The role of inbreeding in the reproductive fitness of kakapo (*Strigops habroptilus*)

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Kaitlyn White

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Abstract

As populations decline, inbreeding becomes increasingly unavoidable. Increased genome-wide homozygosity for inbred individuals can result in reduced survival and reproductive fitness (*i.e.* inbreeding depression), via the expression of deleterious recessive alleles and reduced heterozygosity at over-dominant loci. The tendency for deleterious recessive alleles to drift to high frequencies in small populations means that inbreeding depression can be particularly severe for threatened populations. Therefore, the genetic consequences of small and isolated populations are becoming of increasing concern to conservation biologists.

The kakapo (*Strigops habroptilus*) is a critically endangered, flightless, nocturnal parrot that now survives only on predator-free island sanctuaries in New Zealand. The recent population bottleneck of 51 individuals, lek mating system and insular origin of all but one of the surviving kakapo, render them particularly susceptible to inbreeding depression. Low productivity of kakapo has been reported and potentially attributed to, an aging population, diet and inbreeding. For this reason, the present study investigated the relationship between inbreeding and reproductive fitness in kakapo.

The preferred method of assessing inbreeding is to use multi-generational pedigree information. However, this is currently unavailable for kakapo, therefore molecular estimates of relatedness were used as a surrogate for pedigree-derived inbreeding coefficients. Internal relatedness and pairwise relatedness were calculated using 25 polymorphic microsatellite loci. The link between relatedness estimates and variation in early life history traits was investigated using heterozygosity-fitness correlations. This was achieved through the use of generalised linear mixed modelling, with an information-theoretic approach and model averaging where necessary. The reproductive traits investigated were female fecundity (clutch size), egg fertility (probability of an egg being fertilised by an individual male), hatching success of fertile eggs (proportion of fertile eggs that a female hatches) and sperm quality (concentration, motility and morphology).

Neither variation in female fecundity nor egg fertility could be attributed to homozygosity with any confidence. Hatching success was determined to be strongly reduced for the more homozygous females and the more homozygous males were determined to have significantly higher proportions of abnormal sperm. Therefore, the present study demonstrated that inbreeding depression is a contributing factor towards reduced reproductive success in kakapo. These findings are consistent with known detrimental effects of inbreeding, in particular for New Zealand endemic species that have experienced severe

population bottlenecks. To minimise these effects, and hence reduce potential impacts on population growth and species recovery, kakapo managers should aim to prevent further erosion of genetic diversity and to breed from the descendants of the genetically-distinct Fiordland male Richard Henry. The present study highlights the importance of considering the genetic components of populations in any conservation management program.

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Chapter 1: General Introduction

1.1 General background

Worldwide, direct and indirect human impacts, such as habitat fragmentation, introductions of invasive species, overexploitation and climate change, are severely reducing population sizes (Caughley 1994; Kerr & Currie 1995). Small, fragmented or isolated populations have a greater extinction risk than larger populations due to increased susceptibility to the combined effects of demographic, environmental, catastrophic and genetic stochasticity (Frankham 1998; Frankham 2005). Conservation biologists are becoming increasingly aware of the importance of genetic stochasticity in these small populations (Hedrick & Kalinowski 2000; Frankham *et al.* 2002; Jamieson *et al.* 2006). As populations decline, genetic variation is lost through two major genetic components; genetic drift and inbreeding (Frankham *et al.* 2002; Briskie & Mackintosh 2004; Frankham 2005; Bouzat 2010). In small populations, random genetic drift is increased relative to selection, intensifying the potential for beneficial alleles to be lost and deleterious alleles to reach high frequencies (Lynch *et al.* 1995; Reed & Frankham 2003). Genetic drift can have long-term impacts on fitness through a population's inability to respond to changing selection pressures (Reed & Frankham 2003; Frankham 2005). Comparatively, inbreeding can have more immediate impacts that are capable of lowering individual survival and reproductive success, leading to reduced population growth (Coltman *et al.* 1999; Da Silva *et al.* 2006; Hanski & Saccheri 2006; Gage *et al*. 2006; Grueber *et al.* 2010). Therefore, understanding the consequences of inbreeding depression is an important component of conservation management.

1.2 Inbreeding depression

1.2.1 Definition

The detrimental effects that can result from matings between related individuals have been known for some time. Darwin first recognised such effects in 1868 after selectively breeding domesticated animal stocks and experimenting with self-fertilisation in plants (Darwin 1868). With the advent of Mendelian genetics in the nineteenth century, the genetic consequences of inbreeding began to be understood as a form of homozygosis (Wright 1977). Inbreeding depression can now be defined as the general decline in fitness for inbred individuals, relative to offspring from randomly mated individuals (Hedrick & Kalinowski 2000; Allendorf & Luikart 2007).

The decline in fitness associated with inbreeding stems from a genome-wide increase in homozygosity for offspring from genetically-similar parents, due to the inheritance of a higher proportion of alleles that are identical by descent (IBD) (Charlesworth & Charlesworth 1987; Hansson & Westerberg 2002). The decline in fitness related to increased homozygosity can be attributed to two main genetic mechanisms at the loci level. The first is the increased probability of deleterious recessive, or partially recessive, mutations becoming expressed (Charlesworth & Charlesworth 1987; Lynch *et al.* 1995). In a large outbred population, these deleterious mutations are maintained at low frequencies by the selection-mutation balance, and are partially masked by dominant alleles in heterozygous individuals (Charlesworth 1990; Kirkpatrick & Jarne 2000; Frankham 2005). Increased homozygosity for inbred individuals means they are more likely to express these deleterious recessive mutations, thereby suffering reduced fitness (Charlesworth & Charlesworth 1987). The second mechanism involves loci with overdominance, where greater fitness is attained by traits that are heterozygous, compared to either dominant or recessive homozygote loci. Inbred individuals suffer reduced fitness as a result of increased homozygosity at these loci with overdominance (Charlesworth & Charlesworth 1987, 1999; Hansson & Westerberg 2002; Coltman & Slate 2003). It is difficult to distinguish between these two mechanisms of fitness decline in inbred individuals, however the expression of deleterious recessive alleles has been reported to be the more common cause of inbreeding depression (Charlesworth & Charlesworth 1999).

1.2.2 Fitness impacts and magnitude of effects

Inbreeding depression can manifest as a number of detrimental impacts on a wide variety of traits (Charlesworth & Charlesworth 1987; Keller Waller 2002). It is more often detected in traits related to fitness rather than traits less tightly linked with fitness, such as morphological traits. The most common fitness consequence of inbreeding is a reduction in reproductive fitness (Keller & Waller 2002). Directional selection on reproductive traits, owing to direct fitness consequences, predisposes such traits to more severe impacts of inbreeding depression (DeRose & Roff 1999). As a result, reproductive traits such as fertility, embryogenesis, and early survival and growth rates are more commonly detected to be affected by inbreeding depression than phenotypic traits less tightly linked with fitness (Keller & Waller 2002; Charlesworth & Willis 2009). Other traits have also been detected to be reduced by inbreeding depression. For example, territory size and song structure were linked to levels of heterozygosity of male subdesert mesite (*Monias*

benschi) in Madagascar (Seddon *et al*. 2004). Higher levels of heterozygosity were also "preferred" by female Antarctic fur seals (*Arctocephalus gazella*) (Hoffman *et al.* 2007), suggesting that inbreeding levels can also affect mate choice.

In general, the strength of inbreeding depression can be viewed as a function of a population's genetic lineage and environment (Pray *et al.* 1994; Hedrick & Kalinowski 2000; Chapman *et al.* 2009). Bottleneck events, when a large population is very rapidly reduced to a small effective population size (N_e), can result in the effects of inbreeding depression being expressed within a few generations (Groombridge *et al.* 2000; Amos & Balmford 2001). For these bottlenecked populations, the greater the reduction in size, the more severe the magnitude of inbreeding, since the number of genetically dissimilar, individuals is reduced to a point where mating between related individuals becomes inevitable (Keller & Waller 2002; Briskie & Mackintosh 2004; Bouzat 2010). In addition, if population growth is slow following a bottleneck event, the effects of inbreeding depression should accumulate faster due to the greater effects of genetic drift (Kirkpatrick & Jarne 2000). Island populations present a good example of how inbreeding can affect populations differently. Endemic island species are often more inbred than non-endemic island species, as a reflection of their extended period of isolation since the time of their foundation (Frankham 1998). Furthermore, if the ancestral population or founding individuals had a large genetic load, inbreeding depression will be exacerbated due to the tendency for deleterious mutations to become fixed in smaller populations (Hedrick 1994).

The negative effects associated with inbreeding can also become apparent, or more prominent, under stressful and competitive environments (Pray *et al.* 1994; Coltman *et al.* 1999; Armbruster & Reed 2005). In a meta-analysis of 34 laboratory studies by Armbruster & Reed (2005), where inbreeding depression was assessed under various environmental conditions, inbreeding depression was found to be significantly greater under more stressful environments. Increased inbreeding depression under stressful environmental conditions has also been identified in wild populations (Coltman *et al.* 1999; Marr *et al.* 2006). An example can be found in a study by Coltman *et al.* (1999), where more homozygous Soay sheep (*Ovis aries*) carried larger parasitic worm burdens, contributing to reduced overwinter survival. Marr *et al.* (2006) also detected environmental dependence of inbreeding depression on the reduction of hatching success in Mandarte Island song sparrows (*Melospiza melodia*). As a result, detecting inbreeding depression may be dependent on the environmental conditions experienced by the population under study, and, detecting these effects may be difficult under benign environments.

Isolated populations may not display clear signs of inbreeding depression when deleterious mutations have become fixed in the population and individuals all experience the same fitness reductions (Lynch *et al*. 1995; Hedrick & Fredrickson 2010). It is not until progeny produced by crosses between populations display greater fitness than progeny from either population, known as heterosis, that the negative impacts of inbreeding become apparent (Keller & Waller 2002). Heterosis is more likely to occur when deleterious mutations are of small effect, since these alleles are less exposed to selection, and therefore, more likely to drift to fixation (Kirkpatrick & Jarne 2000; Keller & Waller 2002). Consequently, the magnitude of inbreeding depression can vary between species, populations, and even among lineages within a population.

1.3 Estimating relatedness

1.3.1 Pedigree estimates

In order to understand the impact of inbreeding on fitness, models relating the genetic composition of individuals to a fitness-related trait are necessary (Charlesworth & Charlesworth 1987). The first step in developing these models requires quantifying the degree of inbreeding in individuals. The level of inbreeding within an individual can be quantified using the expected increase in homozygosity, relative to that expected by Hardy-Weinberg proportions, termed the inbreeding coefficient (*f)* (Wright 1922). Calculating *f* requires knowledge of the relatedness between both parents, and is obtained using pedigree-derived genealogical relationships (Frankham *et al.* 2002; Taylor *et al.* 2010). Construction of accurate, complete and multi-generational pedigrees has been proven to be the most reliable measure of inbreeding depression (Pemberton 2008; Grueber *et al.* 2011a). In particular, the multi-generational records kept by captive breeding programs have enabled numerous studies of inbreeding depression (Lacy *et al*. 1993 and references therein).

Although captive populations have provided the basis of knowledge for inbreeding depression, highly selective breeding and standardised environmental conditions also limit extrapolation of conclusions to wild populations, since expression of deleterious alleles can be conditional to certain environmental influences (Pray *et al.* 1994). Immigration, extra-pair fertilisation and the logistical restraints of long-term population monitoring have also meant that comprehensive pedigrees are often not able to be constructed (Hansson

&Westerberg 2002; Pemberton 2008). Therefore, our understanding of the influence of inbreeding under natural conditions has previously been limited, especially when these populations are threatened.

1.3.2 Molecular estimates

The recent development of molecular markers has enabled indirect estimation of relatedness, using measures of homozygosity by descent (Frankham *et al*. 2002). Indexes of multi-locus heterozygosity are more frequently being used as estimates of pedigree-derived inbreeding coefficients (*f*) when pedigree information is unavailable (Coltman *et al.* 1999; Amos *et al.* 2001; Slate & Pemberton 2002 Aparicio *et al.* 2006). There are two main types of molecular methods currently used to estimate inbreeding (Frankham *et al*. 2002; Grueber *et al.* 2011a). The first uses shared neutral markers between individuals as an indication of shared ancestry. The second uses the expected increase in homozygosity for inbred individuals, relative to outbred individuals, to estimate inbreeding within an individual.

There are also two main types of estimators for measuring molecular relatedness between a pair of individuals (Wang 2002). The first is the maximum-likelihood that a pair falls under a certain relationship category, such as first and second-order relatives (*e.g.* Goodnight & Queller 1999; Thomas & Hill 2000). The second is a method of moment estimator, where estimates are on a continuous scale and are based on the probability of alleles being identical-by-descent (*e.g.* Queller & Goodnight 1989; Wang 2002). Because the maximum-likelihood approach can be biased for small sample sizes and often requires a very large numbers of markers (Lynch & Ritland 1999; Wang 2002; Milligan 2003), this category of estimators can be unreliable for threatened populations. Consequently, moment estimators seem most appropriate for detecting inbreeding depression in small populations.

There are a number of microsatellite-derived estimators of within individual heterozygosity, none of which are most suitable, but each performs well under different population genetic structures (Slate & Pemberton 2002; Aparicio *et al*. 2006). Initially, methods used for estimating individual relatedness with genetic markers were rudimentary, with the proportion of heterozygous loci used as a crude estimate of heterozygosity (Amos *et al.* 2001; Aparicio *et al.* 2006). This method was flawed when small numbers of microsatellite markers were used and the frequency of alleles at different loci varied (Aparicio *et al.* 2006). Multi-locus heterozygosity measures have recently been improved to measure heterozygosity by taking allele frequencies into account and weighing loci

accordingly (Amos *et al.* 2001; Aparicio *et al.* 2006). Currently, the two most used estimators are: internal relatedness (IR) (Amos *et al.* 2001), which weighs loci according to the frequency of alleles in the population, *e.g.* a homozygote sharing rare alleles is weighted higher than one sharing common alleles; and heterozygosity by loci (HL) (Aparicio *et al.* 2006), which weighs loci by their contribution to overall homozygosity, while taking into account the allelic variability at each locus.

1.4 Heterozygosity-fitness correlations

The link between inbreeding and fitness has rapidly gained recognition as an important component of evolutionary biology and conservation management (Frankham 1995; Hedrick & Kalinowski 2000; Jamieson *et al.* 2006). In order to quantify this link, multi-locus heterozygosity at selected molecular markers can be correlated with variation in fitness-related traits using a method termed heterozygosity-fitness correlations (HFCs) (Chapman *et al.* 2009). Due to the difficulty of examining the multiple genes involved in variation of quantitative fitness characters (*e.g.* reproductive fitness) selectively-neutral molecular markers are used to generate estimates of genome-wide variation in heterozygosity (Frankham *et al.* 2002; Pemberton 2004; Grueber *et al*. 2008). HFCs have become more commonly used by researchers to infer inbreeding depression, particularly for natural populations where obtaining pedigree information is logistically difficult (Hansson & Westerberg 2002; Balloux *et al.* 2004; Grueber *et al.* 2008; Chapman *et al.* 2009). Significant effects of inbreeding have been identified in a number of reproductive traits, such as reduced sperm quality (Gage *et al.* 2006), litter sizes (Liberg *et al.* 2005), hatching success (Brekke *et al.* 2010) and survival (Küpper *et al.* 2010). Relationships between outbreeding and fitness have also been detected with HFCs, for example in juvenile survival of Arabian oryx (*Oryx leucoryx*) (Marshall & Spalton 2000), but are less common (Chapman *et al.* 2009).

HFCs can be difficult to detect, and are often only weak effects (Coltman & Slate 2003). This is due to the limited ability of marker-based methods to measure inbreeding to a degree that rivals the power of a multi-generational pedigree (Balloux *et al.* 2004; Grueber *et al.* 2008). As a result, the genetic mechanisms behind HFCs have been debated (Hansson & Westerberg 2002; Balloux *et al.* 2004; Grueber *et al.* 2008; Chapman *et al.* 2009; Grueber *et al.* 2011a). In light of this, Hansson and Westerberg (2002) proposed three competing hypotheses to explain the potential mechanism behind HFCs. First, the

"direct effect hypothesis" proposes that heterozygote advantage is a product of functional overdominance of the markers under study; a hypothesis mostly applicable to functional markers such as allozymes. Second, the "local effect hypothesis" refers to apparent heterozygote advantage for the markers (typically neutral microsatellites) due to linkage disequilibrium with homozygous fitness loci. Third, the "general effects hypothesis", which supports the theory that lowered marker heterozygosity reflects a genome-wide increase in the frequency of homozygotes.

Further difficulties in detecting HFCs are encountered when inbreeding is severe and has occurred over an extended period of time. Under these circumstances, genetic diversity has been eroded to the point where it is difficult to distinguish a homozygous individual due to inbreeding from a homozygous individual due to random mating (Grueber *et al.* 2008). Continued research using HFCs will help to increase understanding of their ability to detect inbreeding depression (Balloux *et al*. 2004) and hence, provide a valuable new tool for conservation genetics.

1.5 Kakapo ecology and conservation status

The kakapo (*Strigops habroptilus*) is a critically endangered (IUCN 2010), flightless, nocturnal parrot, that is endemic to New Zealand (Powlesland *et al.* 2006). Breeding is synchronous with years of high rimu (*Dacrydium cupressinum*) fruit abundance, a phenomenon that occurs at two to seven year intervals (Merton *et al.* 1984; Powlesland *et al.* 1992; Harper *et al.* 2006). Kakapo are lek breeders (Merton *et al.* 1984) with males establishing and occupying "track and bowl systems" (TBS), during the months of January and February (Eason *et al.* 2006). From these arenas, males advertise to attract prospective mates by making a booming noise from an inflated thoracic air sac (Merton *et al.* 1984). Males are within audible, but not visual, proximity to other males in a spatial distribution that can be described as an "exploded lek" (Gillard 1963). After copulation, females assume sole responsibility for raising any subsequent offspring.

Kakapo were once abundant throughout the three main islands of New Zealand, however, population decline began as a result of the ecological changes that accompanied the Polynesian settlement of New Zealand approximately 650 years ago (Powlesland *et al.* 2006). These changes involved forest clearances, hunting for the kakapo's ornamental feathers and the first introduction of mammalian predators, namely the Pacific rat (Kiore, *Rattus exulans*) and the domestic dog (*Canis familiaris*). These impacts were accelerated

by the arrival of Europeans in the nineteenth century, who also brought with them a suite of mammalian predators, including three mustelid species (stoats, *Mustela ermine;* ferrets, *M. furo*; and weasels, *M. nivalis*), feral cats (*Felis catus*), and two more rat species (Norway rat, *R. norvegicus;* and ship rat, *R. rattus*). Having evolved in the absence of mammalian predators, the flightless and odorous nature of kakapo rendered them particularly vulnerable to predation by these introduced mammals (Lloyd & Powlesland 1994; Innes *et al*. 2010). This ultimately resulted in a population size reduction to approximately 100-200 kakapo by 1980 (Powlesland *et al*. 2006). These remaining birds survived in remote areas of Stewart Island, with the exception of one survivor from the Fiordland population on the mainland, named "Richard Henry". Continued mortality of this surviving population necessitated translocations onto predator-free offshore islands (Lloyd & Powlesland 1994), where the average mortality rate was successfully reduced to *c.*1.3% per annum (Powlesland *et al*. 2006). The lowest population census of 51 individuals was reached in 1992; of these founding individuals 21 were females (Powlesland *et al*. 2006).

1.5.1 Kakapo monitoring and management

Kakapo are currently spread between a number of predator-free islands but are essentially managed as one population, with birds often translocated between islands for breeding purposes. Kakapo are intensively monitored by the New Zealand Department of Conservation (DOC), which has also enabled an extensive collection of monitoring data to be available for analysis. Monitoring of kakapo has been on-going since 1977, after the Stewart Island population was discovered. Since 1997, all birds have been fitted with radio-transmitters, allowing each bird to be located using triangulation of the transmitter signals. A piece of technology called a "SNARK" (radio frequency scanner and logger) (Eason *et al.* 2006) has been used to automatically record the radio frequency and the arrival and departure time of any bird that comes within a 20 metre radius of it. Placement of SNARKs near each male's track and bowl systems allows the recording of the intensity of male courtship and identifies any females that approach a male's arena. In addition, during the breeding season the daily whereabouts of each female can be monitored using radio-tracking. A female kakapo usually has a home range that does not overlap with the male lek arenas (Walsh *et al.* 2006; Farrimond *et al.* 2006; Whitehead *et al.* 2012); therefore movement of a female into the vicinity of a male can be closely monitored to record any matings (Eason *et al*. 2006). Feather clusters left within the track and bowl

arena after copulation can be used to confirm matings, these are distinguished from fighting signs by the ratio of down to contour feathers (>3:1 down to contour feathers) (Powlesland *et al.* 2006). This combination of monitoring techniques has enabled information to be recorded for most matings events, including the occurrence, timing and identity of each individual involved in the mating.

Post-copulatory monitoring of females allows for the detection of nest locations, determined by a female remaining in the same location for at least seven days, followed by visual confirmation of a nest (Eason *et al.* 2006). Nest monitoring occurred according to the methods described in Eason *et al*. (2006). An infrared camera was installed to allow monitoring of the nest contents from a nearby tent. Infrared laser beams were also set up across the entrance route to the nest in order to determine when the female was absent, and thus, when it was appropriate to inspect the nest contents. A battery-powered heat pad was also placed over the eggs and chick to prevent chilling during the female's absence.

With the principal threat of predation removed, conservation efforts for kakapo are now focusing on recruitment into the population (Clout & Merton 1998; Elliott *et al.* 2001). A number of management techniques have been employed to improve kakapo productivity. This has included: fitting all kakapo with a back-pack radio transmitter to monitor movements, survival, health, mating and nesting attempts (Elliott *et al.* 2006); supplementary feeding to maintain general health of kakapo and improve breeding condition, particularly for nesting females (Elliott *et al*. 2001; Robertson *et al.* 2006); nest video monitoring, nest intervention (when necessary), and subsequently, artificial incubation and captive rearing techniques (Elliott *et al.* 2001). Despite these techniques, recruitment remains limited, with low nesting frequency and hatching success remaining a significant hurdle for kakapo recovery (Elliott *et al.* 2006; Powlesland *et al.* 2006). Artificial insemination (AI) was recently introduced to the recovery program to attempt to improve fertilisation success, with the first successful AI chicks produced in 2009 (Robertson *et al.* 2011a). However, in order to effectively utilise this new tool in kakapo management, the genetic consequences of the severe demographic bottleneck need to be addressed.

1.5.2 Genetic diversity of kakapo

The genetic composition of the extant kakapo population is considerably lacking in diversity (Miller *et al.* 2003; Robertson 2006; Robertson *et al.* 2009). Robertson (2006) suggested two likely reasons for this low genetic diversity. Firstly, all but one kakapo are

descended from the already genetically-similar Stewart Island population. With the exception of Richard Henry, all extant kakapo are derived from a founding population discovered in a remote area of Stewart Island. Island populations, such as those that would be found on Stewart Island, have naturally low diversity due to their having been founded by only a small number of birds (Frankham 1998; Caughley 1994). Consequently, the surviving Stewart Island kakapo are severely lacking in genetic diversity (Miller *et al.* 2003; Robertson 2006; Robertson *et al.* 2009). Second, the lek mating system of kakapo could also contribute to the low diversity, as it allows for a limited number of males to contribute their genes to the subsequent generations. This mating system acts to reduce the effective population size, so the loss of allelic diversity through genetic drift is intensified (Miller *et al*. 2003). Lek mating can also increase the degree of inbreeding, because individuals in the next generation are more likely to be related as most will be descended from only a few males.

By 1992, all birds had been translocated to predator-free islands and the population size had been reduced to its lowest count of only 51 individuals (Powlesland *et al.* 2006). This severe population bottleneck is likely to have caused a further loss of diversity. Evidence of this is seen when the genotypes of Stewart Island birds are compared to Richard Henry who differs in his genetic composition, and has higher levels of heterozygosity (Miller *et al*. 2003; Robertson *et al.* 2011b). Since Richard Henry is the only survivor from a larger, more diverse, mainland population, it is clear that a large degree of diversity has been lost to kakapo. Consequently, inbreeding depression is likely to be impacting on kakapo population fitness, but could be difficult to detect because of their relatively low genetic diversity (*e.g*. Grueber *et al.* 2008).

1.6 Research aims

The small population size, combined with a lek mating system and naturally low fecundity (Lloyd & Powlesland 1994), make kakapo highly susceptible to genetic drift and inbreeding depression. Even if a population is increasing in size, inbreeding depression has the potential to slow the rate of increase, extending the time the population is at a vulnerable size. Therefore, understanding the potential impacts of inbreeding and the conditions under which population persistence is compromised, is important in conservation management. Understanding how inbreeding might be impacting on individual and population fitness in kakapo could aid in improving population recruitment

and therefore species recovery. The overall aim of my research is to improve kakapo productivity by determining how inbreeding depression is influencing kakapo reproductive fitness. I will address this aim through the following approaches:

- Identifying the relationship between hatching success and inbreeding using molecular methods as an estimate of individual heterozygosity and genetic compatibility between pairs.
- Identifying the relationship between sperm quality and male heterozygosity to determine if inbreeding is reducing male fertility.
- Understanding the role that inbreeding plays in the reproductive success of kakapo, so that management actions can be tailored to increase population recruitment.

1.7 Thesis structure

The body of this thesis is represented by two main chapters (chapters two and three). These chapters have also been written as stand-alone papers, and as such, the chapters contain a degree of repetition, notably in the introduction and methods sections.

Chapter Two analyses the role of inbreeding depression in the early life history stages of kakapo. This involves the use of heterozygosity-fitness correlations relating female fecundity (clutch size), egg fertility (probability of an egg being fertilised by an individual male), and hatching success of fertile eggs (proportion of fertile eggs that a female hatches) with variation in molecular relatedness.

Chapter Three assesses the influence of inbreeding on male reproductive success. Since kakapo display a lek mating system, inbreeding effects on male reproductive success are most likely to be detected in sperm quality. This chapter describes how male heterozygosity influences three components of sperm quality: morphology, concentration and motility.

Chapter Four highlights the relevance of the knowledge obtained from this research by comparing the findings of the present study with those from similar research addressing inbreeding depression. Management recommendations are also proposed according to the research findings, so that kakapo reproductive success might be improved.

Chapter 2: The Effects of Inbreeding on Kakapo Fecundity, Egg Fertility and Hatchability

2.1 Introduction

Small populations inevitably experience increased incidences of inbreeding and a loss of genetic variation (Frankham *et al.* 2002). Inbreeding leads to the accumulation of alleles that are identical by descent, thereby increasing genome-wide homozygosity for inbred individuals, relative to more heterozygous individuals (Charlesworth & Charlesworth 1987; Hansson & Westerberg 2002). Increased homozygosity can have negative consequences on fitness related traits, due to the expression of lethal recessive alleles and/or lowered heterozygosity at over-dominant loci (Charlesworth & Charlesworth 1987; Lynch *et al.* 1995). The detrimental impacts of inbreeding are particularly conspicuous for early life-history traits (Keller & Waller 2002; Briskie & Mackintosh 2004; Cordero *et al*. 2004) as strong directional selection on these reproductive and developmental stages mean they can have direct fitness consequences (DeRose & Roff 1999). Therefore, disruptions to the major genes responsible for these traits are often more detectable than in traits less tightly linked with fitness (Keller & Waller 2002). For this reason, inbreeding depression frequently manifests as reduced survival and reproductive fitness in wild populations (Crnokrak & Roff 1999; Keller & Waller 2002). Lowered reproductive success can directly impact on population growth, having the potential to further increase the risk of extinction for endangered species (Bouzat 2010). As such, detecting and quantifying the effects of inbreeding depression is an important part of any conservation management program (Jamieson *et al.* 2006; Allendorf *et al.* 2010).

Inbreeding depression is most reliably measured using accurate, complete and multi-generational pedigrees (Pemberton 2008; Grueber *et al.* 2011a). When pedigrees are unavailable or unreliable, molecular methods can measure heterozygosity by descent to generate indirect estimates of an individual's inbreeding coefficient (Frankham *et al.* 2002; Pemberton 2004; Grueber *et al.* 2008). There are two main measures of heterozygosity by descent (Frankham *et al*. 2002; Grueber *et al.* 2011a): the first uses shared neutral markers between individuals as an indication of shared ancestry; the second uses the expected increase in homozygosity for inbred individuals, to estimate inbreeding within an individual. Heterozygosity by descent can then be correlated with variation in fitness related traits to infer inbreeding depression, in a method known as heterozygosity-fitness correlations (HFCs) (Hansson & Westerberg 2002; Pemberton 2004). The ability of HFCs to detect inbreeding depression is subject to much recent debate (Hansson & Westerberg 2002; Pemberton 2004; Grueber *et al.* 2008). This debates stems from the underlying

effect that heterozygosity has on fitness, and the ability to detect this effect using neutral markers (Chapman *et al.* 2009; Szulkin *et al.* 2010; Grueber *et al.* 2011a). For example, detecting HFCs in a severely inbred population might be difficult when genetic diversity is eroded to the point where variance in heterozygosity is small (Grueber *et al.* 2008). This was seen in the study of New Zealand takahe (*Porphyrio hochstetteri)* where pedigree measures succeeded in detecting inbreeding depression, whereas molecular measures did not (Grueber *et al*. 2011a).

The critically endangered kakapo (*Strigops habroptilus)* is a large, flightless, nocturnal parrot that is endemic to New Zealand (Powlesland *et al.* 2006). Due mostly to the introduction of mammalian predators into New Zealand, the kakapo population recently suffered a severe population bottleneck, with the total population size reaching a nadir of 51 individuals (Powesland *et al*. 2006). Intensive management on predator-free islands managed to halt the population decline and allow recovery (Powlesland *et al.* 2006). However, poor reproductive success and low productivity has meant recovery has been slow (Elliott *et al.* 2006). The low productivity has been attributable to the dependence on the nutrient-rich rimu fruit that is available in abundance only every two to seven years (Eason & Moorhouse 2006; Harper *et al.* 2006). The reason behind the reduced reproductive capabilities for kakapo has been debated by managers. Dietary problems, an aging population and inbreeding have been suggested as potential mechanisms (Clout & Merton 1998; Jamieson *et al*. 2006; Robertson 2006). With previous estimates of hatching rates for kakapo only as high as 62% (Elliott *et al.* 2006), compared to the average of 90% for most wild bird populations (Koenig 1982), there is a very high possibility that inbreeding depression is disrupting one or many of the genes involved in the early life history stages.

2.1.1 Study aims

This study aimed to determine the role of inbreeding in the reproductive success of kakapo. Determining the role of inbreeding in kakapo will guide future management decisions in the attempt to minimise such effects. The current absence of a multigenerational pedigree means that molecular methods were required to investigate inbreeding depression in kakapo. This also presented a good opportunity to increase our understanding of the ability of molecular methods to detect incidences of inbreeding depression in a bottlenecked population, while also aiding in the management of a critically endangered species

2.2 Materials and methods

2.2.1 Monitoring data

Monitoring of kakapo has been on-going since 1977 by the New Zealand Department of Conservation (DOC). The present study used only those monitoring records collected between 1997 and 2011, when all known birds in the population were radio-tagged, to ensure the collection and accuracy of the data was consistent between years.

Information was collected for each egg laid, including the identity of the mother; the date the egg was laid; and the number of eggs in the clutch according to the methods detailed in Eason *et al*. (2006). Eggs were classified as fertile or infertile using the candling method, with embryonic development able to be detected from day five of incubation (Daryl Eason pers. comm.). If no development could be detected, the cap of the egg shell was removed and the germ cell investigated for any signs of development or fertility. SNARKs (radio frequency scanner and logger) were used at all the track and bowl systems to record any matings (Eason *et al.* 2006). Using these records, putative paternity could be assigned to clutches when the male had exclusive access to a female. All instances of paternity were then verified using microsatellite markers (Robertson *et al.* 2009). In this way, paternity could also be assigned to offspring with multiple potential fathers. Since extraction of DNA from infertile eggs was not possible, paternity could not be assigned to infertile eggs from clutches derived from multiple matings, and so, these clutches were excluded from the analysis.

Kakapo generally lay one clutch in the breeding season, but can lay a replacement clutch if the first clutch fails or managers remove eggs early in the breeding season (Powlesland *et al.* 2006). The stressful nature of replacement clutches (Hansson *et al.* 2000) has the potential to exaggerate the effects of inbreeding (Pray *et al.* 1994; Coltman *et al.* 1999; Armbruster & Reed 2005). However, replacement clutches occurred too infrequently to be able to identify or control for this effect and therefore replacement clutches were omitted from the analysis.

Parental age is only known for those birds that hatched after the Stewart Island population was discovered in 1977 (Powlesland *et al*. 1995). All the birds were described as adults at the time of discovery. Therefore, the minimum age for all founding birds was estimated using the number of years since discovery plus five juvenile years.

2.2.2 Molecular methods

The genotypes for all founding adults and subsequent chicks, including the embryos that died during development, were determined using genomic DNA extracted from blood or embryonic tissue, using a 5% Chelex protocol (see Walsh *et al*. 1991). Samples were genotyped using 25 of the 30 polymorphic microsatellites developed by Robertson *et al*. (2009); three sex-linked loci (Strhab04, Strhab23 and Strhab44) and two loci that presented problematic allele coding (Strhab02 and Strhab42) were excluded to ensure accuracy across the markers (Robertson *et al.* 2009; Robertson, unpublished data). Multiplex amplification of microsatellite loci was done by polymerase chain reaction (PCR) carried out in ten-microlitre reactions containing c. 100*ng* of template DNA, 0.04*pmol* of M13-labelled, locus-specific forward primer, 0.16pm of locus specific reverse primer, 0.16*pmol* of M13 primer 5′-end labelled with an Applied Biosystems dye (VIC, FAM, NED or PET), 200μm each of dATP, dGTP, dTTP and dCTP,16mm $(NH_4)_2SO_4$, 67mm Tris-HCl, pH 8.8, 0.01% Tween-20, 1.5mm $MgCl_2$ and 0.2 units of Taq DNA polymerase (BIOTAQ, Bioline USA Inc.). The thermal cycling parameters were an initial 2 minute denaturation at 94°C, followed by 10 cycles of 94°C/25 sec, 60°C/50 sec (minus 1°C each cycle) and 72°C/50 sec and then 30 cycles of 94°C/25 sec, 48°C /50 sec and 72°C/50 sec and then a final 30 min hold at 60°C. Following amplification, PCR products were size-fractionated on an ABI3100 Genetic Analyzer (Applied Biosystems Inc). Peak-calling was done using the program GeneMapper (Applied Biosystems Inc.), but all peak sizes were visually checked for accuracy. Genotypes for all individuals were done twice at all loci to detect any genotyping errors (Hoffman & Amos 2005) and none were detected (Robertson *et al.* 2009; Robertson unpublished data). No significant linkage disequilibrium has been detected among these microsatellite loci and parentage is determined with 99% probability (Robertson *et al.* 2009).

2.2.3 Estimating relatedness

The software package COANCESTRY (Wang 2010) was used to calculate the Wang (2002) method of moment relatedness estimates for every pair combination of kakapo. Wang estimates the relatedness between two individuals using the probability that two alleles at a locus are identical by descent, given the frequency of alleles in a population. Population allele frequencies were calculated using all kakapo that were alive between 1997 and 2011. A plot between the Wang and Queller-Goodnight (a similar relatedness estimator: Queller & Goodnight 1989) estimators was used to assess the ability

of Wang to calculate pair relatedness (Fig. 2.1), with highlighted relationships between known first-order relatives, and between Richard Henry and the Stewart Island founder birds (unrelated, Robertson 2006). The Wang (referred to as "pair relatedness" hereafter) method appeared to perform well for the kakapo genotypes and so was deemed a reliable substitute for pedigree measures of relatedness between putative parents.

Both internal relatedness (IR, Amos *et al.* 2001) and heterozygosity by loci (HL, Aparicio *et al.* 2006) are appropriate measures of heterozygosity for populations with high incidences of inbreeding because rare, homozygous alleles are weighted higher than the more common homozygous alleles (Amos *et al.* 2001; Aparicio *et al.* 2006). Aparicio *et al.* (2006) suggests HL outperforms IR when the study population has rare alleles and/or immigrations, and when low numbers of microsatellite markers are used. For kakapo, twenty-five microsatellites seemed a sufficient number to reliably estimate heterozygosity using IR, however, Richard Henry may be considered an "immigrant' since he possesses some distinct alleles (Miller *et al.* 2003) and therefore HL might generate more accurate estimates of heterozygosity. Therefore, both measures of heterozygosity were used in the analysis and calculated using the IRMacroN4 developed by Amos *et al.* (2001). For both estimators, negative values were indicative of more heterozygous individuals and positive values represented more homozygous individuals (Amos *et al.* 2001; Aparicio *et al.* 2006).

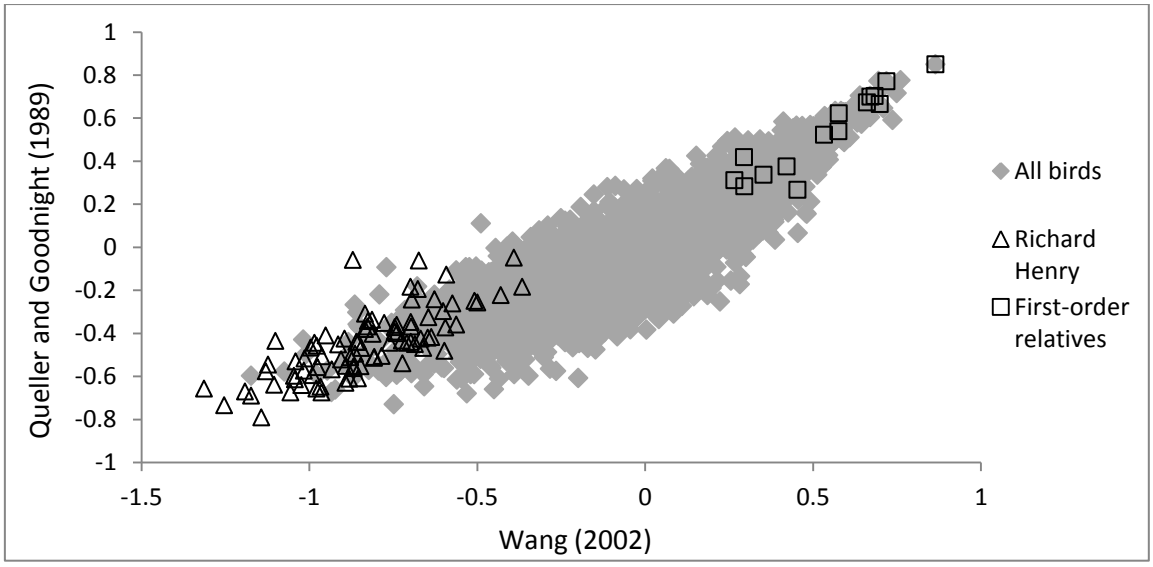

Figure 2.1: The performance of two relatedness estimators, Wang and Queller & Goodnight, to calculate relationships between kakapo. Allele frequencies used to estimate relatedness were calculated using twenty-five microsatellite DNA markers (Robertson *et al*. 2009). Known relationships are included to highlight the ability of these estimators to determine relatedness, including all relationships between Richard Henry and Stewart Island (unrelated, triangles $n = 86$) and first order relatives (related, squares $n = 14$).

2.2.4 Model fitting

Generalised linear mixed models (GLMM) were used to assess how inbreeding might be impacting on three early life-history stages: female fecundity (clutch size), egg fertility (probability of an egg being fertilised by an individual male) and hatching success of fertile eggs (proportion of fertile eggs that a female hatches). The GLMMs were fitted using the *lmer* function in the *lme4* package (Bates & Maechler 2009) in R (R Core Development Team 2009). Global models were generated using all potential explanatory variables. The global model was standardised using the standardize function in the *arm* package (Gelman *et al.* 2009) so that parameter estimates are comparable for all predictors. Using functions available in the *MuMIn* package a subset of the models was produced from the standardised global models. These models were ranked according to Akaike's Information Criterion (AIC_c) values. A natural average method (Burnham and Anderson 2002) was used to model-average the top $2\Delta AIC_c$ models of the subset. Model-averaging produced weighted parameter and standard error estimates for each predictor (Nakagawa & Freckleton 2010; Grueber *et al.* 2011b). If there was only one top model in the top 2ΔAICc, model-averaging was not necessary and parameter estimates were taken from that model.

2.2.4.1 Fecundity

Female fecundity was analysed using clutch size as the response variable (n = 81 clutches). Clutch size followed a normal distribution and so the GLMM was fitted with a Gaussian error structure. Maternal IR was the genetic variable of interest and was fitted as a fixed effect. Year was entered as a fixed effect to account for variation across breeding seasons. This is particularly relevant for kakapo, as productivity is known to have some dependence on rimu fruit abundance (Eason & Moorhouse 2006). Other covariates that also had the potential to influence clutch size and were included in the model were female age (Reid *et al.* 2003) and the Julian dates when the clutch was laid (lay date) (Rowe *et al*. 1994). Female ID (n = 29) was included as a random effect to account for multiple clutches laid by the same female.

2.2.4.2 Egg Fertility

A male kakapo could be assigned as the putative father based on exclusive access to a female who subsequently laid eggs (see above). The ability of a male to fertilise those eggs was analysed using the proportion of fertile eggs, per male, as the response variable. The GLMM was therefore fitted with a binomial error structure, entered as an egg being either fertile (1) or infertile (0). All eggs (both fertile and infertile) in clutches produced by females that had mated with multiple males were excluded from the analysis, since paternity of any infertile eggs in these clutches could not be determined. Paternal IR was fitted as a fixed effect. Year and male age were also entered as fixed effects, as they had the potential to influence male breeding condition (Cockrem 2006, Eason *et al.* 2006). Kakapo are believed to follow an annual cycle of gonadal growth and regression (Cockrem 2006), therefore, the Julian date of the first mating by the male in a breeding season (first mating) was also entered as a fixed effect in an attempt to control for variation in peak male breeding condition. Male ID (n = 24 males) was included as the random effects variable to account for breeding over multiple seasons by some males.

2.2.4.3 Hatching

The investigation of hatching success was performed using only the eggs that had been successfully fertilised. Since some clutches were fathered by multiple males, hatching success was analysed on a per egg basis (n = 136 eggs), using a GLMM with a binomial error structure. The response was entered as either hatched (1) or unhatched (0). Eggs that failed to hatch due to damage inflicted by the female, interference by sooty shearwaters (*Puffinus griseus*), chilling after being rolled from the nest, or due to artificial incubation

failures, were excluded from the analysis, as they were not reflective of genetic impacts. Both pair relatedness, of the mother and putative father, and female IR were entered as fixed effects. Male IR was not included as the male kakapo has no further contribution to the raising of offspring beyond fertilising the egg (Powlesland *et al*. 2006). To control for multiple eggs per pair/female both pair ID (n = 54 pairs) and female ID (n = 27 females) were entered as random effects. Covariates that were included in the model have been detected to influence hatching in other bird species. These included clutch size (Reid *et al.* 2000), year (Eason & Moorhouse 2006), and maternal age (Ortego *et al.* 2010; Grueber *et al*. 2010).

During the nesting period, fertile eggs were often transferred to more capable nesting females and/or to an artificial incubator when necessary, for example, during years of low rimu fruit supply when females were forced to increase their time spent foraging (Elliott *et al.* 2001). Ideally, the effect of inbreeding on hatching would take into account those eggs that were manipulated and those that were naturally hatched. However, most eggs were manipulated in some way and this manipulation was not exclusive to just one incubation technique. For example, some eggs were transferred between foster mothers, mothers and the artificial incubator multiple times. As a result, the influence of management could not be analysed. In saying this, the intensity of management is likely to have largely removed maternal influences on the nesting environment, and consequently hatching failure should be largely under genetic control. For this reason, hatching success was still assessed in spite of the inability to control for management interference.

2.3 Results

Models assessing individual heterozygosity were performed using both HL and IR. These two metrics produced very similar results when entered into the models. IR and HL estimates also correlated very well (R^2 = 0.89), suggesting there is little difference in their ability to estimate heterozygosity, given the kakapo allele frequencies. This is consistent with Chapman *et al.* (2009) who could find no significant difference between these heterozygosity metrics. Therefore, only results produced by models that used IR are reported for simplicity. The IR values generated displayed a reasonable range of between -0.5 and 0.5 for both male and female kakapo (Fig 2.2). Hence enough variation in heterozygosity was present to assess the relationship between IR and the fitness-related traits.

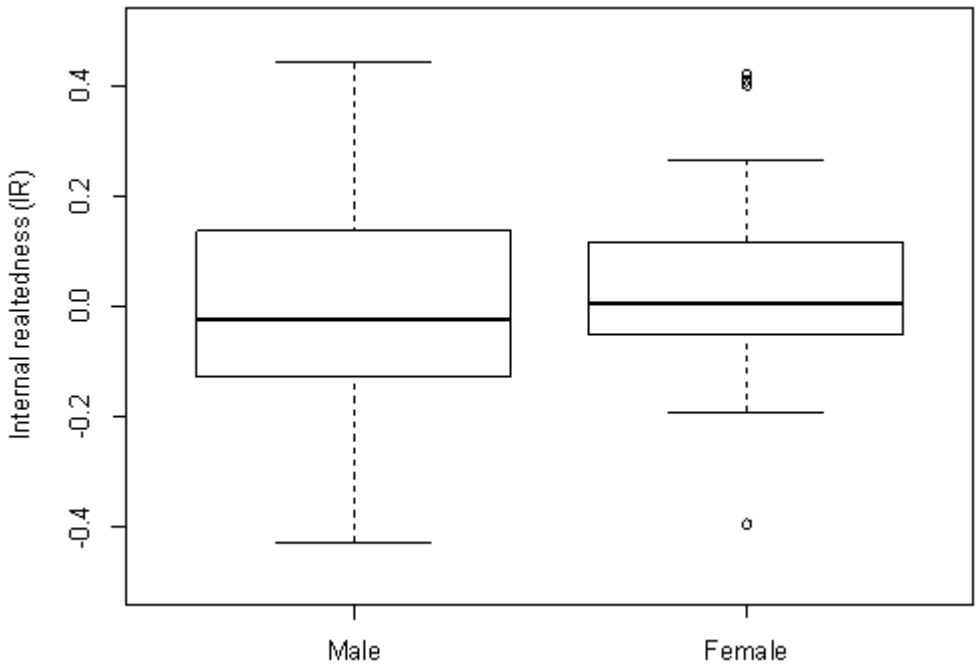

Figure 2.2: Box plot showing the median, upper, and lower quartile values for the levels of internal relatedness (IR, Amos *et al.* 2001) in breeding male (*n* = 24) and female (*n* = 29) kakapo.

2.3.1 Fecundity

The average clutch size for kakapo in this study was 2.5 ± 0.7 eggs. When modelling the effect of maternal IR on clutch size, only one model ranked in the top $2\Delta AIC_c$ and so that model was taken to best explain variance in clutch size (Table 2.1). This model included lay date and maternal IR as the explanatory variables. Lay date displayed a relatively strong negative effect size (Table 2.2), suggesting that females have significantly larger clutch sizes when they lay their clutches earlier in the year (Fig 2.3). Female IR had a weaker, although still significant, effect on clutch size (Table 2.2), with more homozygous females having smaller than average clutches (Fig 2.4). However, there did appear to be an outlier in the data that looked as if it could be driving this trend (Fig 2.4). This data point was from a female named "Ellie" who had a high IR value and laid two clutches with just one egg each. Removing Ellie from the analysis resulted in a considerably weaker and a non-significant relationship between clutch size and female IR (β = -0.184 [SE = 0.134], p>0.05). Neither age nor year featured in the top model and so did not appear to influence kakapo fecundity to a degree that was detectable by this analysis.

Table 2.1: The top ten models, ranked according to ΔAIC_c (the difference in AIC_c from the strongest model), assessing the effects of inbreeding on kakapo clutch size. Female ID was included as the random effects variable. "Female IR" was the internal relatedness value for each female, "Lay date" was the Julian date in which the clutch was laid, "Age" was the estimated age of each female kakapo and "Year" was the year in which the clutch was laid. The table also includes the AIC_c value, the degrees of freedom (df) and the Akaike weight. The selected model is in bold.

Rank	Model	AIC_c	ΔAIC_c	df	Weight
1	**Lay date + Female IR**	**162.0**	**0.00**	**5**	**0.509**
2	Lay date + Female IR + Age	164.1	2.09	6	0.179
3	Lay date + Female IR + Year	164.3	2.33	6	0.159
4	Lay date	166.4	4.44	4	0.055
5	Lay date + Female IR + Age + Year	166.4	4.47	7	0.054
6	Lay date + Year	168.6	6.65	5	0.018
7	Lay date + Age	168.6	6.67	5	0.018
8	Lay date + Age + Year	170.9	8.93	6	0.006
9	Female IR	175.5	13.57	4	0.001
10	Female IR + Year	177.8	15.83	5	0.000

Table 2.2: The standardised parameter estimates (β) and standard errors (SE) taken from the top ranked ΔAIC_c model from the generalised linear mixed model with Gaussian error structure, analysing the effect of inbreeding on kakapo clutch size. Female ID was fitted as the random effects variable. Fixed effects included: "Maternal IR" as the internal relatedness value for each female; "Lay date" as the Julian date in which the clutch was laid; "Age" as the estimated age of each female and "Year" as the year in which the clutch was laid. Test statistics (*t*) and associated p-values and 95% confidence intervals (CI) are also included in the table. Significant parameter estimates ($p<0.05$) are in bold.

	β	**SE(β)**	***t***	***95% CI***	***P***
(Intercept)	**2.543**	**0.068**	**37.20**	**2.679 to 2.407**	**<0.001**
Lay date	**-0.557**	**0.138**	**-4.18**	**-0.281 to -0.833**	**<0.001**
Female IR	**-0.359**	**0.138**	**-2.64**	**-0.083 to -0.635**	**<0.01**

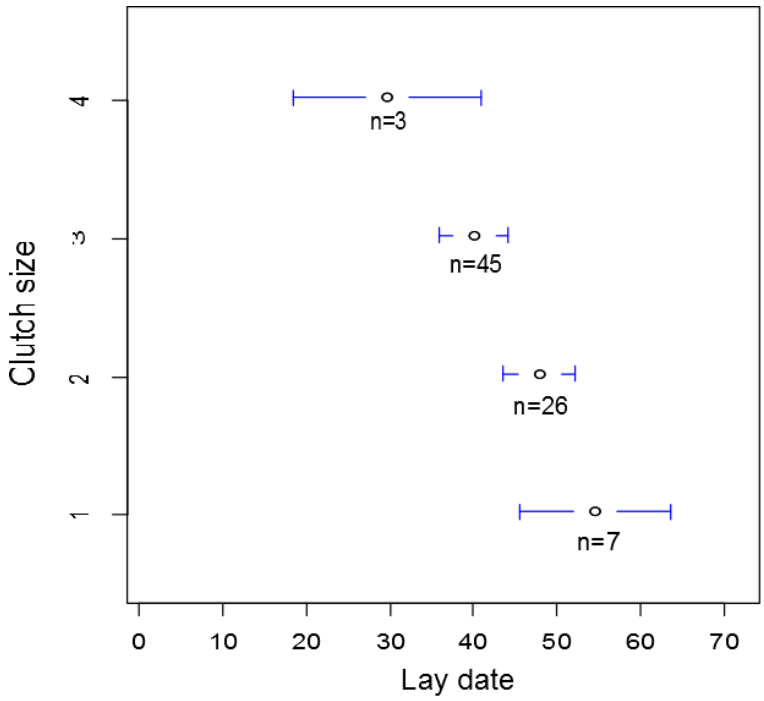

Figure 2.3: The relationship between kakapo clutch size and Julian date of the year that the clutch was laid. Mean lay dates are plotted for each clutch size. Blue bars represent 95% confidence intervals around the means. Sample size (n) for each clutch size is included. Replacement clutches within a breeding season were excluded from the analysis.

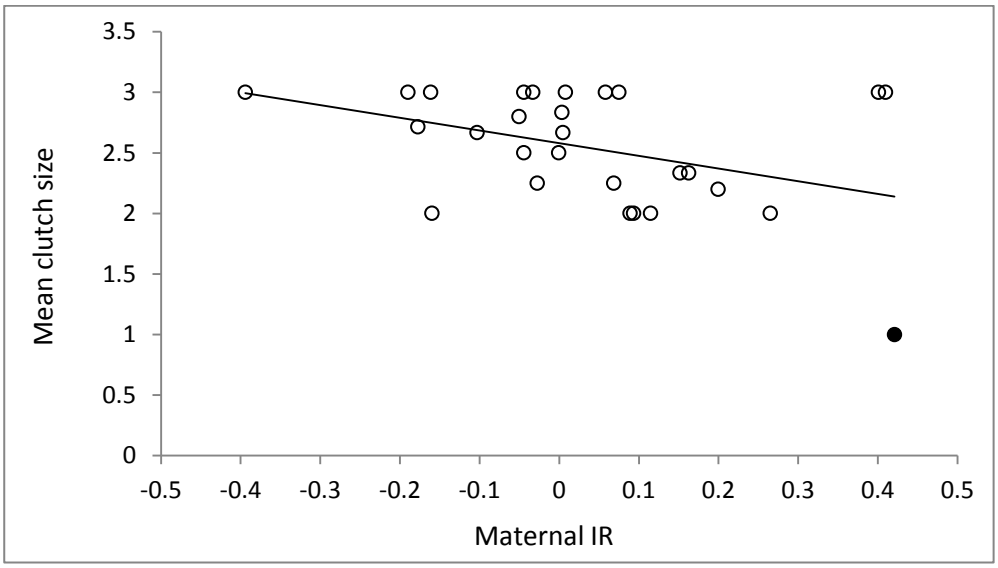

Figure 2.4: The relationship between kakapo clutch size and maternal internal relatedness (IR, Amos *et al*. 2001). IR ranges between -0.5 and 0.5 and positive IR values represent more homozygous females and negative IR values represent more heterozygous females. Replacement clutches within a breeding season were excluded from the analysis. The solid data point represents the mean clutch size for "Ellie" that was removed from the analysis (see text for details).

2.3.2 Egg fertility

When analysing the effect of inbreeding on the ability of males to fertilise eggs, three models were ranked in the top $2\Delta AIC_c$, including the date of first mating of the year and age as explanatory variables (Table 2.3). The next best model in the subset included male IR as a fixed effect. Although this model had a ΔAIC_c value higher than two, IR was the variable of interest and so was included to determine how male IR affects egg fertility.

The near-significant, large effect size for "Date first mating" indicated that the proportion of fertile eggs was higher for males that began mating earlier in the breeding season (Table 2.4). Despite featuring in the top models, male age only had a weak positive effect on egg fertility, which was not statistically significant (Table 2.4).When controlling for this seasonal variation in fertilisation success, and the slightly positive effect of age, male IR produced a weak positive effect size (Table 2.4) suggesting that the males that are more homozygous produce a higher proportion of fertile eggs. However, this was not significant due to the very large standard error, and the data did not appear to follow any such trend (Fig 2.5).

Table 2.3: The top ten models, ranked according to ΔAIC_c (the difference in AIC_c from the strongest model), assessing the effects of inbreeding on the proportion of eggs successfully fertilised by male kakapo. Male ID was included as the random effects variable. "Male IR" was the internal relatedness value for each male, "Date first mating" was the Julian date when the male was first recorded mating, "Age" was the estimated age of each male kakapo, and "Year" was the breeding year. The table also includes the AIC_c value, the degrees of freedom (df) and the Akaike weight. The selected model is in bold.

Rank	Model	AIC_c	ΔAIC_c	df	Weight
1	**Date first mating**	**152.7**	**0.00**	**3**	**0.215**
2	**Constant**	**153.1**	**0.43**	**2**	**0.173**
3	**Date first mating + Age**	**154.6**	**1.91**	**4**	**0.083**
4	**Date first mating + Male IR**	**154.7**	**2.05**	**4**	**0.077**
5	**Date first mating + Year**	**154.8**	**2.14**	**4**	**0.074**
6	Age	155.0	2.29	3	0.068
7	Male IR	155.1	2.38	3	0.065
8	Year	155.2	2.54	3	0.061
9	Date first mating + Age + Year	156.4	3.74	5	0.033
10	Date first mating + Age + Male IR	156.7	4.00	5	0.029

Table 2.4: The standardised parameter estimates (β) and standard errors (SE) produced by model-averaging the top 2.2ΔAIC$_c$ models from a subset of generalised linear mixed models with binomial error structure, analysing the effect of inbreeding on the fertilisation success of male kakapo. Male ID was fitted as the random effects variable. Fixed effects included male IR, the Julian date in the breeding season that the male first mated (First mating), male age, and year. Test statistics (*z*) and associated p-values, 95% confidence intervals (CI) and relative importance (RI) are also included in the table. Significant parameter estimates (p<0.05) are in bold.

	β	**SE(β)**	***z***	***95% CI***	***P***	***RI***
(Intercept)	-0.365	0.502	0.726	0.639 to -1.369	0.468	
Date first mating	0.857	0.527	1.627	1.911 to -0.197	0.104	0.68
Age	0.357	0.715	0.499	1.787 to -1.073	0.618	0.15
Male IR	0.275	0.884	0.311	2.043 to -1.493	0.756	0.14

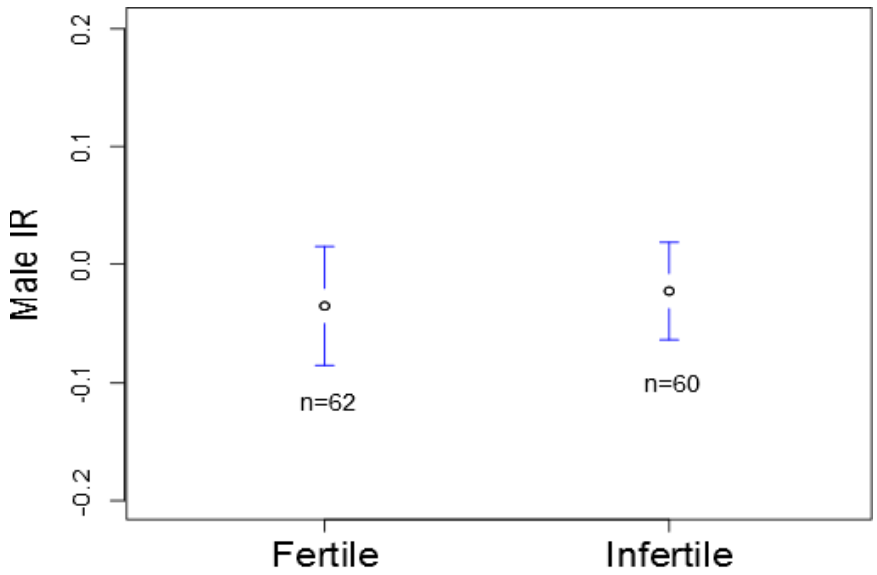

Figure 2.5: The difference in mean male heterozygosity (IR, Amos *et al*. 2001) fertile (*n* = 62) and infertile (*n* = 60) kakapo eggs. IR ranges between -0.5 and 0.5, with positive IR values represent more homozygous females and negative IR values represent more heterozygous females. Blue bars represent 95% confidence intervals around the means. Clutches produced by females that mated with multiple males were excluded the analysis as paternity was unable to be determined.

2.3.3 Hatching

When analysing the effect of inbreeding on the hatching rate of fertile eggs, female IR featured in all the models of the top ten ranked models and so clearly had a large influence on hatching (Table 2.5). The top five ranked models included female IR, as well as each of the other fixed effects independently: clutch size, age, year and pair relatedness (Table 2.5) were either close to or lower than $2\Delta AIC_{c.}$ Therefore, model averaging was performed on the models ranked within the top $2.2\Delta AIC_c$ in order to understand the effect of female IR in relation to variables contributing towards variation in hatching success.

Female IR produced a very strong negative effect size (Table 2.6), suggesting that the more homozygous females have significantly lower hatching success than the more heterozygous females (Fig 2.5). The next strongest effect was produced by clutch size (Table 2.6) and, although the large standard error meant this result was not statistically significant, it implies that hatching rates are higher for eggs in larger clutches. Parental relatedness, female age and year all appeared to have very weak influences on hatching rates of fertile eggs, compared to that of female IR (Table 2.6)

Table 2.5: The top ten models, ranked according to ΔAIC_c (the difference in AIC_c from the strongest model), assessing the effects of inbreeding on the proportion of fertile eggs that successfully hatch. Female ID and Pair ID were included as the random factors. Both maternal IR and a Wang pair-wise relatedness estimator (Wang 2002) were entered as the genetic factors of interest. "Female IR" was the internal relatedness value for each female, "Wang" was the pair-wise relatedness estimate for each pairing, "Clutch size" was the size of the clutch, "Age" was the estimated age of the mother, and "Year" was the breeding year in which egg was laid in. The table also includes the AIC_c value, the degrees of freedom (df) and the Akaike weight. The selected model is in bold.

Rank	Model	AIC_c	ΔAIC_c	df	Weight
1	**Female IR**	**139.0**	**0.00**	**4**	**0.245**
2	**Female IR + Clutch size**	**141.1**	**1.61**	**5**	**0.109**
3	**Female IR + Wang**	**141.4**	**1.93**	**5**	**0.093**
4	**Female IR + Age**	**141.4**	**1.95**	**5**	**0.092**
5	**Female IR + Year**	**141.6**	**2.12**	**5**	**0.085**
6	Female IR + Clutch size + Age	143.0	3.51	6	0.042
7	Female IR + Clutch size + Wang	143.0	3.57	6	0.041
8	Female IR + Clutch size + Year	143.3	3.79	6	0.037
9	Female IR + Age + Wang	143.4	3.92	6	0.035
10	Female IR + Age + Year	143.6	4.10	6	0.032

Table 2.6: The standardised parameter estimates (β) and standard errors (SE) produced by model-averaging the top 2.2ΔAIC$_c$ models from a subset of generalised linear mixed models with binomial error structure, analysing the effect of inbreeding on the hatching success of fertile eggs. Both maternal IR and a Wang pair-wise relatedness estimator (Wang 2002) were entered as the genetic factor of interest. Female ID and Pair ID were included as the random factors. Covariates included the year the egg was laid, the age of the female and clutch size. Test statistics (*z*) and associated p-values, 95% confidence intervals (CI) and relative importance (RI) are also included in the table. Significant parameter estimates (p<0.05) are in bold.

	β	**SE(β)**	***z***	***95% CI***	***P***	**RI**
(Intercept)	**1.303**	**0.251**	**5.200**	**1.805 to 0.801**	**<0.001**	
Female IR	**-1.287**	**0.481**	**2.676**	**-0.325 to -2.249**	**<0.01**	**1.00**
Clutch size	0.350	0.453	0.773	1.256 to -0.556	0.440	0.18
Age	0.235	0.500	0.470	1.235 to -0.765	0.639	0.15
Wang	-0.275	0.557	0.493	0.839 to -1.389	0.622	0.15
Year	0.107	0.501	0.214	1.109 to -0.895	0.831	0.14

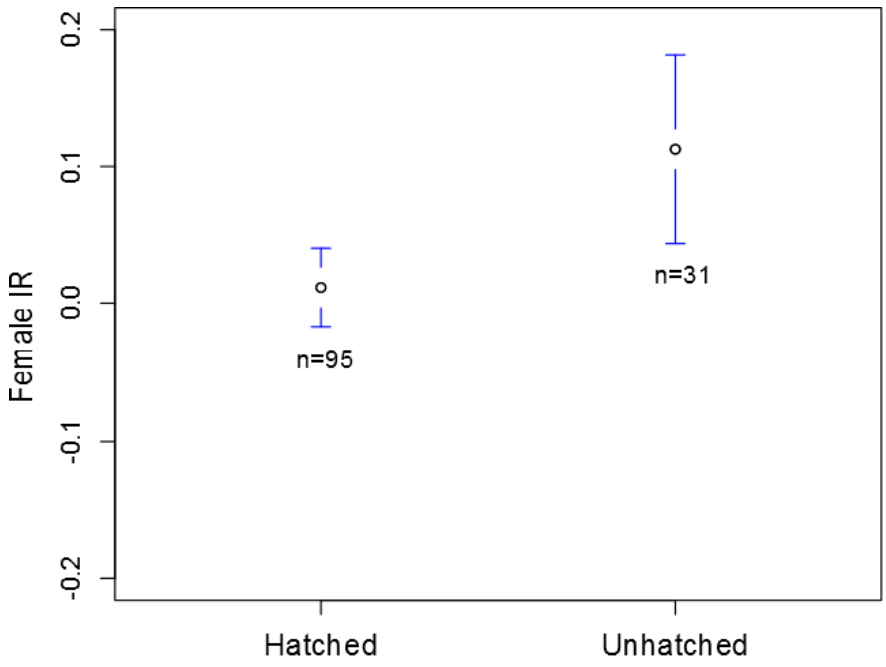

Figure 2.6: Difference in mean female heterozygosity (IR, Amos *et al*. 2001) between fertile kakapo eggs that hatched (n=95) and fertile eggs that failed to hatch (n=34). IR ranges between -0.5 and 0.5, with positive IR values represent more homozygous females and negative IR values represent more heterozygous females. Blue bars represent 95% confidence intervals around the means.

2.4 Discussion

Here I identify which early life-history stages are being influenced by inbreeding in kakapo, and, in doing so, show strong evidence for severe inbreeding depression in this species.

2.4.1 Fecundity

Fecundity was the first early life-history stage investigated, using variation in clutch sizes. Clutch size appeared to be reduced in more homozygous females. However, this trend was largely driven by one bird, "Ellie", who had a high level of homozygosity and laid two clutches comprising of just one egg each. In light of this trend being dependent on one individual, I concluded that inbreeding was not influencing clutch size in the population in general. Nevertheless, a number of studies have determined clutch size to be positively related to heterozygosity (Ortego *et al.* 2007; Garcia-Navas *et al.* 2009; Olano-Marin *et al*. 2011; Wetzel *et al.* 2012). Kakapo generally have low variation in clutch size, typically laying clutches consisting of two to three eggs, and only rarely laying clutches of one or four eggs (Eason *et al.* 2006). It is possible that the degree of variation in clutch size for kakapo is too small to detect any strong effect of heterozygosity on clutch size. The factor that had most influence on fecundity was lay date, with clutches laid later in the season consisting of slightly fewer eggs. This is a well-known relationship in bird species and is reflective of an increased fledging and survival probability for offspring that hatch earlier in the season, due to factors such as food availability and environmental conditions (Verhulst & Nilsson 2008).

2.4.2 Egg fertility

The second early life-history stage investigated was the ability of male kakapo to fertilise the eggs. Male homozygosity showed no detectable effect on the proportion of eggs that males successfully fertilised in a breeding season, and again seasonal variation in breeding times appeared to have more of an influence on reproductive success. Failure to detect a relationship between multilocus heterozygosity and male fertilisation success may have been hindered by the skewed male reproductive success that is typical of a lek mating system (Robertson 2006; Eason *et al.* 2006). It is possible that the highly homozygous males are missing out on breeding entirely, due to a lowered "attractiveness" to females (*e.g.* Aparicio *et al*. 2001) or reduced competitive ability on the lek (*e.g.* Höglund *et al.* 2002).

The apparent lack of reduced fertilisation ability for the more inbred males may also be because such an effect was being masked by very early death embryos. In this study, the technique used to determine if eggs were fertile was candling and, reportedly, it can be difficult to distinguish between infertility and embryos that die within the first few days of development using this technique (Kempaenears *et al.* 1996; Birkhead *et al.* 2008). More reliable methods of determining egg fertility in early stages requires potentially viable eggs to be sacrificed (Small *et al.* 2000; Birkhead *et al.* 2008) and would not be an appropriate method to use on a critically endangered species such as kakapo.

Sperm quality is a fitness trait that is often reduced for inbred males (Gage *et al.* 2006; Asa *et al*. 2007). Obtaining information on sperm quality, in relation to levels of male heterozygosity (see next chapter), is necessary to more accurately determine whether male kakapo have compromised fertilisation abilities, without confounding factors masking the effects.

2.4.3 Hatching

The third early life-history stage investigated was hatching success, analysed as the proportion of fertile eggs that successfully hatched. The relatedness between pairs did not have a detectable influence on hatching success. This result is in contradiction to a number of studies that have found hatchability to be reduced for genetically similar parents (Hansson 2004; Spottiswoode & Møller 2004). It is possible that the level of between allelic diversity within the population was not sufficient to identify related individuals. For example one pair of randomly selected first order relatives were detected to have a pair relatedness estimate close to zero (Fig. 2.1). Perhaps when multi-generational pedigrees become available, pedigree determined estimates of relatedness can clarify if there is the trend between pair relatedness and hatching success. Alternatively, assessing the genotypes of eggs that fail compared to eggs that hatch might be more revealing as to whether the more homozygous, or inbred, progeny are suffering from reduced fitness due to inheritance of alleles identical by descent.

In contrast, maternal IR did explain a significant level of variance in hatching success, an effect that was far stronger than any of the other fixed effects entered in the model. This result indicates that high levels of homozygosity in female kakapo can disrupt the major genes involved in embryogenesis, causing embryo mortality and lowered hatching success for those females. Lowered hatching success is consistent with known detrimental effects of inbreeding in birds (Greenwood *et al.* 1978; Kempenaers *et al*. 1996;

Jamieson *et al.* 2003; Cordero *et al*. 2004; Spottiswoode & Møller 2004; Brekke *et al.* 2010; Hagen *et al.* 2011). In only a few cases have inbred females been found to be responsible for reduced hatching rates in wild bird populations; for example, in great tits (van Noordwijk & Scharloo 1981) and song sparrows (Keller 1998). However, determining maternal effects on kakapo hatching is consistent with the large investment by female kakapo in their offspring (Clout *et al.* 2002), and the strong evolutionary force associated with such an investment (Reinhold 2002). Therefore, it can be deduced that embryo failure is likely due to the disruption to one, or many, of the maternal effect genes, due to the expression of deleterious recessive alleles (Charlesworth & Charlesworth 1987, 1999; Hansson & Westerberg 2002; Coltman & Slate 2003).

There are multiple stages of embryogenesis that might be impaired by increased maternal homozygosity. It is possible that more homozygous females are less capable of incubating eggs compared to more heterozygous females, and therefore, need to invest more energy into foraging (Eason & Moorhouse 2006). Although this may be the case, it is unlikely to be the mechanism detected by this analysis, given that most kakapo were provided with supplementary food (Eason & Moorhouse 2006), and eggs were often artificially incubated, kept warm with heat pads on the nest, or transferred to more capable females (Eason *et al.* 2006). A more likely explanation for embryo failure is lower quality of the maternal provisions of the egg, such as egg size, composition and androgen contribution (Royle *et al.* 1999; Tschirren *et al.* 2009). However, further investigation into which of these maternal investments are affected is required before robust conclusions can be made.

Maternal age did not appear to influence hatching success for female kakapo. This is in contrast to a number of studies that detected a positive relationship between age and hatchability (*e.g.* Schiegg *et al.* 2002; Ortego *et al*. 2010; Grueber *et al*. 2010). The failure of this study to detect this trend may be due to the relatively unknown age of most of the breeding kakapo (Horn *et al.* 2011). Since age was estimated from the date the bird was found on Stewart Island it is unlikely to be an accurate representation of the true age of a female.

2.4.4 Using HFCs to detect inbreeding depression

The relationship between molecular measures of maternal multi-locus heterozygosity and hatching success was a strong effect compared to the other potential explanatory variables entered into the model. This is in contrast to the majority of

literature that predicts that, even when significant, heterozygosity-fitness correlations are generally weak due to the weak relationship between pedigree and molecular measures of inbreeding (Balloux *et al.* 2004; Pemberton 2004; Slate *et al*. 2004; Chapman *et al.* 2009; Szulkin *et al*. 2010). In general, inbreeding depression can be well represented by HFCs when there is high variance in inbreeding levels in the population, and when a large number of polymorphic markers are used (Balloux *et al.* 2004; Slate *et al.* 2004). The use of 25 polymorphic microsatellites in this study most likely enabled a more reliable proxy of the underlying inbreeding coefficients of kakapo.

This study provides support for the use of HFCs to assess the impacts of inbreeding depression in conservation research when detailed pedigrees are unavailable. The ability to use HFCs for analysing kakapo data opens the possibility for assessment of other fitness related traits potentially impacted by inbreeding depression (*e.g.* immune response or mating success). In saying this, caution should be taken as to the ability of HFCs to reveal inbreeding depression in kakapo as no comparisons between multilocus heterozygosity and pedigree estimates have been made, and therefore, the reliability of the microsatellites markers as a proxy to genome-wide homozygosity in kakapo is still relatively unknown (Balloux *et al*. 2004; Slate *et al.* 2004; Grueber *et al*. 2008). In addition, the ability of HFCs to detect inbreeding is species and population specific (Alho *et al*. 2009), and although they may be useful for kakapo, this may not be the case for a species with a different genetic make-up and history of inbreeding.

2.5 Conclusions

This study provides evidence that inbreeding depression has the potential to reduce hatchability for kakapo. Maternal heterozygosity was found to be a significant predictor of embryo mortality in kakapo. In contrast, no relationship between female heterozygosity and fecundity or between male heterozygosity and fertilisation success could be detected. It is most likely that the maternal effect genes responsible for egg provisioning are being disrupted by deleterious recessive alleles in the more inbred females, causing failed embryogenesis and lowered hatching success. These findings were determined using HFCs, adding to the mounting evidence for the usefulness of molecular methods to detect inbreeding depression when pedigrees are either unavailable or unreliable. The deleterious impact of inbreeding on hatching success has the potential to decrease population growth, thus prolong the length of time kakapo are at risk of extinction. Using this information,

kakapo conservation managers should attempt to minimise matings between related individuals and prevent further erosion of genetic diversity.

Chapter 3: The Effects of Inbreeding on Kakapo Sperm Quality

3.1 Introduction

An extensive repertoire of research has revealed that inbreeding depression can have adverse effects on individual survival and reproduction (Coltman *et al.* 1999; Keller & Waller 2002; Briskie & Mackintosh 2004; Cordero *et al*. 2004). The genetic basis underlying inbreeding depression is a genome-wide increase in homozygosity for inbred individuals (Charlesworth & Charlesworth 1987, 1999; Hansson & Westerberg 2002). A lack of heterozygosity can have deleterious effects on fitness through two recognised mechanisms: first, the increased probability of recessive lethal alleles becoming expressed (Charlesworth & Charlesworth 1987); second, through the lesser occurrence of heterozygosity at over-dominant loci (Charlesworth & Willis 2009). Directional selection on traits with large fitness consequences means they are more susceptible to the negative impacts of inbreeding (DeRose & Roff 1999). As such, lowered reproductive success, via disruptions to the genes responsible for fertility and fecundity, has frequently been linked to inbreeding (Keller & Waller 2002).

Despite sex-specific consequences of inbreeding having been highlighted in a number of studies (Jamieson *et al*. 2003; Brekke *et al.* 2010; see also Chapter 2), thus far there has been little documentation of the impacts on male reproductive success. This is most likely attributable to evaluation of maternal effects, such as fecundity and offspring survival, being easier in natural populations (Roldan & Gomendio 2009). This is particularly true in species where females mate with multiple males, making determining the paternity of infertile eggs difficult (Roldan & Gomendio 2009). When the role of inbreeding in male reproductive success has been analysed, fitness-related traits such as measures of mating success (Höglund *et al.* 2002) or the number of sired offspring (Seddon *et al*. 2004; Zajitschek *et al.* 2009; Olano-Marin *et al.* 2011) were used. The ability of these approaches to accurately reflect the male's ability to sire offspring are limited, as highly homozygous males can suffer reduced mating success as a result of poor sperm quality or the males themselves being less "attractive" (Aparicio *et al*. 2001); that is to say, female effects can be hard to disentangle from male effects (Roldan & Gomendio 2009). One way to bypass these inaccuracies in measuring male reproductive success is to assess male sperm quality.

Spermatogenesis is a highly specialised and complex physiological process that has a strong multi-locus component and has large fitness consequences (de Kretser *et al.* 1998). For this reason, spermatozoa are prone to the negative impacts of inbreeding

(DeRose & Roff 1999; Gage *et al.* 2006). The effects of inbreeding on sperm quality have been documented experimentally (Margulis & Walsch 2002; Michalczyk *et al.* 2010), in captive populations (Roldan *et al.* 1998; Asa *et al.* 2007; Ruiz-Lopez *et al.* 2010) and in the wild (Gage *et al.* 2006). In addition, Keller & Waller (2002) reported compromised fertility as a consequence of inbreeding depression for over half the reviewed studies. Therefore, poor sperm quality has the potential to lower fertilisation success, with severe consequences for population growth and persistence. As a result, it is important to identify if sperm quality is impacted by inbreeding, particularly for populations where breeding is intensively managed, for example using artificial insemination.

The kakapo (*Strigops habroptilus*) is a large, flightless, nocturnal parrot that was once found throughout New Zealand (Powlesland *et al*. 2006). Kakapo are highly vulnerable to introduced mammalian predators (Lloyd & Powlesland 1994), and were thought to be functionally extinct until the discovery of a remnant population in a remote area of Stewart Island in 1977 (Powlesland *et al*. 2006). The species was reduced to a total of just 51 individuals by 1992 (Powlesland *et al.* 2006) and now only survive on predator-free islands. Therefore, the genetic diversity of the extant kakapo is very low; with the exception of a male named "Richard Henry" who was the sole survivor from the Fiordland population (mainland New Zealand). Richard Henry is now deceased, but sired three offspring in his lifetime, two of which were male and one was female (Miller *et al*. 2003).

Kakapo breeding is synchronised with years of high rimu fruit abundance, which occurs every two to seven years (Merton *et al.* 1984; Powlesland *et al.* 1992). Kakapo are lek breeders (Merton *et al.* 1984), with males establishing and occupying "track and bowl systems" (TBS) during breeding years, typically during the months of January and February (Eason *et al.* 2006). Consistent with lekking behaviour there is no pair bond formed and male kakapo provide no further investment in their offspring following insemination (Eason *et al.* 2006). Therefore, male reproductive success is entirely dependent on the male's ability to sire offspring (Trivers 1972).

Using a method known as heterozygosity-fitness correlations (HFCs) (Hansson & Westerberg 2002; Pemberton 2004), multi-locus heterozygosity at neutral molecular markers can be correlated with fitness-related traits to infer inbreeding depression. Although pedigrees are the preferred method for the quantification of inbreeding depression, multi-generational pedigree data are often not available for natural populations (Frankham *et al.* 2002; Pemberton 2008; Grueber *et al.* 2011a). HFCs have allowed inbreeding depression to be identified in populations without available pedigrees and

where identifying the impacts of inbreeding is important for conservation management (Frankham *et al.* 2002). In addition, HFCs have already proven useful for kakapo, with detection of reduced hatchability for more inbred females (see Chapter 2).

3.1.1 Study aims

It was the aim of this research to identify whether inbreeding is causing sperm quality to be compromised in kakapo. This aim was investigated by correlating morphological abnormalities, concentration and motility of spermatozoa with molecular estimates of individual homozygosity. The use of molecular estimates was necessary due to the lack of multi-generational pedigree data for kakapo, but also provides an opportunity to increase our understanding of the ability of HFCs to detect inbreeding depression.

3.2 Methods

3.2.1 Sperm data analyses

Sperm data were collected and assessed by the New Zealand Department of Conservation (DOC). Collection of kakapo semen began in 2005 for the purpose of artificial insemination and assessment of male kakapo fertility (Daryl Eason pers. comm.). All studied males were at breeding age (more than five years old) and displayed courtship behaviour (booming) at the time of ejaculate collection. Sperm samples were obtained from male kakapo *in situ* using a species-specific massage technique of the lower back, perineum and cloaca for approximately 0.5 to 2 minutes (Blanco unpublished data). The cloaca was then everted and if the technique proved successful, semen was ejaculated. In an attempt to avoid contamination from faeces, urine and bacteria, semen was collected directly into a 75μL capillary tube. This process was repeated several times, depending on the bird's behaviour and semen production. The collected semen was diluted 1:20 with Lactated Ringers Saline solution. Sperm motility and concentration were assessed immediately after dilution. The motility was either a visual estimate of the percentage of moving sperm in a sample or a count of 100 sperm. Any movement was considered motile. Concentration was determined on a gridded counting chamber using normal sperm counting methods.

A smear was made by mixing 2μL of this solution with 3μL of eosin/nigrosin morphology stain (Bakst & Cecil 1997; Björdahl *et al*. 2004) on a microscope slide. This

slide was dried and a coverslip mounted with DPX glue (Leica Microsystems). Morphological examination was carried out at a later date, using 400 and 1000 x magnification. The glass slides were kept at room temperature as bird sperm generally does not suffer cold shock as mammal sperm does (Parks & Lynch 1992). Diagrams and descriptions of specific kakapo sperm morphology were recorded to maintain consistency in the comparison between samples. Some variation in sperm traits was inflicted by the sampling process. For example, contamination by urine, uric acid, bacteria, blood, rain and debris, caused damage to the sperm either at the time of collection or during processing of the morphology slides. Abnormalities due to contamination could be defined by the morphological characteristics of the abnormality; for example, damage to the sperm membranes, bent flagellum, or when uric acid crystals could be identified. Any abnormalities caused by contamination were omitted from the calculation of the percentage of abnormalities per sample. This also removed any variation caused by refinement of the sampling technique over the years and collection by different samplers.

Determining sperm motility and concentration involved all sperm, including abnormalities and contamination. Any abnormalities that resulted from the sampling process were excluded when calculating the percentage of sperm abnormalities. Morphological abnormalities were then spilt into subcategories according to the location of the defect. These included; head (abnormal shape, bent/buckled, double/forked, giant, microcephaly, abnormal acrosome) mid-piece (proximal or distal cytoplasmic droplet, lateral attachment of head to midpiece, giant, micro, swollen, coiled/wrapped) and flagellum (multi-flagellation) (see Appendix A). The *in situ* nature of sperm sampling meant that collected data was opportunistic and all sperm characteristics could not be assessed for every sample.

3.2.2 Molecular methods

Twenty-five polymorphic microsatellite markers were used to genotype male kakapo according to the methods outlined in Chapter 2. No significant linkage disequilibrium has been detected among these microsatellite loci (Robertson *et al.* 2009). No errors were detected after genotyping all individuals twice (Hoffman & Amos 2005; Robertson *et al.* 2009, Robertson unpublished data) Both internal relatedness (IR, Amos *et al.* 2001) and heterozygosity by loci (HL, Aparicio *et al.* 2006) are appropriate measures of heterozygosity for kakapo, but they also correlated well (see chapter 2), hence only IR was used for the analysis of inbreeding on sperm quality. Male IR was calculated using the

IRMacroN4, developed by Amos *et al.* (2001). Negative IR values are indicative of more heterozygous males and positive values of more homozygous males; values ranged between -0.5 and 0.5 (Amos *et al.* 2001).

3.2.3 Model fitting

Generalised linear mixed models were used to assess the influence of male heterozygosity on sperm concentration ($x10^4 \mu L$), motility (% of moving sperm in a sample) and morphological abnormalities (% of abnormal sperm in a sample). Each sperm characteristic was entered as the response variable into independent models and fitted with a Poisson link. Male IR was entered as the explanatory variable of interest. Male ID was included as a random effect to account for multiple samples taken for some males. Year was also included as a random effect to allow for variation in sperm characteristics between years, as annual variation in rimu fruit levels (Eason & Moorhouse 2006; Cockrem 2006) and other external cues could also influence sperm production. The Julian date the semen was collected was also included as a fixed effect to account for seasonal variation in sperm traits (Penfold *et al.* 2000). Male age (Kidd *et al.* 2001; Pizzari *et al.* 2008) was also included as a fixed effect to control for demographic variation in sperm quality. However, since all except three of the males (Sinbad, Gulliver and Stumpy) hatched on Stewart Island before the population was discovered in 1977, age was unknown for most male kakapo in this study. Therefore, minimum age was estimated by summing of the number of years since discovery with five juvenile years.

Global models were run using the *lmer* function in the *lme4* package (Bates and Maechler 2009) in R (R Core Development Team 2009). Each model was standardised using the standardize function in the *arm* package (Gelman *et al.* 2009) so that effect sizes were comparable. Using functions available in the *MuMIn* package, a subset of models was produced for each standardised global model. These models were ranked according to Akaike's Information Criterion (AICc) values. If there was clearly only one model ranked in the top 2ΔAICc then this model was taken as the top model. If more than one model ranked in the top 2ΔAICc then a natural average method (Burnham & Anderson 2002) was used to model-average these models to produce weighted parameter and standard error estimates for each predictor (Nakagawa & Freckleton 2010; Grueber *et al.* 2011b). A summary of parameter estimates, standard errors, and associated p-values was produced for each model.

3.3 Results

3.3.1 Concentration

The average sperm concentration for the kakapo sampled was approximately 46.6 x$10^4 \mu L$ (± 54.9, n = 33). When modelling the effect of male heterozygosity on sperm concentration, only two models fell within the top 2ΔAICc of the subset of ranked models (Table 3.1). Both models contained the date of semen collection; the second ranked model also contained IR (Table 3.1). Therefore, the model containing these two variables was taken to best explain variation in sperm concentration. The resulting parameter estimates predicted that sperm concentrations were significantly higher for samples collected later in the breeding season (Table 3.2). The effect of IR on sperm concentration was stronger than the sperm collection date, but not significant due to the high standard error and the inclusion of zero in the 95% confidence interval (Table 3.2). However, the effect was in contrast to that predicted; that is more homozygous males had higher sperm concentrations.

Table 3.1: The generalised linear mixed models assessing the effects of inbreeding on sperm concentration ($x10^4 \mu L$). Male ID and year were included as the random factors. Male IR (Amos *et al.* 2001) was entered as the genetic factor of interest. "Collection date" was the Julian date the semen was collected and "Age" was the estimated age of the male. Models are ranked according to ΔAIC_c (the difference in AIC_c from the strongest model). The table also includes the AIC_c value, the degrees of freedom (df) and the Akaike weight. The selected models are in bold.

Rank	Model	AIC_c	ΔAIC_c	df	Weight
1	**Collection date**	361.6	0.00	4	0.479
2	**Collection date + IR**	362.6	0.98	5	0.293
3	Collection date + Age	364.0	2.36	5	0.147
4	Collection date + IR + Age	365.2	3.58	6	0.080
5	Constant	399.2	37.62	3	0.000
6	IR	399.9	38.34	4	0.000
7	Age	401.3	39.73	4	0.000
8	Age + IR	402.2	40.65	5	0.000

Table 3.2: The standardised parameter estimates (β) and standard errors (SE) produced by model-averaging the top $2\Delta AIC_c$ models from a subset of generalised linear mixed models with Poisson error structure. Models were analysing the effect of inbreeding on sperm concentration ($x10^4 \mu L$) for male kakapo. Male ID and year were fitted as the random effects variable. Fixed effects included male IR, male age and the Julian date the sample was taken. Test statistics (z) and associated p-values, 95% confidence intervals (CI) and relative importance (RI) are also included in the table. Significant parameter estimates (p<0.05) are in bold.

	β	**SE(β)**	***z***	**95%CI**	***P***	**RI**
(Intercept)	1.884	0.789	2.386	3.462 to 0.306	0.017	
Collection date	**0.573**	**0.097**	**5.894**	**0.767 to 0.379**	**<0.001**	**1.00**
IR	0.821	0.595	1.378	2.011 to -0.369	0.168	0.38

3.3.2 Motility

The sampled male Kakapo had a mean of 34.3% motile sperm (± 27.8, n = 36). Modelling the effect of inbreeding on sperm motility resulted in only one model ranking in the top 2ΔAICc, of which included semen collection date as the only fixed effect (Table 3.3). The next two best models in the subset contained the semen collection date as well as the fixed effects: age then IR, respectively (Table 3.3). Although these two models were ranked with ΔAICc slightly higher than two, IR was the variable of interest and so both models were included in the top ranked models.

Age and IR had the strongest effect sizes, indicating that sperm motility decreases as males increased in both age and homozygosity, but in neither case were the conclusions robust as both effects were associated with large standard errors and 95% confidence intervals included zero (Table 3.4). Collection date had a moderate effect size with the percentage of motile sperm significantly higher when samples were collected later in the breeding season (Table 3.4). Although semen collection date was the only significant effect size, it was the weakest out of the three variables (Table 3.4).

Table 3.3: The generalised linear mixed models assessing the effects of inbreeding on sperm motility (% of moving sperm) in kakapo. Male ID and year were included as the random factors. Male IR (Amos *et al.* 2001) was entered as the genetic factor of interest. "Collection date" was the Julian date that the semen was collected and "Age" was the estimated age of the male. Models are ranked according to ΔAIC_c (the difference in AIC_c from the strongest model); The table also includes the AIC_c value, the degrees of freedom (df) and the Akaike weight. The selected models are in bold.

Rank	Model	AIC_c	ΔAIC_c	df	Weight
1	**Collection date**	**291.4**	**0.00**	**4**	**0.561**
2	**Collection date + Age**	**293.5**	**2.11**	**5**	**0.195**
3	**Collection date + IR**	**293.8**	**2.41**	**5**	**0.168**
4	Collection date + IR + Age	296.0	4.67	6	0.054
5	Constant	299.1	7.77	3	0.012
6	Age	301.2	9.80	4	0.004
7	IR	301.3	9.95	4	0.004
8	Age + IR	303.5	12.10	5	0.001

Table 3.4: The standardised parameter estimates (β) and standard errors (SE) produced by model-averaging the top $2.5\Delta AIC_c$ models from a subset of generalised linear mixed models with Poisson error structure. Models were analysing the effect of inbreeding on sperm motility for male kakapo. Male ID and year were fitted as the random effects variable. Fixed effects included male IR, male age and the Julian date the semen sample was taken. Test statistics (*z*) and associated p-values, 95% confidence intervals (CI) and relative importance (RI) are also included in the table. Significant parameter estimates ($p<0.05$) are in bold.

	β	**SE(β)**	***z***	***95% CI***	***P***	**RI**
(Intercept)	2.939	0.420	7.004	3.779 to 2.099	<0.001	
Collection date	**0.246**	**0.077**	**3.198**	**0.400 to 0.092**	**<0.01**	**1.00**
Age	-0.443	0.564	0.790	0.685 to -1.571	0.429	0.21
Male IR	-0.332	0.607	0.547	0.882 to -1.546	0.585	0.18

3.3.3 Morphology

Mean number of sperm abnormalities per sample was 24.1 % (± 22.4, n = 60). When analysing the effect of inbreeding on the percentage of sperm abnormalities, two models were ranked in the top $2\Delta AIC_c$ (Table 3.5). The first contained IR as the only fixed effect; the second contained just the constant. Therefore, the top ranked model, with IR as a fixed effect, was taken to best explain variation in sperm morphology. This model determined IR to be a strong predictor of sperm morphology with the percentage of abnormal sperm significantly higher for more homozygous males (Fig. 3.1; β = 0.656 [SE = 0.321], 95%CI = (1.298, 0.014), p<0.05). The majority of morphological abnormalities were concentrated in the head of the spermatozoa (19.8% ± 18.2, n = 60), with a very small number of abnormalities detected in the mid-piece (1.8% ± 2.9, n = 60) and flagellum (0.9% ± 2.0, n = 60) sections. The relationship between heterozygosity and sperm abnormalities was further supported by the low percentages of abnormalities for Richard Henry's offspring (Fig. 3.1 & 3.2).

Inspection of the data (Fig 3.1) revealed a bird, known as "Lionel", to have a negative IR value but the highest percentage of sperm abnormalities of any other male. Further investigation revealed that 10% of his sperm had multi-flagellation, a proportion that was considerably higher than the sample mean of 0.9% (Table 3.1). This high degree of multi-flagellation appears to be the reason Lionel has comparatively high levels of sperm abnormalities, as Lionel does not stand out as an outlier when only head abnormalities plotted against male IR (Fig 3.2). Since Lionel stands apart from the normal trend in this way, the analysis was repeated with Lionel excluded. In doing so, the relationship between homozygosity and sperm abnormalities was strengthened (β = 0.772 [SE = 0.289], 95%CI = (1.350, 0.194), p<0.01).

Table 3.5: The generalised linear mixed models, ranked according to ΔAIC_c (the difference in AIC_c from the strongest model), assessing the effects of male heterozygosity on sperm abnormalities (%). Male ID and year were included as the random factors. Male IR (Amos *et al.* 2001) was entered as the genetic factor of interest. "Collection date" was the Julian date that the semen was collected and "Age" was the estimated age of the male. The table also includes the AIC_c value, the degrees of freedom (df) and the Akaike weight. The selected model is in bold.

Rank	Model	AIC_c	ΔAIC_c	df	Weight
1	**IR**	**220.1**	**0.00**	**4**	**0.387**
2	**Constant**	**221.6**	**1.53**	**3**	**0.180**
3	IR + Age	222.2	2.13	5	0.133
4	IR + Collection date	222.4	2.31	5	0.122
5	Age	223.8	3.74	4	0.060
6	Collection date	223.8	3.78	4	0.059
7	IR + Age + Collection date	224.6	4.53	6	0.040
8	Age + Collection date	226.1	6.06	5	0.019

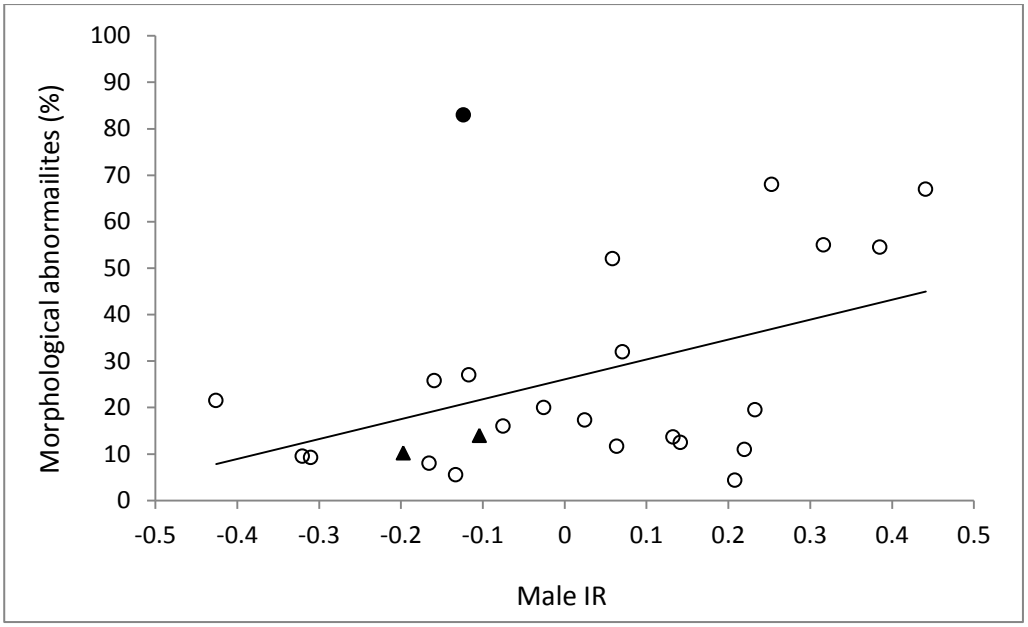

Figure 3.1: The relationship between male homozygosity (IR, Amos *et al*. 2001) and the percentage (%) of morphological abnormalities in their sperm. IR ranges between -0.5 and 0.5 and positive IR values represent more homozygous males and negative IR values represent more heterozygous males. The filled circle data point was a bird named "Lionel" that was an outlier due to a larger number of flagellum abnormalities. The filled triangles are the two offspring sired by the Fiordland bird "Richard Henry".

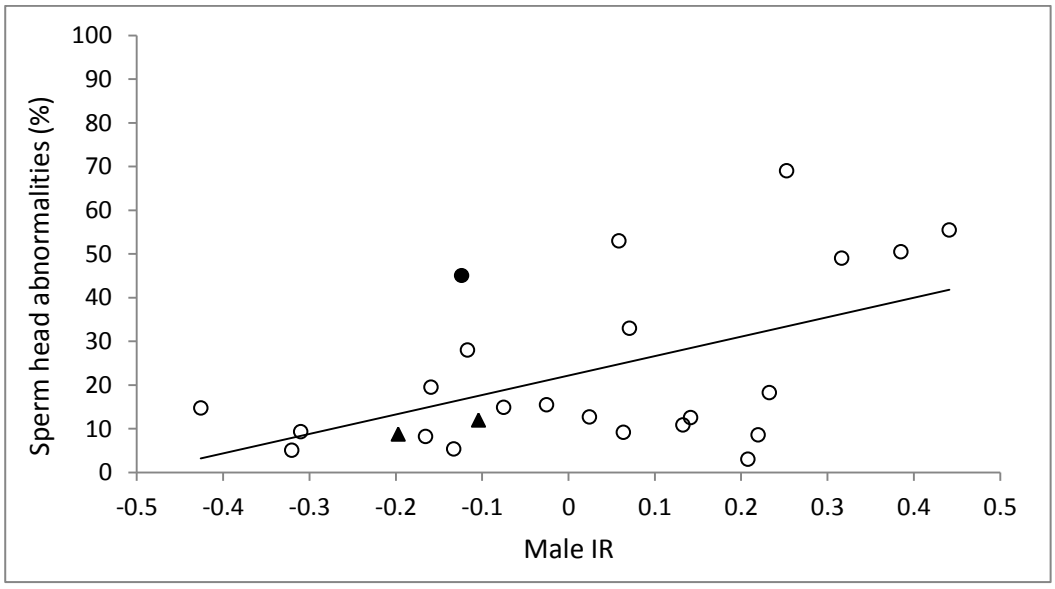

Figure 3.2: The relationship between male homozygosity (IR, Amos *et al*. 2001) and the percentage (%) of sperm head abnormalities. IR ranges between -0.5 and 0.5 and positive IR values represent more homozygous males and negative IR values represent more heterozygous males. The filled data point was a bird named "Lionel" who was an outlier due to a larger number of flagellum abnormalities but conforms to the general trend here. The filled triangles are the two offspring sired by the Fiordland bird "Richard Henry".

3.4 Discussion

Here I identify which sperm quality traits are being influenced by multi-locus heterozygosity levels in kakapo. In doing so, I show strong evidence for inbreeding depression in kakapo.

3.4.2 Sperm concentration and motility

When analysing the effect of inbreeding on sperm motility and concentration, neither were found to be influenced by male heterozygosity. However, this was most likely due to smaller sample sizes for these two sperm characteristics limiting the statistical power of the models, and the limitations associated with measuring sperm traits in the field. As such, failing to detect a relationship between these sperm traits and multilocus heterozygosity does not necessarily mean they are not affected by inbreeding, particularly as both the average sperm concentration and percentage of motile sperm for kakapo were considerably lower than that found in other avian species (*e.g.* Hispaniolan parrots, *Amazona ventralis*, Brock 1991; northern pintail ducks, *Anas acuta*, Penfold *et al.* 2000; and blue rock pigeons, *Columba livia*, Sontakke *et al.* 2004). Burrows (2006) also detected a decline in sperm motility with increasing severity of population bottleneck in introduced New Zealand birds. Therefore, it is still possible that, like the introduced birds of New Zealand, inbreeding depression is having deleterious effects on both these sperm traits in kakapo and an increased sample size would potentially enable the detection of such an effect.

Both sperm motility and concentration were determined to be higher in semen collected later in the breeding season. This likely reflects the annual gonadal development cycle of kakapo, during which maximum sexual behaviour is reached when testosterone levels peak (Cockrem 2006). Similarly, in northern pintail ducks, semen quality (concentration and morphology) was found to increase as testosterone concentrations increased, reaching a peak approximately two weeks after the onset of sperm production (Penfold *et al.* 2000). This peak in sperm quality was thought to be related to a critical level of testosterone being reached, coinciding with the female's egg-laying period. Although peak testosterone levels will vary between individual male kakapo (Cockrem 2006), sperm concentration and motility for kakapo appears to be related to peak levels of testosterone being reached later in the breeding season. To confirm this trend, hormone levels in kakapo would need to be quantified throughout the breeding season and correlated with semen quality.

3.4.2 Sperm morphology

The investigation of the effect of inbreeding on kakapo sperm morphology revealed a higher percentage of abnormal spermatozoa for the male kakapo with higher levels of marker-derived homozygosity. Therefore, deleterious recessive alleles appear to be causing disruptions to the complex process of spermatogenesis in the more homozygous male kakapo. This trend is in agreement with a number of studies that have linked sperm morphology to levels of inbreeding (van Eldik *et al.* 2006; Gage *et al.* 2006; Asa *et al.* 2007; Ruiz-Lopez *et al.* 2010). However, this is the first documentation of the negative impact of inbreeding on sperm morphology in an avian species.

The majority of abnormal morphologies detected in kakapo were in the head of the spermatozoa, with very few abnormalities detected in the mid-piece and flagellum. Ruiz-Lopez *et al.* (2010) reported a similar trend in the spermatozoa from three *Gazella* species. In their study, sperm DNA fragmentation was more prevalent as inbreeding increased, and this in turn was associated with higher numbers of sperm head abnormalities and lower motility. They concluded that the increased expression of deleterious recessive alleles that results from inbreeding caused damage to sperm DNA. Such damage can cause problems with DNA compaction, leading to abnormal sperm head morphology (Miller *et al.* 2010; Ruiz-Lopez et al. 2010). Poor DNA integrity in human sperm has also been associated with abnormal morphology, as well as lowered concentration and motility (Schlegel 2006). This relationship has also been detected in birds, with a high prevalence of excess DNA in the spermatozoa of the threatened Houbara bustard (*Chlamydotis undulate*) (Wishart *et al.* 2002). Therefore, the production of fragmented DNA seems a likely explanation for the large number of sperm head abnormalities in kakapo. Should this be the case, inbred males may also be compromising the viability of their offspring after passing on deficient DNA (Roldan & Gomendio 2009). Investigation into the integrity of the DNA within the abnormal kakapo sperm would help to confirm whether this is the true mechanism behind the high prevalence of sperm head abnormalities.

In this study, multi-flagellation was the only abnormality able to be detected in the sperm flagellum. An unusually large proportion of these mutli-flagellated sperm were detected in the individual named "Lionel". Multi-flagellation has been described in the sperm characteristics of a knockout mouse model for the gene $Hrb^{-/-}$ (HIV-1 Rev-binding/interacting protein) (Juneja & Van Deursen 2005). Similarly, a domestic bull (*Bos*

primigenius) was suspected to have a genetic defect relating to the $Hrb^{-/-}$ gene, with 60% of sperm displaying multiple flagella (Kopp *et al.* 2007). Although the presence of this gene has not been reported for avian species, it is possible that Lionel has a mutation in this, or a similar, gene that is causing his sperm to display a high percentage of flagellum abnormalities. Therefore, it appears that this study has potentially detected a recessive mutation in Lionel. Fortuitously, Lionel has already been removed from the breeding population after failing to fertilise multiple clutches (Eason pers. comm.) and will not be permitted to pass on this mutant gene. As Lionel was one of the founding kakapo from Stewart Island, his parents are unknown. As a result, it is not currently possible to determine whether Lionel has siblings among the founding birds, and therefore, whether other birds are also carrying this allele in the heterozygous form. If other kakapo are carrying this deleterious allele, the small population size and low genetic diversity of the kakapo population means that genetic drift and further inbreeding would have the potential to bring this allele to fixation (Charlesworth & Charlesworth 1987).

3.4.3 Consequences for male reproductive success

Lowered sperm quality has the potential to lower male fertilisation success (Oettlé 1993; Bonde *et al.* 1998; Gomendio *et al.* 2000; Malo *et al.* 2005; Asa *et al.* 2007). With inbreeding having deleterious effects on sperm morphology, and potentially on motility and concentration, the ability of inbred male kakapo to sire an egg is likely to be severely compromised. This does appear to be the case for kakapo, with 58% of the eggs laid between 1990 and 2002 failing to hatch, with approximately 40% of these classified as infertile (Elliott *et al.* 2006). Although male homozygosity failed to predict egg fertility (see Chapter 2), the assessment of male fertilisation ability in the absence of maternal effects points toward inbreeding depression playing a role in the high infertility rates for kakapo (see Chapter 4 for further discussion).

The lek mating system of kakapo is also responsible for a highly-skewed male reproductive success, with females more frequently selecting just a few males (Robertson 2006). Lowered sperm quality could also further reduce the chances of some males contributing to the gene pool due to the lowered competitive ability of their sperm (Denk *et al.* 2005; Zajitschek *et al.* 2009). Inbred males may not suffer total reproductive failure, but sperm from a more outbred male will likely outcompete that of an inbred male when mating with the same female (Roldan & Gomendio 2009). Therefore, reduced sperm quality in kakapo has the potential to further restrict male reproductive success as, for

example, an ‘attractive’, yet subfertile male, could reduce the number and fitness of offspring produced in a breeding season.

3.4.4 Usefulness of HFCs for kakapo

The consequences of lowered male fertility for the kakapo population are of great concern for conservation management, as population recruitment and therefore population growth are directly reduced by such effects. The use of HFCs in this study helped to identify the role of inbreeding in sperm quality and this information can now be used to support artificial insemination decisions; for example, to avoid inseminating a female with sperm of lesser quality. The use of heterozygosity as a surrogate to inbreeding coefficients has been debated (Balloux *et al.* 2004; Slate *et al.* 2004; Grueber *et al.* 2008). However, the strong relationship detected here between multilocus heterozygosity and sperm abnormalities suggests that this is a reliable method for detecting inbreeding depression in kakapo, while a comprehensive pedigree remains unavailable. The usefulness of HFCs for kakapo was reinforced by the positioning of Richard Henry’s offspring in the more heterozygous end of the IR continuum (Fig. 3.1 & 3.2).

3.5 Conclusions

Analysis of sperm characteristics using HFCs demonstrates severe inbreeding depression in kakapo. Sperm quality was significantly decreased in males with higher levels of homozygosity via a high proportion of morphological abnormalities, the majority of which were found in the head of the spermatozoa. It is most likely that fragmentation of the DNA in the spermatozoa, caused as a result of the expression of deleterious recessive alleles, is the mechanism behind these head abnormalities. This study did not detect a relationship between male homozygosity and sperm concentration or sperm motility, despite these two sperm traits being considerably low compared to other species. Sample sizes could have played a role in the failure to detect such a relationship and therefore inbreeding cannot be ruled out as having some role in depressing sperm concentration and motility. The reduction in sperm quality for kakapo is likely to be behind the specie’s low fertility rates and HFCs have enabled this to be identified when pedigree data was unavailable.

Chapter 4: General Discussion

The chapters presented in this thesis examine the impact of inbreeding on the reproductive fitness of kakapo. In this chapter, these findings are summarised and their relevance to conservation genetics discussed. The importance of this knowledge in terms of kakapo recovery is also identified and potential management strategies are recommended so that inbreeding depression in kakapo can potentially be minimised. The usefulness of HFCs to identify inbreeding depression in threatened species such as the kakapo is also discussed.

4.1 Detection of inbreeding depression in kakapo

In this study, the influence of inbreeding on the reproductive fitness of kakapo was assessed by correlating variation in early life history traits with molecular estimates of relatedness. The fitness-related traits that were analysed included; female fecundity (clutch size), egg fertility (probability of an egg being fertilised by an individual male), hatching success of fertile eggs (proportion of fertile eggs that a female hatches) and sperm quality (concentration, motility and morphology). The key findings derived from assessing these traits determined that inbreeding is negatively affecting both hatchability and normal sperm morphology in kakapo. More specifically, female kakapo with higher levels of homozygosity hatched lower proportions of fertile eggs (Chapter 2), and, male kakapo with higher levels of homozygosity displayed an increased proportion of spermatozoa with morphological abnormalities in the head of the sperm (Chapter 3). Based on the results detected in Chapters 2 and 3, it appears that deleterious recessive alleles are becoming expressed in the more inbred individuals. These deleterious alleles are causing disruptions to the complex processes involved in embryogenesis and spermatogenesis in kakapo, thereby causing reduced reproductive fitness. Overall, a significant proportion of the high variation in hatching success experienced by kakapo can be explained by inbreeding depression.

4.1.1 Using HFCs

The negative impacts of female IR on kakapo hatching success, and male IR on sperm morphology, were determined using a method previously described as HFCs (see Chapter 1). As there is current debate as to the underlying genetic mechanisms driving HFCs (Hansson & Westerberg 2002; Balloux *et al.* 2004; Grueber *et al.* 2008; Chapman *et al.* 2009; Grueber *et al.* 2011a), the three hypotheses proposed by Hansson & Westerberg (2002) must be considered as potential drivers of the HFCs detected in the present study.

Direct effects, where the markers themselves are responsible for the relationship between heterozygosity and fitness (Hansson & Westerberg 2002), cannot be ruled out as being responsible for the heterozygosity advantage. However, direct effects are generally limited to markers such as allozymes or MHC loci, and so, considering microsatellite markers were used, are unlikely in the present study (Grueber *et al.* 2011a). Based on Robertson *et al.* (2009), the microsatellite markers used to generate HFCs in kakapo did not have any detectable linkage disequilibria; hence, local effects are also unlikely in the present study.

Distinguishing between local and general effects is difficult (Hansson & Westerberg 2002; Szulkin *et al.* 2010). However, general effects seem the most probable explanation for the relationship detected between multi-locus heterozygosity and reproductive fitness in kakapo. This can be attributed to the kakapo data mostly adhering to the two main criteria, described by the HFC literature, which increase the likelihood of generating HFCs reflective of general effects (Slate & Pemberton 2002; Balloux *et al.* 2004; Slate *et al.* 2004; Grueber *et al.* 2011a). The first is the use of a large number of highly polymorphic markers. The use of 25 microsatellites for determining relatedness in kakapo was a reasonably large number of markers compared to most HFC studies, which have typically used less than 20 markers (see Table 3 in Grueber *et al.* 2011a). The second is a high variance in inbreeding coefficients within the population. The kakapo microsatellites had an average expected heterozygosity of 0.47 and an average of 3.3 alleles per loci (Robertson *et al.* 2009). When compared to other HFC studies (see Table 3 in Grueber *et al.* 2011a), this is not a high degree of polymorphism, but did appear sufficient to generate a range of individual heterozygosity values (Fig. 2.2).

Regardless of the underlying mechanism, the strong HFC effects detected in this study of kakapo suggest that fitness is reduced for individuals with lower levels of heterozygosity. This is in contrast to HFC analysis of the takahe (*Porphyrio hochstetteri*), where effect sizes relating heterozygosity to individual life-history stages were weak and statistically insignificant, despite documented pedigree-derived impacts of inbreeding on hatching success (Jamieson *et al.* 2003; Grueber *et al.* 2011a). Before HFCs can be truly classified as a reliable indicator of inbreeding depression for kakapo, the correlation between multi-locus heterozygosity and pedigree estimates of inbreeding coefficients is required (Pemberton 2008; Grueber *et al.* 2011a). This will only become possible once a multi-generational pedigree is constructed for kakapo. Until this time, HFCs should be viewed as a beneficial tool for the genetic management of kakapo.

4.2 Influence of inbreeding on male fertility

In Chapter 3, lowered sperm quality of kakapo was determined to be a consequence of increased male homozygosity. The trait that was found to be significantly affected was sperm morphology, with more homozygous males having a higher proportion of sperm head abnormalities. Sperm concentration and motility were also low for kakapo compared to other avian species that have had their sperm quality assessed (*e.g.* Brock 1991; Penfold *et al.* 2000; Sontakke *et al.* 2004). However, these sperm characteristics were not significantly linked to male homozygosity in kakapo, most likely due to a smaller sample size for these two sperm traits. Therefore, sperm generally appears to be of low quality for most male kakapo, but only increased morphological abnormalities can be attributed to inbreeding with any confidence.

Low sperm quality is expected to decrease a male's fertilisation ability (Oettlé 1993; Bonde *et al.* 1998; Gomendio *et al.* 2000; Malo *et al.* 2005; Asa *et al.* 2007). Despite this, no relationship could be identified between male homozygosity and egg fertility (Chapter 2). This suggests that lowered sperm quality has no negative fertilisation consequences and that male kakapo might still be transferring sufficient proportions of normal sperm to fertilise the eggs. In domestic dogs (*Canis lupus familiaris*), Oettlé (1993) reported that fertility was strongly suppressed for males with approximately 40% abnormal sperm, but the majority of males with less than 40% abnormal sperm were still fertile. It is possible that kakapo sperm follows a similar threshold influence on fertility, and fertilisation success is only compromised for males with very high proportions of sperm abnormalities. For example, one male kakapo Lionel who had > 80% abnormal sperm (Fig 3.1) and has thus far failed to sire any offspring, despite copulating with multiple females (Eason pers. comm.).

However, it seems more likely that fertilisation abilities are compromised in kakapo for two main reasons. Firstly, approximately 38.9% of eggs laid between 1990 and 2002 were determined to be infertile (Elliott *et al.* 2006), suggesting that fertility was a major contributing factor towards lowered hatching rates. Second, lowered sperm quality has previously been linked to male subfertility in other species (*e.g.* Oettlé 1993; Bonde *et al.* 1998; Gomendio *et al.* 2000; Malo *et al.* 2005; Asa *et al.* 2007), including avian species (Denk *et al*. 2005; Laskemoen *et al.* 2010). Consequently, it is more likely that fertility is compromised in kakapo, but that the relationship between male IR and egg fertility was not able to be detected in the present study.

One potential reason a relationship between male IR and egg fertility was not detected is the use of the candling method to classify an egg as fertile or infertile. Very early death embryos (*i.e.* in the first few days of development) and infertile eggs (no signs of development, see Chapter 2) can be hard to distinguish using the candling method (Kempaenears *et al.* 1996; Birkhead *et al.* 2008). Misclassification of early embryo deaths as infertile eggs would falsely increase the infertility rate for kakapo and might explain why lowered sperm quality did not appear to reduce fertilisation success. Reliably determining egg fertility during the very early stages of development would require sacrificing potentially viable eggs (Kempaenears *et al.* 1996; Birkhead *et al.* 2008). As the kakapo is a critically endangered species, this is not currently possible and therefore fertilisation rates cannot be reliably determined at this point of time.

Another possible reason sperm quality, but not egg fertility, was linked to homozygosity might be due to variation in semen volumes received by the females. Higher semen volume can increase the probability of fertilisation, through the presence of higher sperm counts (Laskemoen *et al.* 2010). In captive bred Houbara bustards (*C. undulata)*, fertilisation success was associated with sperm concentrations, but not with sperm morphology (Wishart *et al*. 2002). Higher sperm quantities, via multiple copulations, can be sought after by females to increase the chances of receiving normal, viable sperm, and therefore insure against infertility (Sheldon 1994). Egg fertility observations in this study were limited to clutches where one male had exclusive access to a female so that the putative paternity of infertile eggs could be determined. However, some female kakapo potentially mated with a male more than once, thereby increasing the sperm quantity stored in the sperm storage tubules for later fertilisation of the eggs. Further investigation controlling for multiple matings by females and variation in male semen volumes might be more revealing as to the role that sperm quantity plays in fertilisation success.

One way to resolve whether inbreeding reduces the fertilisation abilities of male kakapo would be to increase the sample size in the egg fertility analysis (Chapter 2) thereby increasing the power to detect the influence of male IR. Additionally, including an interaction between sperm quality and male IR, as a potential explanatory variable of egg fertility, might also be revealing. It is also possible that fertilisation success in kakapo is more strongly influenced by sperm concentration and motility, than it is by sperm morphology. The results partly support this hypothesis, whereby sperm collected earlier in the season was significantly more motile and higher in concentration (Chapter 3). Concurrently, the strongest effect in the egg fertility analysis predicted that the males that

first began mating earlier in the season sired a higher proportion of fertile eggs (Chapter 2). Therefore, determining which sperm traits have the most influence on male kakapo fertilisation ability would also increase our understanding of the relationship between sperm quality and fertilisation success. Reassessment of the effect of heterozygosity on sperm motility and concentration when a larger sample size is available, would then enable the link between male homozygosity, sperm quality and fertilisation success to be investigated with more confidence.

4.3 Severity of inbreeding depression in kakapo

The results presented in this study suggest that kakapo are suffering from severe inbreeding depression, because large effect sizes were detected for the influence of IR on both hatching success (β = -1.287 [SE = 0.481]) and sperm morphology (β = 0.772 [SE = 0.289]). In small, isolated populations, the effects of random genetic drift and inbreeding are exacerbated and the probability of a recessive gene becoming fixed is much higher (Frankham 1998; Eldridge *et al*. 1999; Kirkpatrick & Jarne 2000). The detection of such strong influences of heterozygosity on both egg hatchability and normal sperm production is consistent with deleterious recessive alleles being at high frequencies in the kakapo population. Therefore, the severity of inbreeding depression is likely to be reflective of kakapo experiencing a population bottleneck. More specifically, the insular origin of the remnant population (all kakapo with the exception of Richard Henry) and the recent bottleneck of the Stewart Island founders have left the population with little remaining genetic diversity (Miller *et al.* 2003; Robertson 2006; Robertson *et al.* 2009), resulting in a large genetic load. Not only that, the kakapo's lek mating system further reduces the effective population size, strengthening the role of drift in reducing genetic diversity and increasing the genetic load. Although we don't have an estimate of the degree to which inbreeding depression is reducing or inhibiting overall population growth in kakapo, nevertheless its negative effects on individual fitness are important to consider in the kakapo recovery, and indeed in any conservation management program.

4.4 Other variables explaining variation in reproductive fitness

Conclusions drawn from HFCs should be tentative, on the understanding that results are correlative and mechanisms other than inbreeding can also drive variation in early life

history traits. For example, seasonal influences accounted for some variation in more than one of the traits investigated by the present study; including sperm concentration, motility (Chapter 3), the likelihood an egg was fertilised and female fecundity (Chapter 2). An explanatory variable that was included in the model but did not appear to have much influence on reproductive traits was kakapo age. This is inconsistent with previous studies that have reported age to have some influence on both female hatching success (Ortego *et al.* 2010; Grueber *et al*. 2010) and male sperm quality (Kidd *et al.* 2001; Pizzari *et al.* 2008). The use of a subjective approximation of age may have led to an underestimation of the effects of age on reproductive success. Therefore, the present analysis may not have identified the true relationship between kakapo age and reproductive success. Unfortunately, the limited data available for known-aged kakapo (Horn *et al*. 2011) meant that estimation of age was the only way to control for it. As a result, it may be beneficial to repeat this analysis, when more breeding data becomes available, using known aged kakapo only. In addition, hatching success can also be influenced by non-genetic variables (Briskie & Mackintosh 2004), which were not included in the models, such as food and nutrient supply, disease and environmental conditions. Therefore, although inbreeding was determined to be a strong influence on both hatching success and sperm quality, it is not the only factor to be considered when attempting to increase the reproductive success of kakapo.

4.4.2 Inbreeding depression under various environmental conditions

Inbreeding depression can become apparent, or more prominent, under stressful and competitive environments (Pray *et al*. 1994; Armbruster & Reed 2005). For example, harsh environmental conditions on the Galapagos Islands increased the severity of inbreeding depression in the cactus finch (*Geospiza scandens*) (Keller *et al.* 2002). Although the present study did not assess environmental influences directly, it is possible that the effects of inbreeding in kakapo could be, or already are, accentuated under certain conditions. For example, inbred females with less suitable home ranges (*i.e.* on a more exposed side of the island) may have higher incidents of hatching failure than an inbred female in a higher quality home range. The influence of environmental conditions on inbreeding may be particularly relevant for the future management of kakapo, because translocation to habitat that varies from the usual habitat on Stewart/Codfish Islands might cause increased inbreeding depression, as was experienced by translocated takahe (Jamieson & Ryan 2000).

Inbreeding depression also might be accentuated when the population becomes less intensively managed. All kakapo currently receive regular health checks and supplementary feeding to ensure physical condition is maintained (Elliott *et al.* 2001; Powlesland *et al.* 2006; Eason pers. comm.). As the population increases, the recovery group aims to begin establishing populations that are less intensively managed (Elliott *et al.* 2001). However, reducing the intensity of management could cause the effects of inbreeding depression to become more severe or cause previously undetected effects to become apparent. For example, should supplementary feeding be reduced or removed, competition for limited resources might result in even further reduced hatching success for female kakapo. Although it is unreasonable to expect that all kakapo will be intensively managed in the future, care should be taken to consider the potential genetic impacts before management decisions are made. Habitat suitability at potential new translocation sites could also be taken into account to minimise inbreeding depression in an establishing population.

4.5 Population bottlenecks in New Zealand endemic species

Many New Zealand endemic species have undergone severe population bottlenecks as a result of extensive habitat modification and introduction of mammalian predators (Innes *et al*. 2010). Genetic bottlenecks were, until recently, thought to have little or no effect on New Zealand endemic species due to this extended history of severe bottlenecks (Pain 2002; Jamieson *et al*. 2006). However, the consequences of genetic bottlenecks experienced by New Zealand endemic birds are being increasingly uncovered (Briskie & Mackintosh 2004; Brekke *et al.* 2010; the present study). According to Briskie & Mackintosh (2004), population bottlenecks where the total number of individuals reaches less than 100-150 are expected to demonstrate severe inbreeding depression. This appears to be the case for kakapo, after passing through a population bottleneck of only 51 individuals (Powlesland *et al*. 2006). The high levels of hatching failure are reflective of this population size reduction and associated inbreeding depression. This is consistent with other endangered New Zealand endemic birds that have also experienced reduced hatchability as a result of inbreeding depression, for example, the black robin *(Petroica traverse)* (Bulter & Merton 1992), takahe (Jamieson *et al.* 2003), hihi *(Notiomystis cincta*) (Brekke *et al.* 2010) and kaki *(Himantopus novaezelandiae)* (Hagen *et al.* 2011). Thus far, the extent to which sperm quality is affecting other New Zealand endemic species has not

been thoroughly assessed. However, Burrows (2006) did determine sperm quality to be reduced in bottlenecks of bird species introduced to New Zealand. Given this finding, it is possible that New Zealand endemic species will follow a similar trend.

Overall, it does appear that New Zealand endemic species that have experienced a population bottleneck tend to experience declines in fitness as a consequence of inbreeding depression. It is now apparent that consideration of the genetic aspects of conservation management should be a high priority. As mentioned earlier (Chapter 1), the severity of inbreeding depression is dependent on the population's genetic lineage and environment (Pray *et al.* 1994; Hedrick & Kalinowski 2000; Chapman *et al.* 2009). Therefore, when determining the effects of inbreeding on New Zealand endemic species, populations should be assessed independently, as effects may vary between locations.

4.6 Management options to minimise inbreeding depression

Detecting reduced fitness in kakapo means that management must be tailored to minimise further inbreeding and retain what genetic diversity still remains in the population. The alternative would be to take no action and given the severity of inbreeding on kakapo reproductive fitness, such a management option would likely compound the influence of inbreeding as the population will remain smaller for a longer period. Consequently, doing nothing is not an option here.

I have mentioned already that consideration of the potential environmental impacts should be taken when making management decisions in the recovery of kakapo. However, there are four more management approaches that could potentially be utilised to manage the genetic impacts of inbreeding in kakapo. These include: (1) purging the population of its genetic load and artificial selection; (2) increasing the effective population size; (3) actively managing matings so that related individuals are not allowed access to each other; and (4) "genetic rescue", whereby introducing one or more unrelated immigrants into the population reduces the genetic load caused by a high frequency of recessive mutations of small effect. The potential advantages and disadvantages of each of these approaches in terms of the management of inbreeding depression in kakapo are discussed below.

4.6.1 Purging the genetic load and artificial selection

Deleterious recessive alleles are more exposed to natural selection in inbred individuals due to their increased genome-wide homozygosity (Keller & Waller 2002). Therefore, it

has been argued that closed populations that experience inbreeding can potentially purge their genetic load, thereby weakening the effects of inbreeding depression (Templeton & Read 1984; Hedrick 1994; Hedrick & Kalinowski 2000). Theoretically, extended periods of close inbreeding should result in most lethal and semi-lethal mutations being purged and a recovery of fitness for the inbred population (Charlesworth & Charlesworth 1987). In a number of experimental studies under controlled conditions, purging has resulted in population fitness rebounds (Saccheri *et al.* 1996; Roff 2002; Swindell & Bouzat 2006). As a result, artificially selecting against these recessive alleles, or purging the genetic load, seems like an appealing approach to minimise the frequency of these alleles in a population.

However, multiple factors create uncertainty on the effectiveness of purging as an option to minimise inbreeding depression. Firstly, purging is not supported by the overdominance theory; one of the two mechanisms behind the detrimental effects of inbreeding (Frankham *et al*. 2002; Keller & Waller 2002). Secondly, there is insufficient evidence supporting purging of deleterious alleles in natural populations that have undergone bottleneck events (Briskie & Mackintosh 2004; Leberg & Firmin 2008). Thirdly, some recessive mutations have only a small effect on fitness (Hedrick 1994). Being effectively neutral, and so under the influence of drift rather than selection, small-effect detrimental mutations would be very difficult to remove from the population (Charlesworth & Charlesworth 1987; Hedrick 1994; Theodorou & Couvet 2002). Additionally, under high levels of inbreeding, these small-effect alleles are more likely to become fixed in the population (Hedrick 1994; Fu *et al*. 1998). Lastly, and most importantly, intentional inbreeding can significantly increase the risk of extinction, especially when populations are small and are under environmentally stressful conditions (Hedrick 1994; Frankham 1995; Fu *et al*. 1998; Wang 2000; O'Grady *et al*. 2006). The use of purging as a means to reduce inbreeding depression is not a suitable option for kakapo. In reality, purging is more likely to increase the genetic load and put the population at further risk of extinction

An alternative to purging is to artificially select against deleterious alleles by removing deleterious allele carriers from the population. Lionel's sperm flagellum abnormalities (Chapter 3) provide a good example of a potential deleterious recessive mutation in kakapo that has severe consequences for individual fitness. Fortunately, due to this mutation causing Lionel to be infertile, he has been unable to pass this allele on to successive generations. However, it is still possible that other male kakapo are carrying

this allele, in the heterozygous form, particularly since the Stewart Island founders have such low levels of genetic diversity and are likely to related to some degree (Robertson *et al.* 2011b). The potential for multiple carriers of this allele would mean that a significant population size reduction would be required to remove it from the population (see Ralls *et al*. 2000). For example, in Californian condors (*Gymnogyps californianus*) even removing high probability carriers for an allele causing lethal chondrodystrophy necessitated a population reduction to approximately half the original size (Ralls *et al*. 2000).

The associated declines in genetic diversity and increased extinction risk, due to stochastic factors, make selecting against Lionel's deleterious allele a non-viable option for kakapo. Even if artificial selection were a viable option for this particular allele, there are potentially many other lethal or semi-lethal alleles in the kakapo population that are reducing fitness, therefore, removing all or most mutations would be near impossible.

4.6.2 Increasing the effective population size

Increasing the effective population size is a relatively easy and effective option for reducing the effects of inbreeding, as this would minimise the probability of alleles drifting to fixation (Kirkpatrick & Jarne 2000; Hedrick & Fredrickson 2010). For species that still contain multiple populations, and/or captive individuals that could potentially be reintroduced into the wild, this may be as simple as translocating individuals between populations to introduce gene flow. For kakapo, however, there is effectively only one population that is managed on two main offshore islands and it is severely lacking in genetic diversity (Miller *et al.* 2003; Robertson 2006; Robertson *et al.* 2009). In addition, the chances of finding any more kakapo surviving in remote areas of New Zealand (*e.g.* Fiordland) are extremely low. Consequently, increasing the effective population size through outsourcing is not an available option to improve population fitness for kakapo.

It is possible to prevent the effects of inbreeding depression becoming more severe by conserving what remains of the genetic diversity in the kakapo population. This can be achieved by maximising the effective population size (Allendorf & Luikart 2007; Fernández *et al.* 2008; Hedrick & Fredrickson 2010) by maintaining gene flow between island subpopulations and attempting to ensure that all the Stewart Island founder birds contribute a high proportion of their alleles to the next generation. The latter might be achieved by minimising variation in reproductive success between individuals (Robertson 2006; Robertson *et al*. 2011b). Creating a goal number of offspring per founder (*e.g.* ten offspring) would mean that each founder has a high probability of passing on their alleles

and could aid in deciding when lineages might be over or under represented in the population.

4.6.3 Management of matings

The third option for managing inbreeding depression in kakapo is to actively manage matings between individuals so that related individuals cannot mate. Although full control of matings is only feasible in captive populations, the use of intensive monitoring and artificial insemination in kakapo means that matings can be managed to some extent. Already, individuals are translocated between islands in an attempt to separate the closely related individuals and over-represented birds are removed from the breeding islands (Elliott *et al.* 2001; Robertson 2006). This practice should be encouraged and will help to minimise inbreeding events.

One way to manipulate matings would be to remove the more homozygous individuals from the breeding population. Males, such as Barnard, Ben and Gumboots, might be considered for removal since they demonstrate high numbers of sperm abnormalities and IR estimates close to 0.4. However, this is not likely to dramatically improve egg fertility, as the link between sperm quality and fertilisation success is still not certain for kakapo. Removing females, such as Ellie, Esperance and Solstice, who have IR estimates greater than 0.4 and low hatching rates, might also be considered. Removal of these females might be expected to improve the breeding prospects of the more heterozygous females, through increased access to resources and higher quality males. However, as mentioned earlier, female kakapo tend not to move to more suitable home ranges when they become available (Whitehead *et al*. 2012). This would also assume that access to high quality males is limited, which is not likely the case for kakapo due to their lek mating system (Powlesland *et al.* 2006). Therefore, removing the more homozygous females is unlikely to improve the breeding for more heterozygous individuals. Consequently, removing more homozygous individuals of either sex may not result in improvements to reproductive fitness. Even more concerning is the potential to increase inbreeding depression with the removal of individuals as the effective population size will be further reduced. Not only that, any rarer alleles that these individuals might still possess would also be lost from the population, further decreasing the genetic diversity in the population.

An alternative approach might be to prioritise management intervention for those offspring produced by the more heterozygous individuals. Currently, the kakapo recovery

program has an emphasis on producing chicks to rapidly increase the population size to minimise the risk of extinction due to stochastic factors and prevent further erosion of genetic diversity (Clout 2006). Given this emphasis, any bird that has shown signs of breeding has been encouraged, with intervention initiated whenever necessary. Prioritising intervention to eggs that are more likely to be fertile and subsequently hatch (*i.e.* eggs produced by more heterozygous individuals) would enable resources to be more efficiently utilised. If left unmanaged, offspring that are homozygous at deleterious alleles can be purged from the population through natural selection

Constructing a pedigree for the kakapo population would greatly help the management and prioritisation of matings. Currently, the use of the pair-wise relatedness estimators, such as the Wang estimator (Wang *et al*. 2002), is the only way to distinguish the relatedness between founding Stewart Island Kakapo. Although this method is suitable to minimise matings between the more related individuals, sometimes the values generated may not accurately reflect the true relatedness between individuals. For example, Fig 2.1 shows known first-order relatives to have a Wang value close to zero. While this method of determining relatedness is suitable for the time being, as the population becomes more multi-generational and complex, distinguishing relatedness, genetic uniqueness and genetically over- or under-represented lineages, would become more straightforward with the construction of a pedigree. Constructing a pedigree will also help to further confirm the effects of inbreeding depression and determine how well multi-locus heterozygosity can estimate inbreeding coefficients.

4.6.4 Genetic rescue

The genetic diversity of a population can be increased with an approach known as "genetic rescue". Genetic rescue is when individuals that contain new alleles are introduced to a population (Edmands 2007; Bouzat *et al.* 2009; Hedrick & Fredrickson 2010). These new alleles can reduce fitness impacts caused by deleterious alleles that have drifted to high frequencies in the population, as a result of them being of small effect and so appear neutral under selection. For example, the introduction of new genes improved hatching rates in a greater prairie chicken (*Tympanuchus cupido pinnatus*) population (Westemeier *et al.* 1998). Similar genetic rescues have only needed a few individuals to reduce the impacts of a high genetic load (Tallmon *et al.* 2004).

A form of genetic rescue is a potential management approach for kakapo, thanks to Richard Henry, the sole survivor from the Fiordland population. Richard Henry's genetic

value was first realised by Miller *et al.* (2003), identifying higher numbers of minisatellites fragments and lower levels of band-sharing compared to 10 putatively-unrelated Stewart Island males. Following the development of the kakapo microsatellite library by Robertson *et al*. (2009), Richard Henry's genetic distinction was more confidently quantified by Robertson *et al.* (2011b), whereby 22% of alleles identified in Richard Henry (using 27 microsatellites) were not found in the Stewart Island birds. In addition, Richard Henry possessed significantly greater mean heterozygosity (0.741) than the Stewart Island birds (0.463).

The introduction of just one immigrant to a Scandinavian wolf (*Canis lupus*) population managed to increase population growth (Vila *et al.* 2003). Therefore, Richard Henry's genetic make-up represents an opportunity to introduce new alleles into the kakapo population. Unfortunately Richard Henry is now deceased, but he did produce two male and one female offspring. Already these offspring show improved heterozygosity, with lesser impacts of inbreeding (see chapter 3, Fig 3.1 & 3.2). Robertson *et al.* (2011b) estimated that approximately 12.5% of Richard Henry's genome-wide alleles may have already been lost, but if each of his offspring produces 10 or more offspring, there is a greater than 99% chance that the remainder of Richard Henry's alleles will be maintained in the kakapo population. Therefore, prioritising breeding from these birds would introduce new alleles into the population, with the benefit of increasing genetic diversity and reducing the population's genetic load. With a reduced genetic load, the effects of inbreeding will be less severe and the population may see an increase in reproductive success.

One risk of prioritising breeding from Richard Henry's offspring is that, although they possess unique alleles from Richard Henry, they also possess alleles from their maternal line, who was a Stewart Island founder. There is potential for this Stewart Island female to be well represented in the population already. Therefore, prioritisation of breeding from Richard Henry's descendants might be further promoting inbreeding. The ability to track Richard Henry's descendants is just another example of why constructing a pedigree is an important goal for kakapo recovery.

4.7 Artificial Insemination (AI) as a tool for genetic management

Artificial insemination (AI) has recently been introduced into the kakapo recovery program (Robertson *et al*. 2011a). If inbred individuals are used in artificial insemination, the reduced sperm quality of highly homozygous males and reduced hatching success of highly homozygous females is likely to result in lowered probabilities of AI attempts successfully resulting in a chick. Therefore, the use of AI using very homozygous kakapo should be avoided. However, this technique does present an opportunity to attempt to maintain genetic diversity and minimise incestuous matings. This can be achieved by breeding from genetically dissimilar pairs and individuals with rare alleles. More specifically, AI can facilitate breeding from Richard Henry's descendants and ensure all Stewart Island founders are contributing to the gene pool equally. This is particularly relevant for kakapo as males that are less successful at the lek can be given equal opportunities to contribute to the next generation. Minimising incestuous matings might be achieved by, when they do occur, inseminating that female with an unrelated male. Although this is not going to guarantee the related male does not sire the offspring, it does reduce the potential for this to occur. AI will also enable managers to increase the likelihood of Richard Henry's offspring having a significant contribution to subsequent generations.

AI seems to be an appealing option to manipulate kakapo breeding so that inbreeding depression might be minimised. However, this technique is reasonably new in kakapo management and still needs further refinement. For example, presently collected semen cannot be stored for multiple days, making crosses between pairs on different islands difficult. In addition, it is dependent on birds being ready to copulate. For males this is relatively straight forward to determine from their courtship displays. However, it is yet to be determined what signals that a female is in breeding condition. As a result, females are currently inseminated after their first mating as this is the only way to tell they are in breeding condition. Clearly more is to be understood regarding the application of AI in kakapo management, but in the meantime, this method seems the most promising to minimise inbreeding depression.

4.8 Future Research

An important next step towards quantifying the role of inbreeding in kakapo is to identify how these effects are influencing population growth and viability. In order to do this, the fitness effects of inbreeding need to be incorporated into a population viability analysis (PVA). PVAs can be used to predict the extinction risk for populations and are very informative for comparing the effectiveness of different management strategies in species recovery (Boyce 1992). This can aid conservation, also allowing the potential outcomes of genetic management approaches (*e.g.* genetic rescue) to be simulated (Tallmon *et al.* 2004; Johnson *et al*. 2011). In a stochastic computer model of 18 mammal and 12 bird species, O'Grady *et al*. (2006) determined that the estimated time until extinction was significantly reduced when levels of inbreeding depression were included in the model.

Inclusion of inbreeding depression in a population viability model would be highly revealing for kakapo, however, it is not entirely straightforward. Firstly, inbreeding depression can accumulate across life-history stages and failing to account for this in a PVA can underestimate the influence of inbreeding on population viability (O'Grady *et al.* 2006; Szulkin *et al.* 2007; Grueber *et al.* 2010), Therefore, an important next step is to determine whether any other life history stages, such as fledging success and/or juvenile survival, are influenced by inbreeding. Subsequently, determining whether these effects accumulate over life-history stages will enable an accurate estimation of how inbreeding is influencing population growth and viability in kakapo.

Secondly, including inbreeding in a PVA requires the calculation of lethal equivalents (LE) (Frankham *et al.* 2002). One LE is equal to a set of recessive alleles that would cause death (Frankham *et al.* 2002). The slope (-*B*) of the relationship between increases in the inbreeding coefficient (*f*) and the log of overall fitness reductions, determines the number of LEs in a population (Keller & Waller 2002). Inbreeding coefficients are not currently able to be calculated using molecular markers, further highlighting the need to construct a pedigree for kakapo. One potential way to compensate for the lack of inbreeding coefficients is to estimate lethal equivalents, using the slope of fitness between the most heterozygous individuals (e.g. IR>0.4) as a proxy for f=0 and least heterozygous individuals (IR<0.4) as a proxy for f=0.25 (see Grueber *et al*. 2011a). However, this method may not be very reliable since IR and f might not be strongly correlated (Grueber *et al*. 2011a).

Understanding the potential improvement to population growth and viability would enable management decisions to be carried out with higher confidence of the desired outcomes (Johnson *et al*. 2011). For instance, predicting how the population might grow if average population heterozygosity increased after genetic rescue (*e.g.* Johnson *et al*. 2011). Therefore, further research to enable a PVA that includes inbreeding will be able to assist in genetic management decisions.

4.9 Summary of Recommendations

The present study highlights the importance of maintaining genetic diversity in populations. Inbreeding depression in kakapo appears to be reducing both hatching success and sperm quality. These fitness impacts have the potential to slow population recovery efforts and even increase the risk of extinction if left unmanaged. Accordingly, preventing further erosion of genetic diversity and minimising inbreeding should be an important component of the kakapo recovery program. As such the following recommendations are made in no particular order of importance:

- Maintain gene flow between island subpopulations so that kakapo continue to be managed as one meta-population to maximise the effective population size.
- Prioritise management intervention for those offspring produced by the more heterozygous individuals so that resources are optimised and spent on eggs likely to be fertile or to hatch.
- Attempt to minimise variation in reproductive success between founders so each are contributing their genes to the next generation (*e.g.* approximately 10 offspring each to maintain genetic diversity).
- Attempt to produce at least 10 offspring from each of Richard Henry's descendants so that what remains of his genetically-distinct alleles are maintained in the population.
- Construct a pedigree for kakapo to manage matings, determine over- and under-represented lineages, and to further assess inbreeding depression once the pedigree gains depth.

- Continue to investigate and develop artificial insemination as a tool to breed between genetically-dissimilar pairs and as a means of counteracting undesirable pairings. Artificial insemination using very homozygous individuals should be avoided to avoid wasteful inseminations.

- Further investigation into the sperm traits that might affect the fertilisation abilities of male kakapo is also recommended. Concurrently, determining how inbreeding might be influencing these traits and, therefore, egg fertility would also be beneficial to recovery efforts, particularly when selecting males for artificial insemination.

- The role of inbreeding depression in kakapo should be further investigated, using HFCs and pedigree data when it becomes available. For example, determine the role of inbreeding on chick survival and/or immune responses. Investigation of the potential for inbreeding depression in kakapo to accumulate across the life-history continuum would be beneficial in the quantification of inbreeding depression in kakapo.

- Incorporate information on the influence of inbreeding on kakapo fitness into a population viability analysis with the aim of identifying how population growth might be affected by inbreeding depression and how management strategies might reduce or intensify inbreeding effects.

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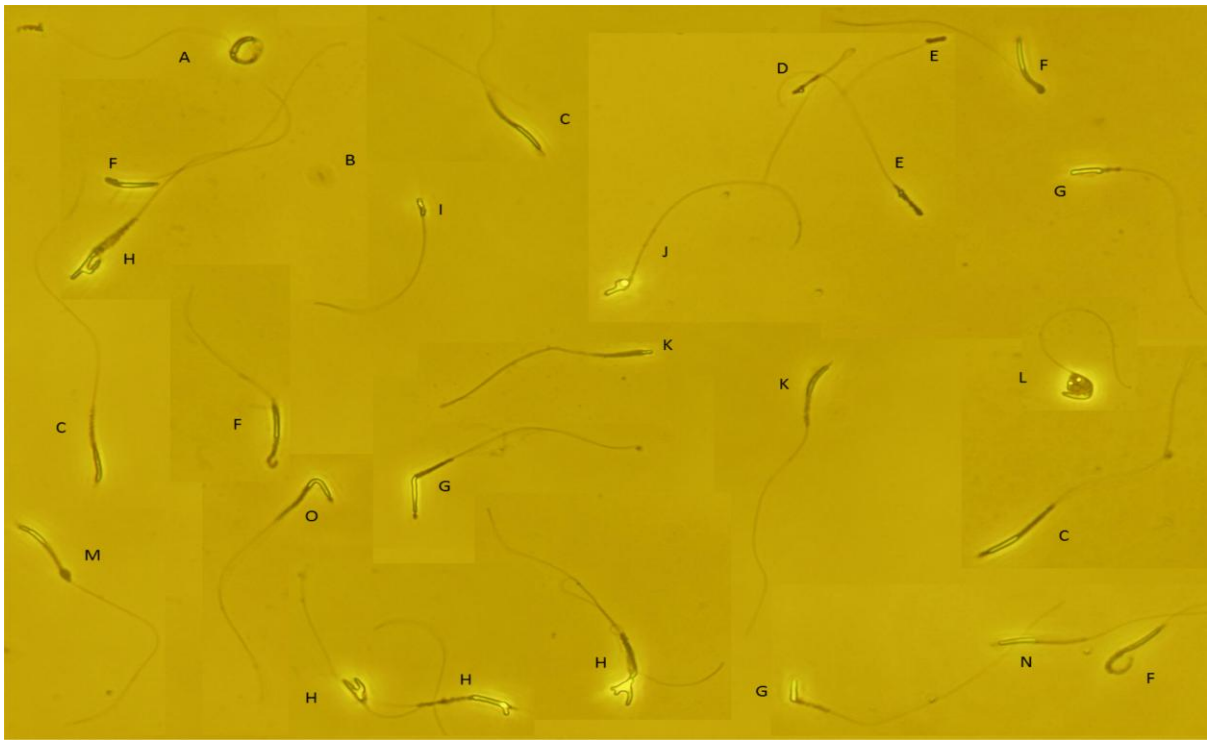
Appendix A:



Examples of some of the sperm abnormalities found in kakapo. Including: (A) Spiraled midpiece and headless; (B) spermatid (immature); (C) normal; (D) microcephaly and bent midpiece (E) missing head (F) bent midpiece; (G) bent neck (head folded back on midpiece);(H) Forked head with swollen midpiece (I) short head bent at neck (head folded back on midpiece) (J) swollen head (due to contamination causing membrane damage and it's bent at neck); (K) microcephaly (L) spermatid (emerging from cell membrane) (M) Distal cytoplasmic drop (N) slightly swollen head; (O) bent head