suitable habitat. From a broader perspective, even comprehensive management strategies are only a means of treating a symptom, and not a solution to the ultimate problem: human overpopulation and destruction of natural habitats for human use (Parkes 1964, Sargent 2008). As long as this problem is not addressed, any conservation effort represents merely a race to compensate the detrimental effects of increasing anthropogenic impact.

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Figure 7.1: South Island robin *Petroica australis* male on Motuara Island