DO SEVERE GENETIC BOTTLENECKS LEAD TO GREATER REPRODUCTIVE FAILURE?

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Table of contents

Abstract 1

Chapter 1: General Introduction

Background 2

Outline of thesis 7

References 10

Chapter 2: Sperm numbers on the perivitelline membrane of birds passing through severe population bottlenecks: can founder sizes explain hatching failure?

Abstract 15

Introduction 16

Methods 19

Results 23

Discussion 25

Tables and Figures 32

References 39

Chapter 3: A preliminary study of sperm abnormalities in introduced birds that have gone through population bottlenecks in New Zealand

Abstract 44

Introduction 45

Methods

Midpiece abnormalities 48

Motility 50

Results 52

Discussion 54

Tables and Figures 59

References 67

Chapter 4: Effect of population bottlenecks on the structure of avian eggshells

Abstract 71

Introduction 73

Methods 76

Results

Eggshell thickness 82

Pore counts 82

Pore shape 83

Discussion 84

Figures 91

References 97

Chapter 5: General Discussion

Summary of results 101

Conclusions 102

Implications for conservation 107

Future research 111

References 115

Acknowledgements 119

Appendices 120

List of tables

Table 2.1. Mean sperm counts on the perivitelline membrane, the differences between expected and observed values when adjusted for body mass and ovum size.

Table 3.1. The frequency of midpiece abnormalities amongst species of introduced New Zealand birds.

Table 3.2. Comparison of motility between New Zealand introduced birds and a variety of other mammals and birds.

List of figures

Figure 2.1. Differential Interference Contrast images of four different species' sperm on the perivitelline membrane of an egg.

Figure 2.2. Linear regression of number of sperm on the perivitelline membrane adjusted for body mass. Species shown are non-bottlenecked species from Europe, and bottlenecked species from New Zealand.

Figure 2.3. Relationship between residual numbers of sperm on the perivitelline membrane adjusted for body mass from the expected values, versus bottleneck size.

Figure 2.4. Linear regression of number of sperm on the perivitelline membrane adjusted for ovum size. Species shown are non-bottlenecked species from Europe, and bottlenecked species from New Zealand.

Figure 2.5. Relationship between residual numbers of sperm on the perivitelline membrane adjusted for ovum size from the expected values, versus bottleneck size.

Figure 3.1. Differential Interference Contrast and fluorescent dye images of sperm from a variety of New Zealand's introduced birds.

Figure 3.2. Differential Interference Contrast and fluorescent dye image of a sperm that has a short midpiece.

Figure 3.3. Correlation between the co-eficient of variation of midpiece size versus bottleneck size in New Zealand's introduced birds.

Figure 3.4. Correlation of mean sperm motility versus bottleneck size in New Zealand's introduced birds.

Figure 3.5. Correltaion of mean sperm motility versus hatching failure rate in New Zealand's introduced birds.

Figure 4.1. Scanning Electron Microscope Images of an eggshell fragment, a round pore, an oval pore and an irregular pore.

Figure 4.2. Linear regression of observed eggshell thickness versus egg mass and linear regression of expected eggshell thickness versus egg mass for New Zealand's introduced birds.

Figure 4.3. Linear regression of the residuals from the observed thickness – predicted thickness versus bottleneck.

Figure 4.4. Linear regression of the observed number of pores versus egg mass and linear regression of the expected number of pores versus egg mass for New Zealand species.

Figure 4.5. (A) Linear regression of the residual observed number of pores from the expected values versus bottleneck size. (B) Linear regression of the residual observed number of pores from the observed linear regression versus bottleneck size.

Figure 4.6. (A) Linear regression of the proportion of round pores versus bottleneck size. (B) Linear regression of the proportion of irregular pores versus bottleneck size.

List of appendices

Appendix I: Raw data for calculating the predicted lines for number of sperm present on the perivitelline membrane adjusted for body mass and ovum size.

Appendix II: Calculation for the co-efficient of variation for the variability in midpiece size in sperm

Appendix III: Calculations for egg surface area and egg volume.

Appendix IV: Raw data for calculating the predicted number of pores per egg adjusted for egg weight.

Abstract

It is generally accepted that populations which experience severe bottlenecks have a reduction in fitness. One of the most frequently reported fitness costs is increased hatching failure in bottlenecked populations of birds. The mechanism responsible for increased hatching failure is unknown. Research on other animals suggest that reduced population numbers cause unavoidable inbreeding that in turn leads to abnormalities in the gametes. In this thesis I examine some of the possible causes for increased hatching failure in severely bottlenecked populations of introduced birds in New Zealand. I look at three traits identified as a cause for infertility or hatching failure previously and determine whether there is a link with the size of a population's bottleneck. It is possible that reduced numbers of sperm reaching the site of fertilisation is a primary cause of hatching failure. I examined the perivitelline membrane of various species of introduced birds and counted the total number of sperm present to compare to how many would be expected in non-bottlenecked species. Although there was no relationship between the size of the bottleneck and the number of sperm present, all species had lower than expected sperm counts. In many species of mammals, a reduction in the quality of sperm is attributed to inbreeding depression bought about by genetic bottlenecks. I next compared the level of sperm abnormalities, variation in midpiece size sperm, and sperm motility with the size of the bottleneck each species passed through when introduced to New Zealand. There was no significant correlation between either the variation in midpiece size or sperm motility with bottleneck size. However, there was a trend for species that passed through more severe bottlenecks to have a slightly higher level of midpiece size and lower motility. Finally, I examined whether there was a link between abnormalities in the eggshell and the size of the respective bottleneck. There was no significant change in eggshell thickness or any change in the number of pores associated bottleneck size. However, there was a decreased number of round pores in severely bottlenecked species, although the consequences of this are unknown. My findings do not directly link a single cause for increased hatching failure in bottlenecked species of birds, but they do highlight the need for monitoring of reproductive traits in endangered species that have experienced a population bottleneck.

Chapter 1:

General introduction

Background

Many of New Zealand's native bird populations are currently experiencing or have recently been through population declines (Holdaway et al. 2001). For most species, population declines are due to invasive pests and large areas of habitat destruction. The populations of some species have been reduced so low that they are experiencing, or have experienced what is termed a population bottleneck. A population (or genetic) bottleneck is when a species or population has a sudden restriction in population size and thus, a subsequent decline in genetic diversity (Frankham et al. 2002). There are two possible genetic consequences for a population that passes through a bottleneck: (a) deleterious alleles are purged from the population (Kirkpatrick and Jarne 2000); or (b) the fixation of deleterious alleles in the population (Lande 1994).

Populations that experience a bottleneck inevitably suffer a loss in genetic diversity (Nei et al. 1975, Kirkpatrick and Jarne 2000). This can occur as bottlenecks increase the chances of individuals mating with genetically similar individuals, even if direct incestuous mating is avoided. When a population has a high proportion of genetically similar individuals, inbreeding increases the level of homozygosity and the expression of deleterious alleles (Frankham et al. 2002). Nevertheless, some models predict that bottlenecks increase the opportunity for deleterious alleles to be removed from the population via natural selection (Kirkpatrick and Jarne 2000). This

phenomenon is referred to purging. Whether purging is effective or not, can depend on the lethality of the deleterious allele (Frankham et al. 2002). Purging can be highly effective for lethal alleles, but much less effective for mildly deleterious alleles and they can become fixed. It is for this reason that inbreeding is generally considered to have negative effects on the fitness of individuals (Charlesworth and Charlesworth 1987, Crnokrak and Barrett 2002).

The fitness costs of inbreeding (and thus bottlenecks) have been the focus of intensive laboratory and field studies in the last few years. Experimental tests on inbred lines of *Drosophila* have shown reduced fecundity, lowered evolutionary potential and a higher chance of extinction (Frankham et al. 1999, Kristensen et al. 2003). Although few studies have been done on wild populations, there is a suggestion that using captive and laboratory populations as models for understanding inbreeding may not be reflective of what happens in wild populations (Jimenez et al. 1994, Crnokrak and Roff 1999). However, reduced fecundity and survivorship, increased susceptibility to disease and the impaired ability to adapt to a changing environment have been reported in some wild populations subject to inbreeding (Frankham et al. 1999, Keller and Waller 2002).

One of the most concerning fitness consequences of bottlenecks is the increased level of reproductive impairment that can occur. Reduced reproduction has been seen in reptiles (Madsen et al. 1995), birds (Bensch et al. 1994, Kempenaers et al. 1996) and mammals, particularly in large carnivores (Wildt et al. 1987, Lindburg et al. 1993) and in ungulates (Roldan et al. 1998). For example, studies on cheetahs (*Acionyx jubatus*) show that increased homozygosity (genetic uniformity) (Merola

1994) from inbreeding leads to greater deformities in sperm, which in turn could lead to reduced litter sizes (Madsen et al. 1995), increased infant mortality (Ralls et al. 1979), infertility, and a greater susceptibility to disease (Merola 1994). This could potentially reduce the overall fitness of the population and its long-term survival may be reduced. This has serious conservation implications and it may put into question the appropriateness of current practises of translocation, as commonly used in New Zealand, which force threatened species into population bottlenecks and thus an increased risk of inbreeding depression.

The study of the effects of inbreeding on reproductive success of birds is limited. Case studies on blue tits (Kempenaers et al. 1996) and the great reed warbler (Bensch et al. 1994) found that inbreeding leads to greater than expected levels of hatching failure. In both studies, the degree of hatching failure was negatively correlated with the level of genetic similarity, even though mating directly with close kin was rare. Many laboratory experiments and models have tried to estimate the degree of inbreeding, which has the least deleterious effects on fitness (e.g. Heschel and Paige 1995, Franklin and Frankham 1998). The models that have been proposed suggest that populations that pass through a bottleneck of 50 individuals will survive for about 100 generations, but will have problems adapting to changing environments (i.e., a reduced evolutionary potential) (Reed and Bryant 2000). Others suggest that a minimum bottleneck size around the size of 500, and up to 5000 may be required to ensure the survival of a population over the long term (Franklin and Frankham 1998).

A comparative study of hatching failure in both native and introduced New Zealand birds found increased rates of hatching failure with population bottlenecks

smaller than about 150 individuals in native species. In the same study, however, increased hatching failure was also observed in species that had been translocated out of their natural habitat. Although the same threshold of about 150 individuals was evident, higher levels of hatching failure were also seen at larger bottleneck sizes of about 600 individuals in the introduced species (Briskie and Mackintosh 2004).

At present, it is not known why there is a high failure amongst birds in New Zealand that have passed through bottlenecks. There is little evidence on whether deformed gametes contribute to the level of hatching failure in birds, although research on ungulates (e.g. *Gazella cuvieri*) suggests that populations that have experienced a bottleneck show greater levels of sperm abnormalities (Roldan et al. 1998). Bottlenecked populations of lions (*Panthera leo*) (Wildt et. al. 1987) also show high levels of abnormalities. Similar effects have been shown in cheetahs (Merola 1994) from captive populations. Animals in zoos appear to have a lower percentage of motile sperm, although the numbers of deformities are about the same as a wild population (Crosier et al. 2006). Similar types of studies have not been done on wild populations of birds, but presumably the same principals would still apply.

In this thesis I explore different fitness traits related to the reduced hatchability of eggs in species that have passed through population bottlenecks. My goal is to explain the possible mechanisms for why populations of birds that have been through a severe bottleneck suffer from increased hatching failure. I use the exotic species of birds introduced to New Zealand as a model system to study this problem. In the 1800's and early 1900's, acclimatisation societies introduced a number of European and Asian passerine birds to New Zealand. The number of individuals introduced

varied from one species to the next. As a result, different species were forced through different sized bottlenecks. Introduced species provide an excellent model system as they permit a comparative approach between New Zealand (post-bottlenecked populations), and the source population (pre-bottlenecked populations) on a number of fitness traits. Such "before" and "after" comparisons are seldom possible with native endangered birds as there are simply no pre-bottlenecked populations left to study. The size of the bottleneck in the introduced species studied in this thesis range from 7 individuals in the cirl bunting (*Emberiza cirlus*) to about 700 individuals in the European blackbird (*Turdus merula*) (Thomson 1922, Lever 1987). This provides a reasonably good range to study, and one that encompasses the number of individuals typically used in translocations by conservation biologists. Throughout this thesis, I assume that population bottlenecks are a reasonable index of the genetic variability present in the post-bottlenecked populations. Some genetic analyses have been done on some of the species studied here such as: house sparrow (Parkin and Cole 1985), greenfinches (Merila et al. 1996), European starlings (Ross 1983), Indian myna (Baker 1987) and chaffinches (Baker et al. 1990, Baker 1992). The work on these species suggests that the populations in New Zealand have suffered a loss in genetic variation but that the amount present today is positively related to the number released. Thus, although the amount of genetic variation is not known for many of the species in my study, work done to date does suggest that the number of individuals released by the acclimitisation societies in the 19th century is a reasonable estimate of genetic variation. I also only used passerine species in this thesis, although other groups of nonpasserine birds were introduced, such as Canada goose (*Branta candensis*); little owl (*Athene nocta*); and a range of game birds such as California quail (*Callipepla californica*); pheasants (*Phasianus colchicus*); turkey (*Meliagris*

gallopave); and chukor (*Alectoris chukar*) (Thomson 1922). As the breeding biology of these species is quite different to passerines (e.g. precocial vs altricial development), this could potentially affect my results. Many game bird populations have also been supplemented by additional releases (often of unknown number) and thus the size of the bottleneck each has passed through are less certain than in the passerines.

Outline of thesis

My thesis contains three chapters that make use of a variety of field and laboratory techniques to understand the mechanism for higher hatching failure in introduced birds and its relationship to bottleneck size. By studying both the eggs and sperm in a range of species that have experienced different sized bottlenecks I hope to understand why hatching failure is related to bottleneck size.

In the next chapter, I start to address the problem of increased hatching failure in bottlenecked populations of birds by examining the hypothesis that fertility of eggs in bottlenecked species of birds is impaired by the numbers of sperm reaching the site of fertilisation. When a female releases an egg from her ovary a membrane is put around the yolk to protect it and hold it together. Sperm have to penetrate this layer, which is known as the inner-perivitelline membrane, to fertilise the egg. As this is happening, a second membrane is laid over the first, trapping any sperm present between the two layers (Birkhead et al. 1994). By counting the sperm present trapped between the two layers, plus the number of holes created by sperm, the total number

of sperm present at the site of fertilisation can be estimated (Wishart 1987). This technique was first developed in the poultry industry by Wishart (1987) and further developed by Birkhead et al. (1994) for the study of sperm competition in passerine species.

In Chapter 3 I look for deformities and abnormalities in sperm of species that have been through population bottlenecks. I use a new technique developed by Birkhead et al (2005) to measure the size and variation in the size of the sperm midpiece. In passerines the midpiece contains only one mitochondria (as opposed to several in non-passerines). As this is where all the energy for the sperm is made, changes in the size or variation in the size of the midpiece could be important in sperm function, particularly if the female has to store sperm for several days before she lays her eggs (Birkhead and Fletcher 1994). I also examine the level of sperm motility in a range of introduced species. Motility is often used as a measure of fertility in males, and although a variety of studies have looked at motility in several populations of mammals (Gerald et al. 2002, Busso et al. 2005) and birds (Birkhead et al. 1998, Hartley et al. 1999, O'Brien et al. 1999), this is the first time it has been examined in New Zealand's introduced species. The results of this chapter are preliminary and work will continue of this aspect of my project this spring.

The fourth chapter is focussed on possible deformities in the avian eggshell and whether such deformities are related to the size of bottlenecks that New Zealand's introduced avifauna experienced. I examine potential changes in pore numbers and shape as well as the thickness of the eggshell that may have occurred when populations of these birds were established. The avian eggshell contains pores that

allow gas (O_2, CO_2 and H_2O) to move from the environment to the embryo and vice-versa (Ar et al. 1974). Any changes in the number or shape of pores, or the thickness of the eggshell could affect the level of gases that are diffused. This could potentially change the hatchability of those eggs (Fox 1976), especially if such changes are more likely to occur in species subject to severe bottlenecks.

In the final chapter I bring the results from the previous three chapters together. I re-examine the potential consequences of population bottlenecks with relation to the data I have gathered and that from the literature on other species, as well as New Zealand native birds. The potential for future research on reproductive failure and its significance for conservation are also discussed. Chapters two, three and four are written as papers to ease publication at a later date. Although there is some degree of repetition it also highlights each aspect of this thesis from the other chapters.

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Chapter 2:

Sperm numbers on the perivitelline membrane of birds passing through severe population bottlenecks: can founder sizes explain hatching failure?

Abstract

In the 19th century, acclimatisation societies introduced a variety of avian species in varying numbers from 7 individuals to about 800 individuals into New Zealand. The rate of hatching failure that these species exhibits is related to their founder size. The mechanism to why species passing through smaller bottlenecks showing higher hatching rates is unknown. I hypothesise that fertility in species passing through smaller bottlenecks may be confounded by sperm reaching the site of fertilisation. By counting sperm numbers on the perivitelline membrane from various introduced species, I was able to determine whether a lack of sperm reaching the site of fertilisation compromised fertility rates. Unexpectedly, I found that sperm numbers were lower than expected across all species when corrected for both ovum size and body mass. The size of the bottleneck in New Zealand's introduced birds was not related to the numbers of sperm reaching the site of fertilisation, nor was rate of hatching failure, but further study needs to be done to understand why all species had lower than expected sperm counts on the perivitelline membrane.

Introduction

In a recent paper, hatching failure was found to increase dramatically with the severity of a population bottleneck among both native and introduced New Zealand birds (Briskie and Mackintosh 2004). This could have serious implications for conservation because many species around the world are currently passing through small bottlenecks as a consequence of human-induced habitat changes. Severe population bottlenecks are thought to reduce genetic diversity in the post-bottlenecked populations. This occurs as individuals are forced to mate with close relatives, leading to an increase in the genetic load and inbreeding depression. This is bought about because there are increased levels of homozygosity which then leads to an increased expression of deleterious alleles (Charlesworth and Charlesworth 1987). Increased homozygosity can also cause a loss in evolutionary potential and subsequently a loss in a population's fitness (Gibbs and Grant 1989, Jimenez et al. 1994, Keller 1998, Keller and Waller 2002).

A variety of fitness traits have been shown to be affected by inbreeding. For example, inbred bighorn sheep (*Ovis canadensis californiana*) have lower recruitment levels in the wild (Whittaker et al. 2004), and white-footed mice (*Peromyscus leucopus noveboracensis*) show lower survivorship after being experimentally inbred in a laboratory and released in the wild (Jimenez et al. 1994). Similar effects have been seen in song sparrows (Keller 1998) and in Mexican jays (Brown and Brown 1998). In adders (*Vipera berus*), inbreeding has deleterious effects on breeding success with smaller litter sizes and high incidences of stillborns (Madsen et al. 1995). In birds, one of the most common effects of inbreeding is an increased rate of

hatching failure (Bensch et al. 1994, Kempenaers et al. 1996, Spottiswoode and Moller 2003, Van de Casteele et al. 2003) .

Although increased hatching failure has been attributed to inbreeding depression and severe genetic bottlenecks, the mechanism for why an increased proportion of eggs fail to hatch is unclear. For example, great reed warblers (*Acrocephalus arundinaceus*) on Lake Kvismaren were founded by a single pair in 1978. Hatching success is higher when parents are less closely related (Bensch et al. 1994). However, even nonincestuous matings in this highly bottlenecked population experience higher than expected levels of hatching failure, presumably due to unavoidable genetic similarity between even unrelated individuals. A similar pattern was found in blue tits (Kempenaers et al. 1996), where matings between siblings and parents/offspring showed higher than expected numbers of unhatched eggs. High hatching failure is usually attributed to increased homozygosity and therefore, increased expression of deleterious alleles (Charlesworth and Charlesworth 1987). However, exactly how deleterious alleles lead to higher hatching failure is unknown but several mechanisms have been suggested and include increased frequencies of addling (bacterial infection in the egg), problems with eggshell development, higher rates of embryo death, and problems with the fertilisation of eggs, either through male (sperm) or female (oviduct) dysfunction.

Determining the mechanism for increased hatching in bottlenecked populations is critical for designing remedial measures when such a problem arises in the conservation of endangered species. In other words, the first step in addressing the problem of hatching failure in endangered birds is to understand what causes the

higher levels of failure. One of the most likely causes of increased hatching failure may be decreased rates of fertilisation, due to either sperm dysfunction or problems in sperm reaching the egg at the time of fertilisation. Using domestic poultry, Wishart (1987) developed a technique that allows an accurate count of the number of spermatozoa present in the infundibulum when fertilization occurs. Fertilization in birds occurs within about 15 minutes of an ovum being released from the ovary. Several spermatozoa penetrate the inner perivitelline membrane, although only one will fertilize the egg (Birkhead et al. 1994). Within minutes a second membrane (the outer perivitelline membrane) is then laid over the inner perivitelline membrane and any spermatozoa present between the two membranes are then trapped. By examining the perivitelline membranes of freshly-laid eggs it is possible to estimate the number of sperm present at the time of fertilisation. This method has been used successfully in behavioural studies of sperm competition (Birkhead et al. 1994, Lifjeld et al. 2000, Michl et al. 2002) but can also be used to study whether increased rates of hatching failure may be due to reduced numbers of sperm at the time of fertilisation. For example, a study of hatching failure in house sparrows (*Passer domesticus*) found that of 22 eggs showing no embryonic development after three days, six were truly infertile as no sperm or holes were detected in the perivitelline membranes (Birkhead et al. 1995). Determining whether hatching failure in bottlenecked populations is a result of infertility due to lower numbers of sperm reaching the site of fertilisation could help conservation managers to understand the mechanism of hatching failure and develop methods for restoring fertility in critically endangered birds.

Sperm is stored for several days in sperm storage tubules in the female before fertilisation occurs and in many monogamous birds copulations peak several days before the first egg is laid (Birkhead and Fletcher 1994). The number of sperm present on perivitelline membrane decreases the longer the time from a female's last copulation (Birkhead et al. 1994, Small et al. 2000). Low counts of spermatozoa on the perivitelline membrane could thus be explained by either low numbers of sperm being ejaculated or poor quality ejaculate in which the spermatozoa struggle to stay alive in the sperm storage tubules.

In this chapter I examine whether birds that have passed through a severe population bottleneck, and show elevated rates of hatching failure, have fewer sperm present at fertilisation. It is hypothesised that bottlenecked species should show reduced counts of sperm on the perivitelline membranes than non-bottlenecked species and that this pattern will be more pronounced the smaller the bottleneck.

Methods

I studied the numbers of sperm on the perivitelline membranes of 10 species of introduced birds in New Zealand. These species were: Song Thrush (*Turdus philomelos*), Blackbird (*T. merula*), European Starling (*Sturnus vulgaris*), Dunnock (*Prunella modularis*), Chaffinch (*Fringella colebs*), Greenfinch (*Carduelis chloris*), European Goldfinch (*C. carduelis*), Redpoll (*C. flammea*), Yellowhammer (*Emberiza citrinella*) and Indian Myna (*Acridotheres tristis*). These species passed through bottlenecks during their establishment in New Zealand, ranging from a low of 66 in the Greenfinch to a high of about 700 to 800 in the Blackbird (Thomson 1922, Lever

1987). As such they provide a model system for examining the effect of different sized bottlenecks on the number of sperm present at fertilisation. A total of three complete clutches were collected for each species except for the Myna, in which only a single clutch could be collected. Eggs were collected from three South Island locations near Christchurch (43°48'58"S, 172°53'54"E), Kaikoura (42°38'66"S, 173°62'54"E) and Ward (41°79'69"S, 174°11'62"E), and one North Island location, near Limestone Downs (37°47'69"S, 174°75'94"E). I found nests of all species during nest building, which allowed me to collect freshly laid eggs. Eggs were collected within 48 hours of being laid (usually on the morning they were laid), and prior to incubation. This is important because the sperm on the perivitelline membrane and the perivitelline membrane itself start to degrade when incubation commences (Birkhead et al. 1995, Small et al. 2000). When an egg was removed it was replaced with a model egg to ensure that the rest of the clutch would be laid as normal. The order of laying is critical to know as the number of sperm trapped in the perivitelline membrane changes throughout the clutch (Birkhead et al. 1994, Lifjeld et al. 2000).

The eggs were gently broken open and the diameter of the yolk was measured using callipers (to the nearest 0.1mm). The yolk was then cut in two relatively equal halves using scissors and the yolk contents washed away with Phosphate Buffered Saline (PBS). This left only the translucent membranes which were then stored in 10% formaldehyde in PBS for up to several months prior to examination under the microscope. Approximately 24 hours before being prepared for microscopic analysis, the membranes were soaked in distilled water to prevent the membrane from breaking when it is mounted onto a slide. It was then cut into smaller pieces, for ease of mounting onto a slide. The fragments of membrane were prepared as a wet mount

onto a glass slide so they remained flat and then covered with a coverslip. PBS was added to the wet mount from time to time to prevent the preparation from drying out. The membranes were viewed at magnifications of 200x – 400x with a Zeiss Axioscope 2 MOT compound microscope using differential interference contrast (DIC). Fragments were carefully scanned for the presence of sperm and holes in the inner perivitelline layer. Both sperm and holes were readily visible using DIC microscopy (Fig. 2.2.1). As each hole was produced by the passage of a sperm, I estimated the total number of sperm present at the time of fertilisation as the sum of sperm and holes combined (hereafter total sperm). All fragments were examined and total sperm calculated as the sum of all sperm and holes on all fragments from a single egg. As the entire membrane was analysed there was no need to estimate the number of spermatozoa based on the number of fields I observed. Because the number of total sperm varies with laying sequence, for the analyses presented here I only used first-laid eggs. Thus sample sizes were three eggs per species (each from a different nest) for all species except the Myna in which only one egg could be collected. Images of sperm on the membrane from each species were captured using a Zeiss Axiocam HRc CCD camera and Axiovision 5.0 software.

To determine whether bottlenecks affect sperm numbers on the perivitelline membranes, I compared the number of sperm for each of the 10 introduced species I studied in New Zealand with that predicted from a regression line between sperm number and egg size from outbred species from Europe. I obtained data on non-bottlenecked species from Birkhead et al. (1994) except that I omitted data from captive populations, as captivity is known to affect the fertility of birds (Swinnerton et al. 2004). As there are known allometric relationships between sperm number, egg

size and body mass, I plotted the relationship between body mass and the number of sperm trapped on the perivitelline membrane, and the size of the yolk with the number of sperm (Birkhead et al. 1994) and then calculated the residual number of sperm observed (i.e., sperm number observed after controlling for either body mass or yolk size). A regression of the total number of sperm on the perivitelline membrane from non-bottlenecked birds allowed me to predict the total sperm counts expected for New Zealand introduced birds, controlling for both body weight and ovum size. Expected sperm counts controlling for body mass were calculated by:

$$\text{Log (total spermatozoa)} = 1.2413 + 0.9624 \times \text{Log (body mass (g))}$$

Expected sperm counts controlling for ovum size were calculated by:

$$\text{Log (total spermatozoa)} = -0.601+3.0913 \times \text{Log (ovum diameter (mm))}$$

A regression of the residuals of sperm number in the introduced species was used to determine if the number of sperm on the perivitelline membrane was related to the size of their population bottleneck, after correcting for both body mass and ovum size. I assumed that the number of individuals introduced was an index of bottleneck size. Data on bottleneck size was obtained from Thompson (1922) and Lever (1987). A single sampled t-test of means was carried out on the residuals versus zero (the expected regression line), to determine whether there was a difference in the number of sperm on the membrane from outbred European populations. All analyses were carried out in Statistica v. 7.0 statistics package.

The collection of eggs was conducted under the approval of the University of Canterbury Animal Ethics Committee. Introduced birds are not protected in New Zealand and no Department of Conservation permit was required for the collection of eggs.

Results

Counts of the total number of spermatozoa on the perivitelline membrane were lower than expected in all species introduced to New Zealand (Table 2.1). The variance and ranges of total sperm number differed from species to species (Table 2.1). The species with the largest variation in sperm number was the chaffinch (SE ±174.51, range 24-550 sperm/egg). Several species had smaller variances in sperm numbers, particularly the European Starling (SE ±10.82, range 31-67 sperm/egg), Dunnock (SE ± 9.17, range 15-45 sperm/egg) and Goldfinch (SE ± 9, range 13-40 sperm/egg; Table 2.1). Four out of ten species examined had counts at least 1000 sperm less than expected for their ovum size, and three species had 1000 less sperm adjusted for body mass (Table 2.1). The single Myna egg examined had only one sperm present on the membrane.

When controlled for body mass, all species introduced to New Zealand fall under the regression line between total sperm number and body size for non-bottlenecked birds (Fig. 2.2). Although three species (Chaffinch, Greenfinch and Redpoll) appear to lie near the expected line, all remaining seven species fall much further beneath the expected number of sperm for their body size. There was no significant relationship between the size of bottleneck each species passed through

during their establishment in New Zealand and the residual numbers of sperm on the perivitelline membrane (Fig. 2.5; $F_{1, 8} = 1.02$, $P = 0.343$, $r^2 = 0.113$). However, overall there was a significant tendency for residual sperm number to be negative, or less than expected (Fig. 2.3). A t-test of residual sperm number against zero (the predicted line) was significant ($t = -3.94$, $df = 9$, $P < 0.004$).

Numbers of sperm on the perivitelline membrane of species introduced to New Zealand was similar when I compared sperm number to ovum diameter. As with body size, all bottlenecked species fell below the expected line (Fig. 2.4). The regression between the residuals for total spermatozoa (adjusted for ovum diameter) and population bottleneck size was not significant ($F_{1, 8} = 0.95$, $P = 0.357$, $r^2 = 0.106$). Again, the trend was for all residuals to be negative (Fig. 2.5). A t-test of residual sperm number adjusted for ovum diameter against zero was significant ($t = -4.45$, $df = 9$, $P < 0.002$).

Finally, I ran a correlation analysis between levels of hatching failure (data from Briskie and Mackintosh 2004) and the residual number of sperm, controlled for both body size and ovum size. Neither the residual number of sperm adjusted for body size ($r = 0.452$, $n = 9$, $P > 0.18$), nor the residual number of sperm adjusted for ovum size ($r = 0.347$, $n = 9$, $P > 0.32$), were significantly correlated with levels of hatching failure observed in New Zealand.

Discussion

I found that most species of birds introduced to New Zealand had fewer sperm on the perivitelline membrane of their eggs than expected based on counts available for non-bottlenecked species. This pattern held for both comparisons based on body size and on egg size. In some cases the number of sperm observed on egg membranes was dramatically less than that expected (Table 2.1). For example, blackbirds in New Zealand had only an average of 200 sperm on their perivitelline membranes, compared to the expected number of 1300-1400. However, these differences between observed and expected numbers of sperm did not appear related to the size of the bottleneck each species passed through during their establishment in New Zealand.

A loss in fitness due to hatching failure should be of great concern to both conservation managers and scientists. Inbreeding causes higher levels of hatching failure and this has been noted in a comparative study (Briskie and Mackintosh 2004) and individual cases (Bensch et al. 1994, Kempenaers et al. 1996, Kruuk et al. 2002). The mechanism of why an increased hatching failure occurs in inbred populations is unknown. Mackintosh (2003) studied the number of sperm on egg membranes in an inbred population of New Zealand robins (*Petrocia australis*) in a similar method to this study, but it was determined that there was no relationship between inbreeding (founder population of 5 individuals) and the number of spermatozoa on the perivitelline membrane. However, there are problems with his study. Sample sizes were small (n = 5), and the technique for counting the total number of sperm was extrapolated from examining a few areas of the egg, instead of surveying the whole membrane. Sperm often have a clumped distribution over the surface of the

pervitelline membrane (Birkhead et al. 1994), thus it is possible Mackintosh (2003) over-estimated the number of sperm by counting in the areas with the most sperm present.

The process by which an egg is fertilised is well studied (Wishart 2002) but very little is known about the consequences of inbreeding on fertilisation success in birds. Birkhead et al. (1995) reported that 75% of house sparrow (*Passer domesticus*) eggs that failed to hatch were in fact, fertilised. This study was a presumed outbred population of sparrows in Spain, but it does indicate that other proximate causes of hatching failure need to be considered. In the bottlenecked populations of New Zealand's introduced birds, only one species exhibited a truly infertile egg: a single Myna egg examined had only one sperm present on the entire membrane. This is a species that passed through a severe bottleneck, with only about 70 individuals being introduced to New Zealand in the North Island (Thomson 1922, Lever 1987). With a sample size of only one, it is too early to conclude this is due to inbreeding depression. Greenfinches passed through a similar sized bottleneck (Thomson 1922, Lever 1987) but the number of sperm of their eggs did not deviate greatly from that expected (Fig. 2.2, 2.4).

It is possible that the numbers of individuals released and recorded by the acclimatisation societies are an under-estimate of the true number released. Although the introduction of exotic birds was an expensive undertaking and carried out primarily by organised societies (Thomson 1922; Lever 1987), some species may have been introduced by private individuals and gone unrecorded. For example, some of the finches (Greenfinch, Goldfinch and Redpoll) were common in the pet trade,

and it is not unfeasible that migrants to New Zealand in the 19th century bought along their pet finches, which escaped or whose offspring were subsequently released. This could change the genetic make-up of those populations and the assumption that these species went through severe bottlenecks may be wrong. Genetic work is needed to confirm that levels of genetic diversity are low in the species that have passed through the most severe bottlenecks, but the release of additional individuals could explain why some species like the Greenfinch do not show reduced numbers of sperm on their egg membranes (Fig. 2.2, 2.4) even though it is a species that is presumed to have gone through a severe bottleneck (66 individuals).

It was unexpected that most of the species in my study would fall well beneath the predicted number of sperm from non-bottlenecked populations (Fig. 2.2, 2.4). Briskie and Mackintosh (2004) found that hatching failure significantly increased at bottleneck sizes of 150 or smaller in introduced birds in New Zealand. In this study however, not a single species, even at the higher bottleneck range, come close to what was expected in sperm counts from their counterparts in non-bottlenecked populations (Fig. 2.3, 2.5). This was confirmed by the statistical tests of sperm count residuals against zero, when adjusted for body mass and ovum size.

Population bottleneck size and its related fitness consequences due to inbreeding and loss of genetic diversity should be considered when managing small or conservation important populations. To date there is still debate over how many individuals are needed to found a population and yet avoid founder effects and inbreeding depression. Generally, the smaller the bottleneck a population experiences, the higher the loss of genetic diversity that can be expected (Montgomery et al. 2000).

Some models suggest that the number of individuals needed when founding a new population could be as low as 50 individuals (Reed and Bryant 2000). Although this number could prevent that population from going extinct in the short term, the reduced genetic diversity of the resulting population may not be sufficient to allow the species to adapt to novel environmental stressors or rapid changes. Other models suggest a more conservative number, suggesting that a range of 500-5000 individuals may be required for founding populations to prevent inbreeding depression and to retain evolutionary potential (Franklin and Frankham 1998). Briskie and Mackintosh (2004) suggested that founding populations would experience higher degrees of hatching failure at about 150 individuals. This number is three times that suggested by Reed and Bryant (2000), but much smaller than Franklin and Frankham (1998). There was no clear point where the number of sperm on the perivitelline membrane was affected by bottleneck size in this study (Fig. 2.3, 2.5). It should be noted that all of the species introduced into New Zealand went through bottlenecks of 1000 individuals or less, and the species in this study went through bottlenecks of approximately 66-700. It is entirely possible that bottlenecks as high as 700-800 individuals still incur a fitness cost of fewer sperm reaching the site of fertilisation. If this is the case, then the bottleneck size where this threshold occurs would have gone undetected in this study. Few species were introduced to New Zealand in numbers higher than 1000 birds, so it would be difficult to test if Franklin and Frankham's (1998) model is more accurate.

A reduction in the number of sperm on the perivitelline membrane could be due to either dysfunction of the sperm (see next chapter) or with the function of the oviduct. Sperm are stored in the female's sperm storage tubules (SST) for several

days prior to laying and have been studied for the role in sperm competition (Briskie and Montgomery 1992, Birkhead and Fletcher 1994). Problems with the ability to store sperm could thus lead to few sperm reaching the egg even in the absence of problems with sperm function. Females of some species are able to store sperm for weeks in a healthy individual (Lake 1975), but this ability could be impaired due to inbreeding depression. Unfortunately, it is unknown at present whether sperm storage can be impaired through inbreeding depression, but this is an area that needs to be examined.

It should be noted that the lower than expected sperm counts may not entirely be a direct result of passing through a bottleneck. Environmental factors might also affect infertility. For example, the presence of toxins, such as fertilisers, pollutants and heavy metals may reduce the fertility of both males and females in both bottlenecked and non-bottlenecked populations (Facemire et al. 1995). The New Zealand environment in which introduced birds now live is not identical to that they experienced in their native range although many of the habitats these species occupy in both areas are highly modified farmland and urban areas. Breeding seasons are generally longer in New Zealand, and seasons are getting warmer as a result of global warming (Coppack and Both 2002). A shift to earlier than normal egg laying might thus lead to higher rates of hatching failure (Coppack and Both 2002, Coppack and Pulido 2004). With limited genetic variation, the ability of these species to adapt to changing seasons and to synchronise breeding could be compromised (Pulido and Berthold 2004). A combination of inbreeding depression and a change in the environment can both have an impact on fitness (Keller et al. 2002), so it is possible

that the reduced number of sperm I observed on egg membranes could be a consequence of both bottlenecks and environmental changes.

Although I cannot rule out some environmental effects, the lower than expected sperm counts on the perivitelline membrane are consistent with the effects expected due to inbreeding after a severe population bottleneck (Fig. 2.2, 2.4). An examination of sperm number on the perivitelline membranes of native birds that passed through severe bottlenecks could help separate the effects of bottlenecks from environmental effects as the native species by and large still live in environments similar to that in which they evolved (or at least more similar than that faced by introduced species in New Zealand). This has not been done and such a study does raise ethical issues as it would involve the destruction of eggs of endangered species. The reduction in number of sperm on eggs on introduced species that have passed through similar bottleneck sizes as many of the endangered native birds nonetheless does raise some concerns for the management of many endangered species. If increased egg hatching failure is caused by reduced numbers of sperm reaching the ovum at the time of fertilisation, then at least part of the explanation for the pattern seen in Briskie and MacKintosh (2004) of bottlenecks <150 having higher hatching failure may be due to reduced sperm reaching the egg. However, more study is required, both here in New Zealand and in Europe, to determine whether the patterns I found hold in species passing through even less severe bottlenecks than the ones I examined.

A reduction in the number of sperm on the egg membranes might be expected to lead to an increase in hatching failure, but I did not find a significant relationship

between residual number of sperm and levels of hatching failure. At present, it is unknown in most species exactly how many sperm are required to ensure a high rate of fertilisation. About 32 sperm are required to ensure fertilisation in zebra finches *Taeniopygia gutta* (Birkhead and Fletcher 1998). Only a couple of species I examined fell below this number (Table 1), suggesting the reduction in sperm number has not been severe enough to explain higher rates of hatching failure that would be due to fertilization failure. Birkhead and Fletcher's (1998) study of zebra finches was conducted on captive and domesticated birds and so it is difficult to make any conclusions regarding the total number of sperm required to ensure fertilization in a wild bird. I was only able to compare the number of sperm found on egg membranes for three species in which there is information both in New Zealand (my study) and the native range (Birkhead et al. 1994). In all three cases the sperm numbers were less in New Zealand than in the native range. Clearly, more species in Europe need be examined and then compared directly with the New Zealand populations to confirm that reduced sperm numbers on the perivitelline membrane of eggs are a direct cause of severe population bottlenecks in birds.

Table 2.1 Mean sperm counts on the perivitelline membrane of introduced birds in New Zealand. SE is calculated standard error, range is lowest and highest sperm counts except for *A. tristis* where n = 1 and no SE or range can be given. Expected counts from body mass and ovum diameter are calculated from regression of non-bottlenecked birds using data in Birkhead et al. (1991). Differences calculated by subtracting the expected mean sperm count from each species' observed sperm count. All values rounded to the nearest whole number.

Species	Observed mean sperm count (sample size)	± SE	Range	Expected sperm count by body mass	Difference from expected (body mass)	Expected sperm count by ovum diameter	Difference from expected (ovum diameter)
Turdus philomelos	184 (3)	90.95	41-353	1040	-856	1185	-1000
T. merula	193 (3)	25.98	142-228	1325	-1132	1463	-1271
Sturnus vulgaris	46 (3)	10.82	31-67	1254	-1208	1519	-1473
Acridotheres tristis	1 (1)	N/A	N/A	1817	-1816	1747	-1746
Prunella modularis	33 (3)	9.17	15-45	326	-293	403	-367
Fringella colebs	201 (3)	174.51	24-550	341	-140	409	-208
Carduelis chloris	139 (3)	69.96	58-278	431	-292	535	-397
Ca. carduelis	22 (3)	9	13-40	251	-229	372	-350
Ca. flammea	56 (3)	13.8	35-82	191	-135	262	-206
Emberiza citrinella	49 (3)	20.5	13-84	416	-367	666	-617

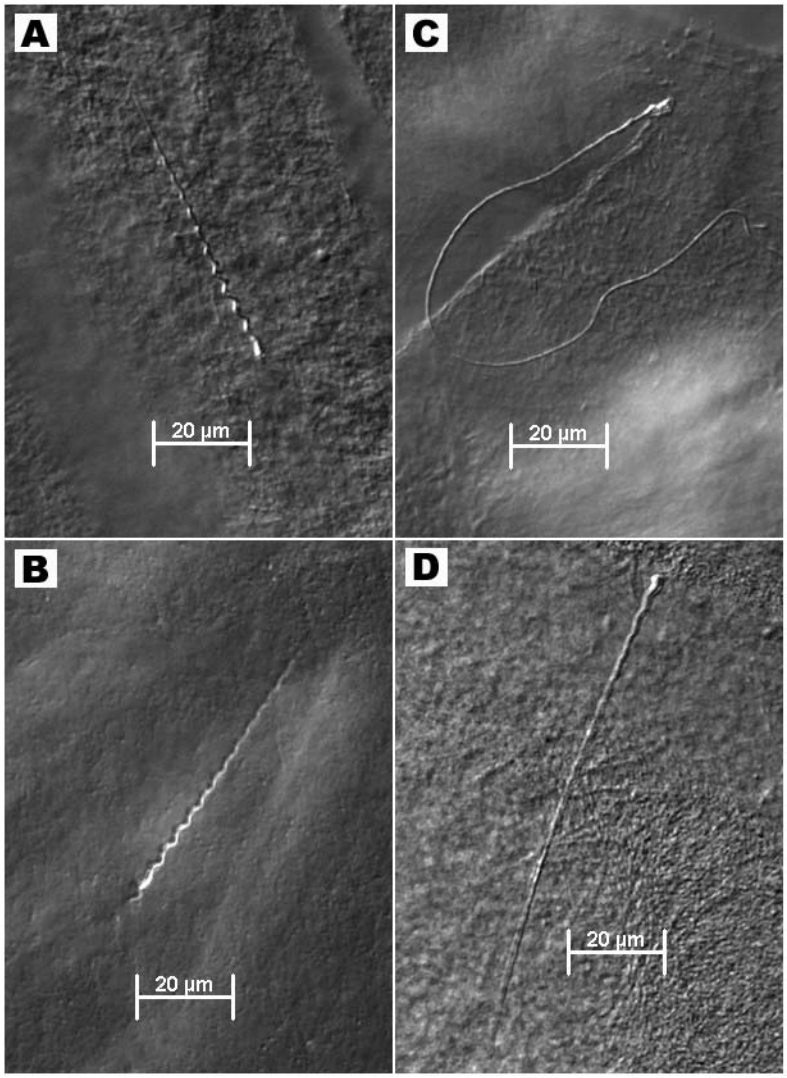

Figure 2.1 Images of sperm from four species viewed under DIC microscope at 400x magnification. Species shown are; (A) *Turdus merula*, (B) *Sturnus vulgaris*, (C) *Carduelis flammea*, (D) *Emberiza citrinella*. Note the difference in structure and size from the different species and the unevenness of the perivitelline membrane layer.

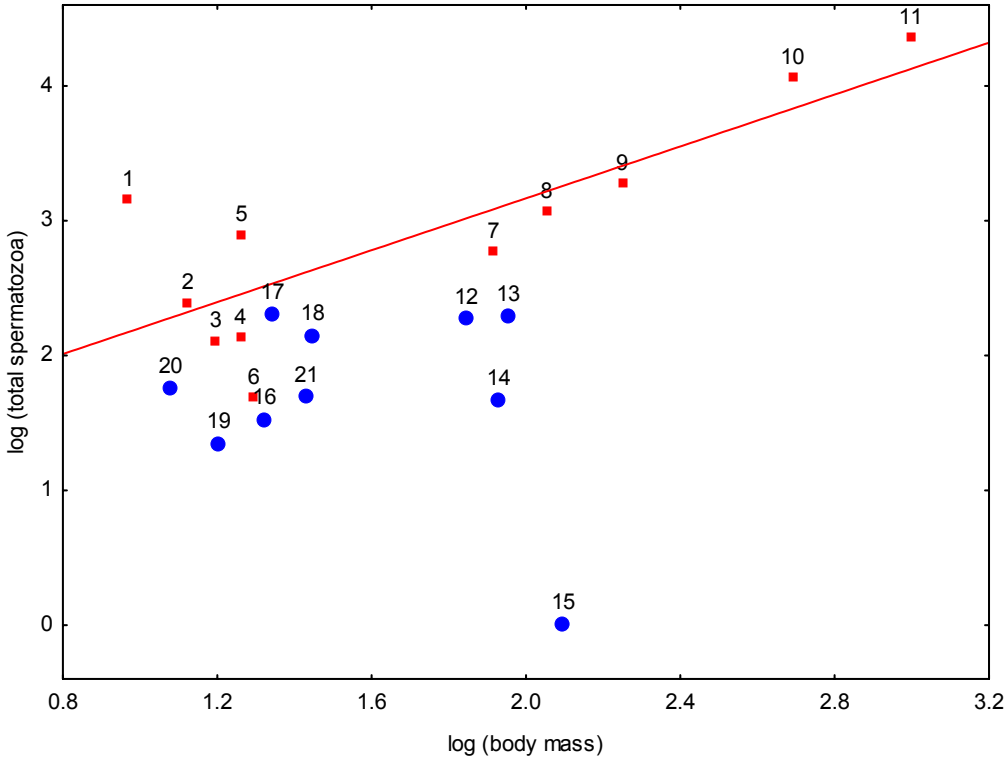

Figure 2.2 Relationship between log (body mass) and log (total spermatozoa) for 21 species of birds. Linear regression line fitted for non-bottlenecked species surveyed in Birkhead et al. (1994) are represented by ( ). This line is significant ($F_{1,9} = 17.84$, $P = 0.002$). Numbers of sperm on perivitelline membranes for species introduced species in New Zealand are represented by ( ). Non-bottlenecked species are (number corresponds to label on graph): 1. *Remiz pendulinus*; 2. *Parus caeruleus*; 3. *Panurus biarmicus*; 4. *Erithacus rubecula*; 5. *Emberiza schoeniclus*; 6. *Prunella modularis*; 7. *Sturnus vulgaris*; 8. *Turdus merula*; 9. *Pica pica*; 10. *Columba palumbus*; 11. *Uria aalge*. Bottlenecked New Zealand species are: 12. *T. philomelos*; 13. *T. merula*; 14. *S. vulgaris*; 15. *Acridotheres tristis*; 16. *P. modularis*; 17. *Fringella colebs*; 18. *Carduelis chloris*; 19. *C. carduelis*; 20. *C. flammea*; 21. *Emberiza citrinella*.

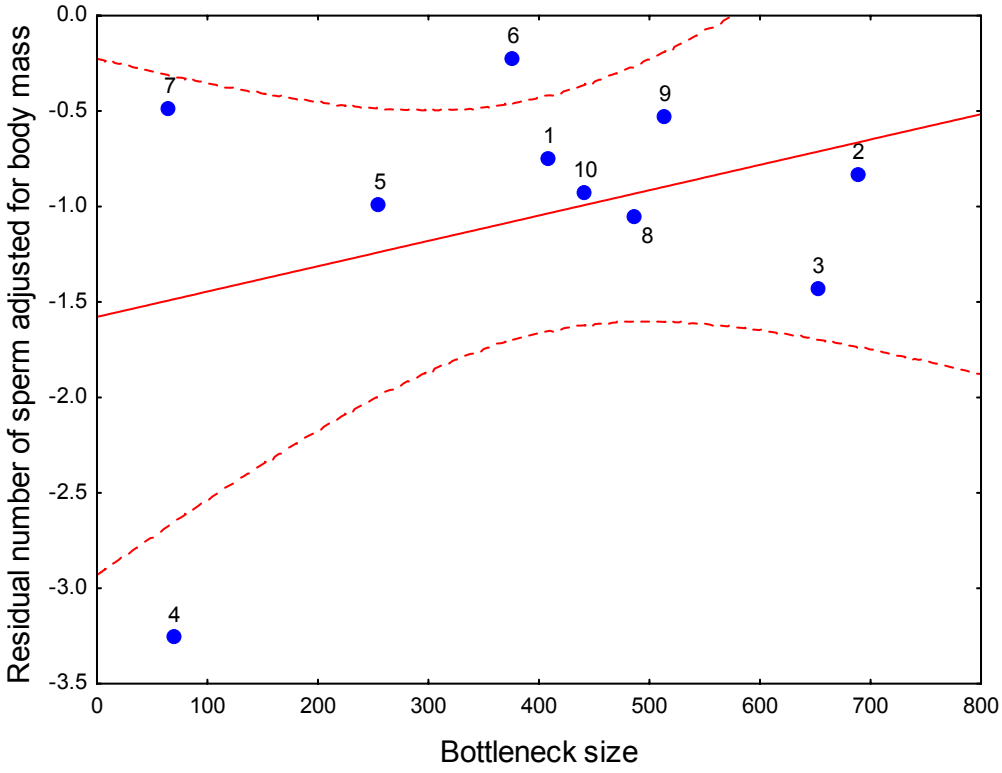

Figure 2.3 Relationship between bottleneck size and residual log (ovum diameter) from predicted regression line from non-bottlenecked birds: $\beta = 0.336$ (SE: ±0.334), $F_{1, 8} = 1.02$, $P = 0.343$. Dotted lines represent 95% confidence intervals. Species are: 1. *T. philomelos*; 2. *T. merula*; 3. *S. vulgaris*; 4. *Acridotheres tristis*; 5. *P. Modularis*; 6. *Fringella colebs*; 7. *Carduelis chloris*; 8. *C. carduelis*; 9. *C. flammea*; 10. *Emberiza citrinella*.

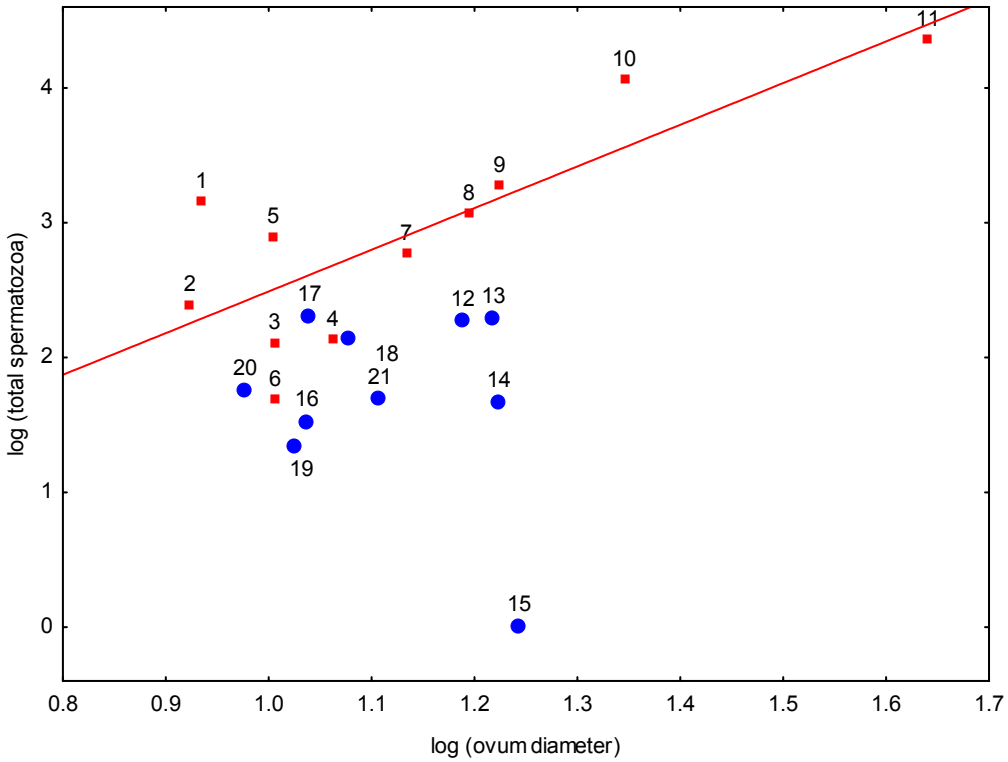

Figure 2.4 Relationship of log (ovum diameter) and log (total spermatozoa) for 21 species. Linear regression line fitted for non-bottlenecked species (Birkhead et al. 1994) is significant ($F_{1,9} = 16.44$, $P = 0.003$). Names and numbers of each species is the same as for figure 2.2.

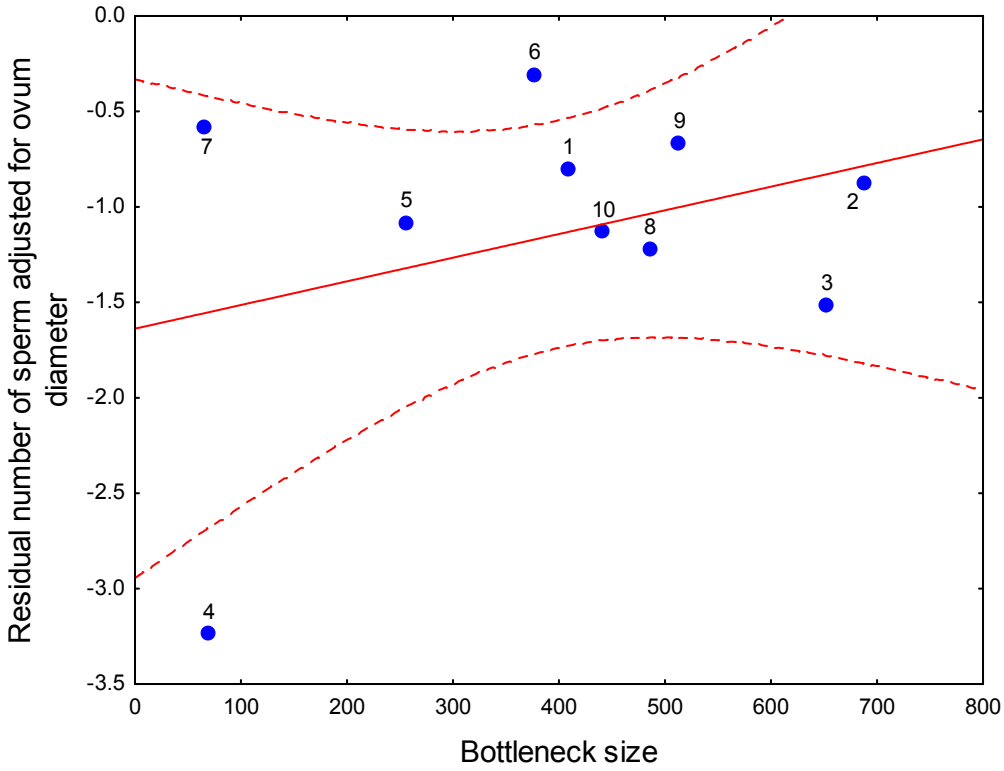

Figure 2.5 Relationship between bottleneck size and residual log (ovum diameter) from predicted regression line of non-bottlenecked birds: $\beta = 0.326$ (SE: ±0.334), $F_{1, 8} = 0.95$, $P = 0.359$. Dotted lines represent 95% confidence intervals. Names and numbers of each species is the same as for figure 2.3.

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Chapter 3:

A preliminary study of sperm abnormalities in introduced birds that have gone through population bottlenecks in New Zealand

Abstract

Decreases in the reproductive performance of males can have serious implications for the survival of endangered species. Studies on a variety of mammals have shown that inbreeding and population bottlenecks can lower male fertility. However, little work has been done on the effects of population bottlenecks on the fertility of male birds. In this chapter, I examine the structure and motility among a variety of introduced species in New Zealand that have passed through bottlenecks of varying severity. I measured variance in the size of the midpiece of a range of species by a fluorescent staining technique. Motility was measured by counting the percentage of motile sperm in freshly collected ejaculates from wild birds. The mean C.V. in midpiece length ranged from 1.7 – 6.9 and sperm motility ranged from 63% – 100%. Preliminary analyses suggest that the size of the population bottleneck each species passed through may affect the coefficient of variation (C.V.) in midpiece length and percent motility. However, sample sizes were small and further work is needed to confirm whether severe bottlenecks impair the motility of sperm and increase the frequency of abnormalities in their structure.

Introduction

Matings between parents that are genetically similar, due to a small population size or a historic bottleneck, have been found to result in higher proportion of unhatched eggs in birds (Bensch et al. 1994, Kempenaers et al. 1996, Briskie and Mackintosh 2004) and higher mortality rates of offspring in mammals (Ralls et al. 1979, Thornhill 1993). The causes of higher hatching failure/mortality are not known, but it is thought that inbreeding, or mating with a genetically similar partner, may unmask deleterious recessive alleles and lead to a loss of heterozygosity (Charlesworth and Charlesworth 1987). Most of the evidence that suggests inbreeding leads to a decrease in fitness is from captive populations, particularly mammals (e.g. (Lindburg et al. 1993, Roldan et al. 1998, Gerald et al. 2002, Busso et al. 2005). However, increasing evidence in the last few years from wild populations suggests that inbreeding effects are not confined to captive populations and in the laboratory (Wildt et al. 1987, Patil et al. 1998, Briskie and Mackintosh 2004, Whittaker et al. 2004, Crosier et al. 2006).

New Zealand has a high number of bird species that are critically endangered and currently exist in small, often isolated and fragmented populations. As a consequence, many species have gone through severe population bottlenecks and rates of inbreeding are likely to be higher than experienced in a pre-bottlenecked population. Thus, it is important to understand the fitness-reducing consequences of passing through a severe bottleneck for an endangered species if it is to be effectively managed for conservation purposes. In practise, it can often difficult to measure such effects on endangered animals because of the low sample sizes and restrictions associated with studying endangered species. If the effects of bottlenecks on fitness

differ between males and females, then this may also necessitate sex specific conservation measures.

An example of how a sex specific approach is needed to understand the effects of population bottlenecks is well illustrated by the increases in hatching failure seen in endangered species of birds. For example, Briskie & Mackintosh (2004) found that species that went through severe population bottlenecks had higher than normal hatching failure rates. This increased failure could be due to either problems associated with females (e.g. problems in production of eggshells; see chapter 4), or with males (e.g. function of sperm). Whether bottlenecks (and presumed higher levels of inbreeding) directly affect male and female fitness differently is poorly understood, especially in birds. The majority of the studies that suggest decreased male fertility is a result of inbreeding or a population going through a bottleneck, has been done on big cats (e.g.(Wildt et al. 1987, Crosier et al. 2006) or ungulates (e.g. (Roldan et al. 1998, Gomendio et al. 2000, Malo et al. 2005).

In chapter 2, I examined whether there was a link between the severity of a population bottleneck and number of sperm present at the site of fertilisation for introduced birds in New Zealand. Although there was no direct relationship between bottleneck size and the number of sperm present on the perivitelline membrane of the egg, all species that I studied had much lower than expected sperm counts. There are two possible reasons why this would be the case: (a) females were unable to store sperm sufficiently long, or transport sperm to the site of fertilization successfully, before the eggs were ovulated and fertlized, or (b) males produced poor quality ejaculates that were unable to reach the egg either through abnormalities or

inadequate production. A reduction in the quality of sperm has been shown in other species that have gone through severe bottlenecks, such as the cheetah (*Acionyx jubatus*) (O'Brien et al. 1987, Menotti-Raymond and O'Brien 1993), but never in a wild population of birds.

In this chapter, I examine two features of sperm that could be important for determining male fertility in New Zealand's introduced birds: (a) the presence of structural abnormalities in the midpiece, and (b) reductions in the motility of sperm. The midpiece of sperm contains the mitochondria, and in passerine birds only a single mitochondria is present that wraps around the axoneme (centrioles that provide the shape for the tail) and provide the energy for movement. Gross abnormalities or increased variation in the size of the midpiece (e.g., over-sized and under-sized midpieces) could thus affect energy production, swimming speed or the lifespan of spermatozoa (Roldan et al. 1998). Sperm motility is a measure of the percent of motile sperm in an ejaculate and has been widely utilised as an index of a male's fertility. For example, the rate of sperm motility is low in species such as cheetahs (*Acionyx jubatus*) (Lindburg et al. 1993, Crosier et al. 2006), lions (*Panthera leo*) (Wildt et al. 1987), and gazelles (*Gazella cuvieri*, *G. dorcas* and *G. dama*) (Roldan et al. 1998, Gomendio et al. 2000). All of these species have been through genetic bottlenecks at some stage in their history. Midpiece length and abnormalities in midpiece structure are highly heritable in mice (Woolley and Beaty 1967), and there is no reason to believe it might be otherwise in birds (Birkhead et al. 2005). If passing through a bottleneck increases midpiece deformities or reduces sperm motility, it could even spread or become fixed in the post-bottlenecked population.

In this part of my thesis, I use a variety of introduced bird species to New Zealand, to determine whether severe population bottlenecks have affected either midpiece morphology or sperm motility. As discussed previously (chapter 1), introduced birds provide an ideal study system for the effects of bottlenecks as the size of the bottleneck each passed through are reasonably well known, and all have non-bottlenecked source populations that can ultimately be used as a control. Here I specifically test the hypothesis that there will be higher variability in the length of midpiece and lower levels of motility in species that have gone through a severe bottlenecks compared to species that have been through larger population bottlenecks.

Methods

Midpiece abnormalities

I obtained sperm samples from 8 species of introduced New Zealand passerines. All samples were collected during the breeding season (October to January). These species were: Song Thrush (*Turdus philomelos*), Blackbird (*T. merula*), Dunnock (*Prunella modularis*), Chaffinch (*Fringella colebs*), Goldfinch (*Carduelis carduelis),* Redpoll (*C. flammea*), Yellowhammer (*Emberiza citrinella*) and House Sparrow (*Passer domesticus*). These species passed through bottlenecks of varying size during their establishment in New Zealand, ranging from a low of about 250 in the Dunnock to a high of about 700 in the Blackbird (Thomson 1922, Lever 1987). Sperm was collected from three South Island locations near Kaikoura (42°38’66”S, 173°62’54”E) and Ward (41°79’69”S, 174°11’62”E), and one North

Island location, near Limestone Downs (37°47'69"S, 174°75'94"E). Samples were collected from live birds by gently massaging the cloacal protuberance. To study the structure of the midpiece, some samples were stored in 10% formaldehyde for further analysis in the laboratory at a later date. I also collected some sperm samples from dead birds (mostly fresh road kills) by extracting sperm from the end of the seminal glomera.

Midpiece size was measured in the laboratory at 400x using a Zeiss Image M1 fluorescent microscope. Sperm were first prepared by washing in phosphate buffer saline (PBS) and centrifuged at 2500 rpm for 2 minutes. They were then stained in Mitotracker Green (Invitrogen, Sydney, Australia) for 20 minutes before being washed again in PBS before mounting. Mitotracker Green is a fluorescent dye specific to mitochondria which allowed me identify and measure the midpiece. The dye was excited at 490 nm and highlights the mitochondria as a bright green section of the sperm (see figure 3.1). Photos were taken of individual sperm using a digital camera. A second photo of each sperm using differential interference contrast (DIC) was also taken to allow me to measure the total length of the sperm more accurately as Mitotracker Green did not consistently stain the flagellum. Photos were captured using AxioCam HRc and Axiovision 3.1 software. The mitochondria of each sperm were then measured using Image Pro Plus v4.5 from the fluorescent images, and the total length of the sperm from the DIC images.

I estimated midpiece abnormalities in two ways. First, I simply calculated the percentage of sperm with obvious midpiece abnormalities (number of sperm with abnormalities divided by total number of sperm sampled per individual). The average

rates of abnormalities from each species were then calculated. Midpiece abnormalities were determined to be a midpiece that appeared either, (a) grossly short or long, (b) broken, or (c) misshapen (see figure 3.2). Second, I then calculated the proportion of total sperm length made up by the midpiece. This was measured on 4 to 30 sperm per individual and then averaged. I next calculated the coefficient of variation (C.V.) for each individual to determine the extent of variation in the proportion of the sperm made up by the midpiece (Zar 1999). The assumption here is that an individual with a high C.V. is producing sperm that are highly variable in midpiece size, and perhaps as a result, produce sperm variable in performance, with those sperm deviating the most, being suboptimal in performance (see Discussion). The mean coefficient of variation for each species was plotted against bottleneck size to determine whether there was any relationship between the size of the introduction of New Zealand's introduced birds and the level of variation in the morphology of their sperm. If severe bottlenecks compromise sperm production, then species passing through the most severe bottlenecks might produce sperm more variable in morphology, including a higher percentage of grossly abnormal sperm, than species passing through less severe bottlenecks.

Motility

I studied the motility of sperm in 6 species of introduced birds in New Zealand. These species were: Song Thrush (*Turdus philomelos*), Dunnock (*Prunella modularis*), Chaffinch (*Fringella colebs*), Greenfinch (*Carduelis chloris*), Yellowhammer (*Emberiza citrinella*) and House Sparrow (*Passer domesticus*). These species passed through bottlenecks during their establishment in New Zealand,

ranging from a low of 66 in the Greenfinch to a high of about 450 in the Yellowhammer (Thomson 1922, Lever 1987). As such they provide a model system for examining the effect of different sized bottlenecks on the level of motility of sperm in each species. Samples were collected from live males by gently massaging the cloacal protuberance (Wolfson, 1954). Sperm was diluted with Dulbecco's Eagle Medium and mounted on a glass slide at 35^{o}C. The sperm was observed under a Leica DMR microscope at 400x magnification and recorded on VHS tape using a JVC TK-C1381 Color Video Camera for analysis later in the laboratory. A total of 3 minutes were recorded for each sample over six fields of view. All sperm that were visible from each frame were counted and the total percentage of sperm that were moving was calculated. The number of sperm counted per individual ranged from 59 to 521. A sperm was determined to be motile if it moved across the screen using its own momentum, i.e., using tail beats, and not through passive movement with water currents.

The mean motility for each species was calculated and plotted against the size of the bottleneck each species went through when introduced to New Zealand. A correlation analysis was carried out to determine if there was a relationship between the size of the genetic bottleneck and the level of sperm motility. I assumed that the number of individuals introduced was a realistic index of bottleneck size. Data on bottleneck size was obtained from Thompson ((Thomson 1922) and Lever (Lever 1987). All analyses were carried out in Statistica v. 7.0 statistics package.

The collection of sperm was conducted under the approval of the University of Canterbury Animal Ethics Committee. Introduced birds are not protected in New

Zealand and no Department of Conservation permit was required for the collection of sperm.

Results

The size and morphology of sperm examined in this study was highly variable between species (Fig 3.1). However, the frequency of gross midpiece abnormalities was low (Table 3.1). I only observed 2 incidences in a single Blackbird male in which the midpiece was much shorter compared to other Blackbirds (Fig 3.2). There were no other examples of gross abnormalities found, such as sperm with multiple tails, bent heads or tails or the lack of an acrosome. Thus, there was no evidence that the bottlenecks each species passed through had any effects on the gross morphology of the midpiece.

Although gross scale abnormalities appeared rare, it is possible that fine scale abnormalities in the length of the midpiece may increase in frequency with severity of a bottleneck across species. The mean C.V. in proportional midpiece length was not significantly with bottleneck size across species ($r = 0.41$; $n = 8$, $p = 0.31$). However, the blackbird appears to be an outlier and when I re-analysed the data with this species remove, the relationship became negative, but still not significant ($r = -0.66$; $n = 7$, $p = 0.1$). Some caution is needed in interpreting either of these comparisons as sample sizes for each species were small (range: $n = 1 – 5$ individuals per species), but to the trend on the graph suggests bottleneck size may have an influence on the level of variability in midpiece size (Fig 3.3).

The percent sperm motility for introduced birds of New Zealand was generally high (Table 3.2). With the exception of the Dunnock, all other introduced species had sperm motility estimates above 75%. There is no data from the source populations for any of the species I examined in New Zealand, so no direct comparisons can be done to determine if there has been a change in either sperm motility since passing through a bottleneck. However, I collected values of sperm motility from the literature and found that the species I studied have levels of sperm motility that are generally higher than many other species (Table 3.2). The sperm motility of the Dunnock was much lower than any other passerine (63.4%) but was still higher than most of the other species of birds for which comparable data is available (Table 3.2).

When the mean percentage of sperm motility for each species was plotted against bottleneck size, the correlation was positive but not significant ($r = 0.41$; $n = 6$, $p = 0.42$). Sample sizes for most species were low (range: $n = 1 – 8$ individuals per species) so some caution is needed in drawing any firm conclusions, but again the trend from the graph suggests that severe bottlenecks may reduce sperm motility (Fig 3.4).

I correlated mean C.V and sperm motility against hatching failure rates from Briskie and Mackintosh (2004). Mean C.V of midpiece length against hatching failure was not significant ($r = -0.22$; $n = 8$, $P = 0.61$) as was motility versus hatching failure ($r = -0.50$; $n = 6$, $p = 0.32$). However, there was a trend for species with higher hatching failure rates to have lower sperm motility (Fig. 3.5). Again, this must be interpreted with caution as sample sizes are small.

Discussion

The presence of gross abnormalities in the midpiece of sperm was rare. I observed only two incidences of an obviously shorter than normal midpiece, both from Blackbirds. The correlation of variation of midpiece proportion in relation to the size of genetic bottleneck was not significant but at this point it is not clear if the relationship between the two is positive or negative (the addition of further species is needed). In contrast, the level of sperm motility in New Zealand's introduced passerines appeared to be similar compared to other species; however, mean sperm motility did appear to decline with increasing severity of population bottleneck size and increased hatching failure rate.

This study is the first to assess the frequency and types of sperm abnormalities with relation to the population bottleneck size across a wide variety of wild bird species. At present the sample sizes are low for most of the species in my study, so robust statistical tests could not be performed and the results should be interpreted with caution. In addition, little work has been done on the characteristics of the sperm from the species I studied in their source population. This makes it difficult to draw conclusions on the effect of population bottlenecks on the quality of sperm. Nevertheless, my preliminary findings suggest the effect of bottlenecks may be manifested in changes in midpiece size and variation, and by decreases in sperm motility.

Previous work on the cheetah (*Acionyx jubatus*) has shown that sperm motility is impaired and the levels of abnormalities are high (Lindburg et al. 1993, Crosier et al. 2006). Cheetahs have been through two historic population bottlenecks (O'Brien et al. 1987) so provide a good example of the effects of bottlenecks on reproductive traits. Nonetheless, most of the work has been done on captive populations of animals, and there is some debate over whether captive animals can provide a realistic assessment of fertility in wild populations of endangered species. Recent work by Crosier *et al* (2006) shows that although the assessment of captive populations underestimates the level of sperm motility, it does not underestimate the percentage of abnormal sperm. A population of African lions in Ngorongoro Crater (Africa) which went through a population bottleneck of around 6 – 15 individuals also shows lower sperm motility and increased sperm abnormalities compared to an outbred population (Wildt et al. 1987). The bird species examined in my study all have percent motility values similar to the big cats which have been through bottlenecks (Table 3.2). However, as passerines and felines are not closely related phylogenetically, it is not the best comparison to use to determine whether New Zealand's introduced birds have been affected by genetic bottlenecks. However, it does illustrate the effect of bottlenecks and inbreeding on reduction in one aspect of reproductive fitness.

My data on introduced birds suggest there could be a link between the size of a population bottleneck and the percentage sperm motility and the level of variation in the proportion of the midpiece in the sperm. However, caution must be taken as sample sizes were small and I was unable to sample some species that have been through the most severe bottlenecks (e.g. cirl bunting). Based on previous work, I was expecting that severe bottlenecks should decrease sperm motility and increase

variation in midpiece size. Both predictions were not supported by my data, but the trends in the graphs could suggest otherwise. At present, it is not possible to say at what bottleneck size such effects are first seen, and so it is not possible to provide an estimate of minimum bottleneck size needed to avoid deleterious effects on sperm function. However, within the range of bottleneck sizes that my study species passed through, the fact that some effects where found suggests further work on these species may be able provide an answer to this problem.

Compared to other species, the Blackbird appeared to show more variability within the proportion of sperm length made from the midpiece than any other species (Fig 3.3). This was unexpected as it passed through the largest bottleneck of all the species in my sample, and presumably inbreeding effects were least severe as a result. However, if is possible that variability in midpiece size could be the reverse of what I was predicting. In other words, rather than severe bottlenecks leading to increase variation in midpiece size (through the deleterious effects of inbreeding), it might be the case that the larger genetic variation present in species passing through the larger bottlenecks could lead to larger variation in the size of the midpiece. At present, it is unknown what effect a large deviation in midpiece size has on sperm function. The obvious assumption is that midpieces that deviate far from the norm are more likely to be dysfunctional (i.e., midpiece length is under stabilizing selection) (Birkhead et al. 2005). However, more work is needed to directly link sperm function (e.g. motility, speed, longevity) within variation in midpiece length before this issue can be resolved.

The Dunnock appeared to have much lower motility than other passerine species studied (Fig 3.4). It is difficult to suggest that this is just an effect of a small founder population, as the Greenfinch, which passed through an even more severe bottleneck, did not show a similarly reduced level of sperm motility. Dunnocks have an unusually complicated polygynandrous mating system, where females and males have multiple partners. Perhaps this could exaggerate any effect on sperm quality if this odd mating system increased the severity of the genetic bottleneck, as might happen if variance in male reproductive success was high (i.e. many males are excluded from breeding and so the number of individuals contributing to the gene pool is much less than the number introduced). All the other species examined in this study, apart from the Dunnock, have similar sperm motility to another passerine, the zebra finch (Table 3.2) (Birkhead et al. 1998), suggesting little effect of bottlenecks at any level. However, zebra finches are domesticated and the levels of inbreeding probably higher than in the wild and so it is unclear if this comparison can shed much light on levels of sperm motility in wild and non-bottlenecked birds.

Although my data is preliminary and sample sizes are small, I found that the techniques I used work well on a range of species (Fig 3.1). The results I found to date suggest that further investigation is clearly warranted, both in regards to morphological abnormalities and to decreases in sperm motility. More replicates of each species are required, and it would be useful to examine further introduced species, such as Cirl Bunting (*E. cirlus*), Indian Myna (*Acridotheres tristis*) and European Starling (*Sturnus vulgaris*). Collecting sperm for the examination of motility and abnormalities is non-invasive and relatively inexpensive so could be readily used on critically endangered species in New Zealand such as Kakapo

(*Strigops habroptilus*), Saddleback (*Philesturnus carunculatus*) and Chatham Island Robin (*Petroica traversi*). The identification of particular males with sperm production problems could then be used to manage populations in such a way that scarce breeding females are not paired to infertile males.

Figures

Table 3.1 The frequency of gross midpiece abnormalities among introduced New Zealand birds. A gross abnormality in midpiece structure was observed in only one species, the Blackbird (*Turdus merula*).

Species	Number individuals sampled	Total number of sperm measured	% sperm with abnormal midpiece
Prunella modularis	2	37	0
Carduelis carduelis	1	21	0
C. flammea	3	28	0
Passer domesticus	1	29	0
Turdus philomelos	1	14	0
T. merula	5	73	2.7
Emberiza citrinella	1	15	0
Fringella coelebs	2	21	0

Table 3.2 Comparison of sperm motility levels in different species of mammals and birds from the literature and this study. C, captive; W, wild; WB, wild born; SDZ, San Diego Zoo; NA, North America; Ser, Serengeti National Park; Ngo, Ngorongoro Crater. Whether a population passed through a bottleneck was defined by the authors of the studies, but in most cases the actual size of the bottleneck was unknown.

Order	Species	Bottleneck	Population	Mean sperm motility (%) ± (S.E)
Rodenta	*Chinchilla lanigera*	No	C[1]	93.9 (0.7)
Primates	*Macaca mulatta*	No	C[2]	17.4 (15.9)
Artiodactyla	*Gazella cuvieri*	Yes	C[3]	52.6 (12.7)
Carnivora	*Acinonyx jubatus*	Yes	C (SDZ)[4]	42.7 (6.7)
		Yes	C (NA)[4]	70.7 (3.5)
		Yes	C (NA)[5]	67.0 (2.0)
		Yes	WB[5]	78.0 (1.4)
	Panthera leo leo	No	W (Ser)[6]	91.0 (4.2)
		Yes	W (Ngo)[6]	83.0 (4.6)
	P. l. persica	Yes	C[6,7]	61.0 (3.7)
	P. tigris	No	C[7]	53.8 (15.8)
	Felis catus	No	C[8]	80.8 (2.0)
Sphenisciformes	*Spheniscus magellanicus*	No	W[9]	59.2 (3.9)
Gruiformes	*Chlamydotis undulata*	No	C[10]	59.7 (5.1)
Passeriformes	*Taeniopygia guttata*	No	C[11]	85.4 (1.8)
	Prunella modularis	Yes	W[12]	63.4 (4.7)
	Passer domesticus	Yes	W[12]	84.9 (3.7)
	Turdus philomelos	Yes	W[12]	77.2
	Fringella coelebs	Yes	W[12]	100
	Emberiza citrinella	Yes	W[12]	95.6 (1.2)
	Carduelis chloris	Yes	W[12]	80.9 (2.5)

[1](Busso et al. 2005); [2](Gerald et al. 2002); [3](Roldan et al. 1998); [4](Lindburg et al. 1993); [5](Crosier et al. 2006); [6](Wildt et al. 1987); [7](Patil et al. 1998); [8] (Kashiwazaki et al. 2005); [9](O'Brien et al. 1999); [10](Hartley et al. 1999); [11](Birkhead et al. 1998); [12]Present Study.

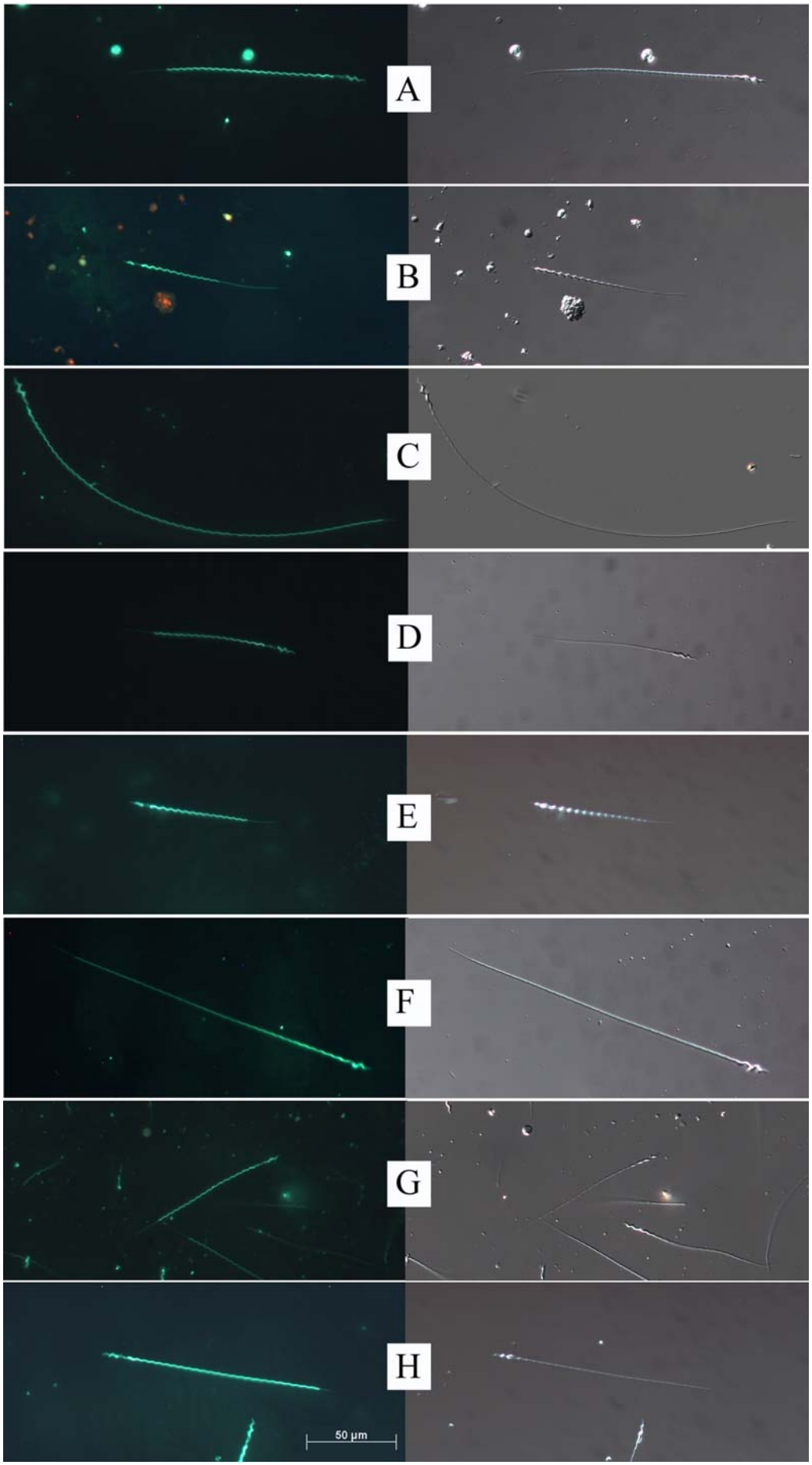
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Figure 3.1 Fluorescent (left) and DIC (right) images of sperm from each species examined. The midpiece in each image on the left shows up as a bright green 'helix'. The acrosome also shows a bright green at the head end of the sperm, while the nucleus and flagella do not stain as readily with Mitotracker Green Fluorescent Dye and appear duller in the images. Species are (A) *Emberiza citrinella*; (B) *Turdus merula*; (C) *Fringella coelebs*; (D) *Prunella modularis*; (E) *T. philomelos*; (F) *Carduelis flammea*; (G) *Passer domesticus*; (H) *Ca. carduelis*.

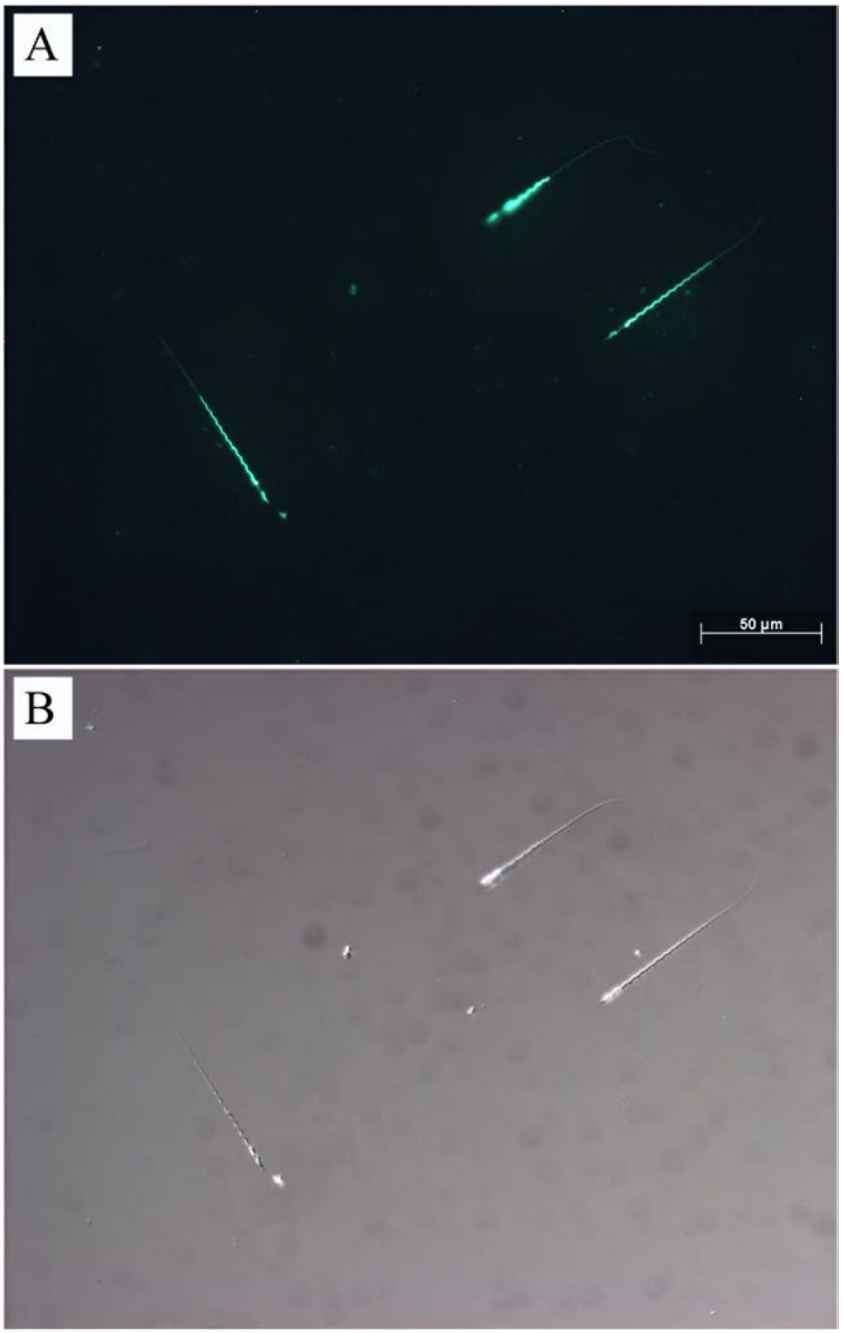

Figure 3.2 (A) Fluorescent microscopy of sperm from a single Blackbird (*Turdus merula*). The top sperm has a distinctly shorter midpiece than the other two, an example of a gross abnormality. (B) The same view from (A) but taken using DIC microscope. The top sperm appears the same as the other two normal sperm. The scale in both pictures is the same.

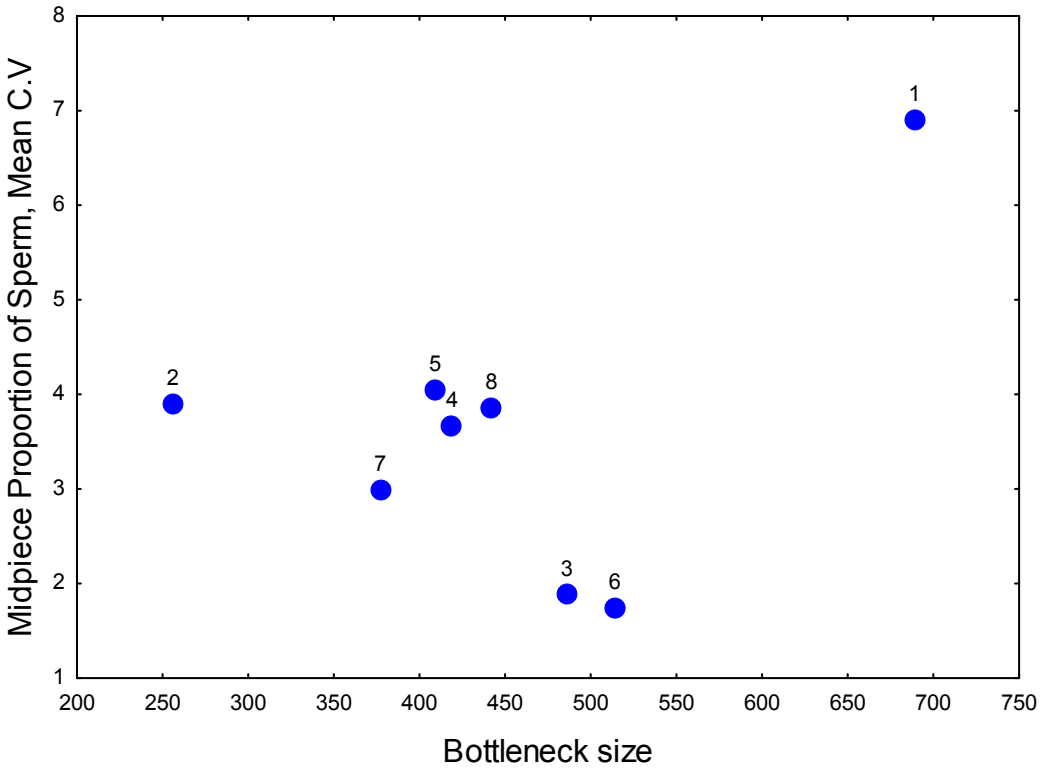

Figure 3.3 Coefficient of variation in midpiece size in relation to the size of the population bottleneck each species passed through. Correlation analysis is not significant r = 0.41; n = 8, p = 0.31. Species are (sample sizes) (1) *Turdus merula* (5); (2) *Prunella modularis* (2); (3) *Carduelis carduelis* (1); (4) *Passer domesticus* (1); (5) *T. philomelos* (1); (6) *C. flammea* (2); (7) *Fringella coelebs* (2); (8) *Emberiza citrinella* (1).

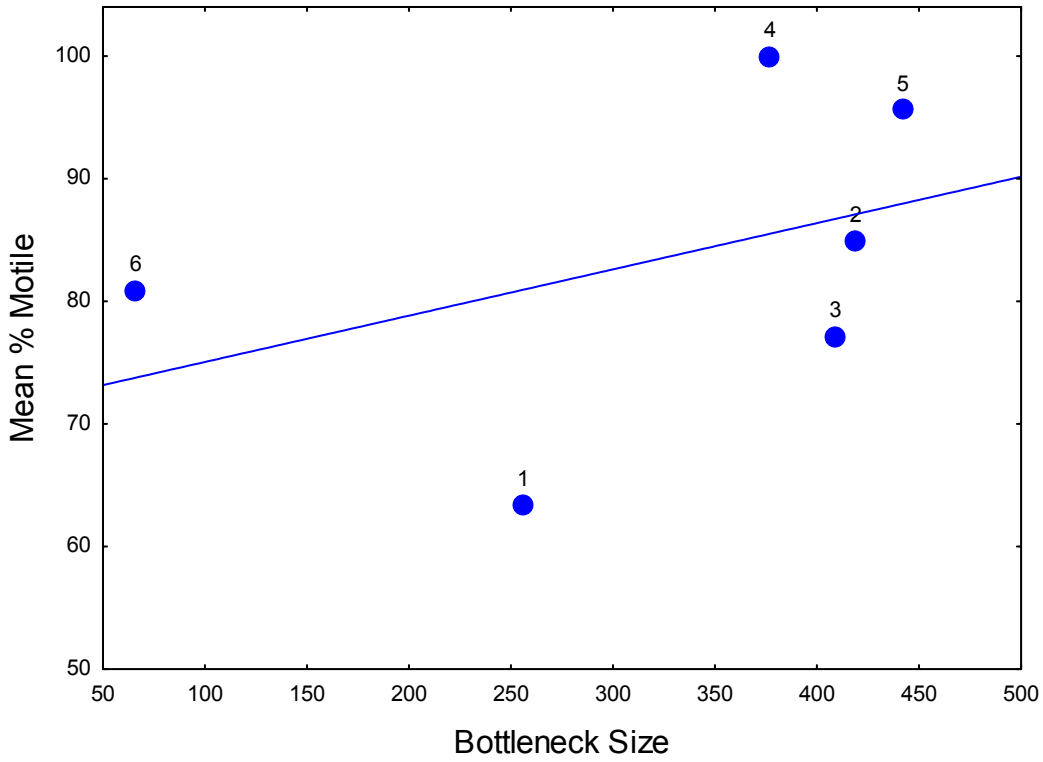

Figure 3.4 Mean sperm motility of introduced birds in New Zealand in relation to population bottleneck size. Correlation analysis is not significant r = 0.41; n = 6, p = 0.41. Species are (sample sizes); (1), *Prunella modularis* (3); (2), *Passer domesticus* (8); (3), *Turdus philomelos* (1); (4), *Fringella coelebs* (1); (5), *Emberiza citrinella* (2); (6), *Carduelis chloris* (3).

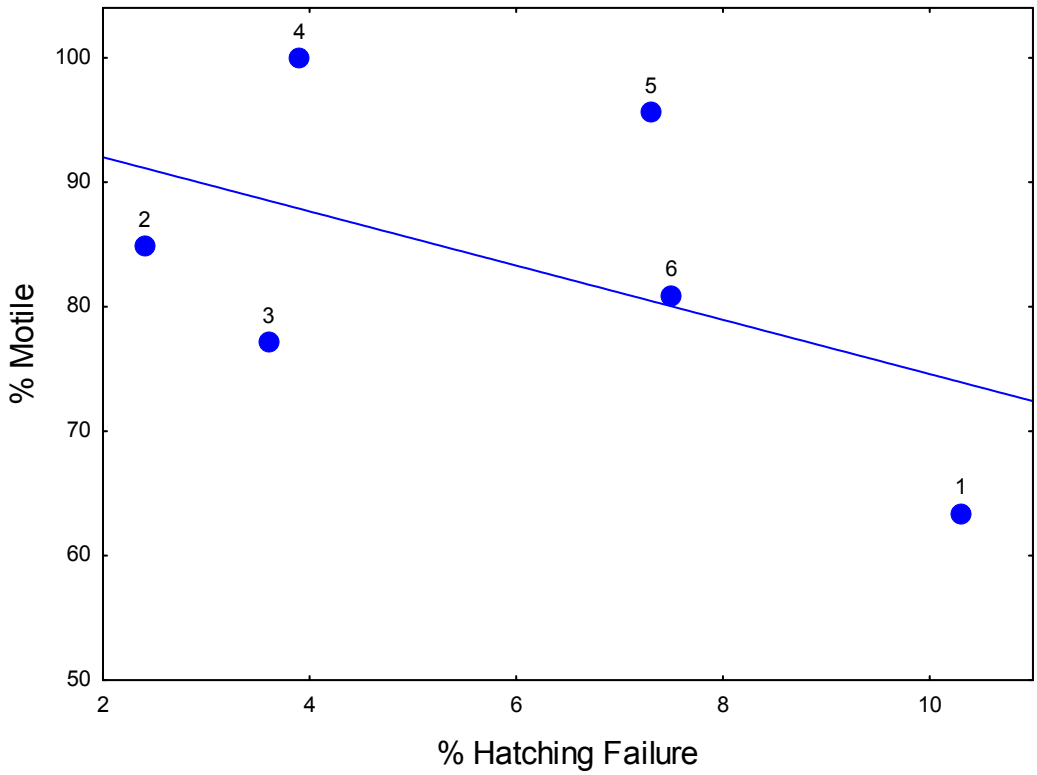

Figure 3.5 Mean sperm motility of introduced birds in New Zealand in relation to hatching failure rate. Correlation analysis is not significant r = - 0.50; n = 6, p = 0.32. Species list is the same as for figure 3.4

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Chapter 4:

Effect of population bottlenecks on the structure of avian eggshells

Abstract

The pores on the surface of the avian eggshell are responsible for controlling the amount of gas that diffuses between the environment and the embryo. In a healthy individual these pores allowed a tightly controlled amount of oxygen in and carbon dioxide and water out of the egg. Changes in the number and thickness of these pores could thus increase hatching failure rates if they impede the efficient transfer of gases. Although there has been research into the effect of pollutants on the shell characteristics, the effects of genetic stressors (such as inbreeding caused by population bottlenecks) on shell characteristics have not been determined. I examine whether populations that have been through population bottlenecks experience changes in the number of pores, the thickness of eggshells and the shape of pores. I found that introduced birds in New Zealand have fewer eggshell pores than what would be expected from non-bottlenecked populations. However, there was no relationship between the size of the bottlenecks and residual number of pores. The thickness of eggshells in New Zealand's introduced birds also did not deviate from what would be expected. Species that have been through more severe bottlenecks had proportionally more irregularly shaped pores than species that have been through larger bottlenecks. Although it is not clear if changes in pore shape negatively affect hatching success, my results indicate that severe bottlenecks have led to some changes

in the structure of eggshell that could reduce reproductive fitness in post-bottlenecked populations.

Introduction

The survival of developing avian embryos is dependent on the ability to exchange gas across the eggshell (Ar et al. 1974, Rahn et al. 1979, Rahn et al. 1987). Gases necessary for metabolism, such as H_20, O_2 and CO_2, are conducted between the embryo and outside atmosphere through pores across the surface of the egg shell (Rahn et al. 1987). These pores do not actively transport gases through the shell, but act as a pathway for passive diffusion (Rahn et al. 1979). In the absence of pores, O_2 would not be able to enter the egg and the embryo would succumb to hypoxia (Fox 1976, Tullett and Deeming 1982, Rahn et al. 1987). Pores are also responsible for the removal of CO_2, which accumulates in the egg as a by-product of embryo respiration (Ar and Rahn 1985; Rahn et al. 1987). In addition to this, H_2O is lost from the egg throughout incubation through evaporation (Simkiss 1980, Kern et al. 1992).

Three characters of avian eggshell pores determine the level of gas exchange (or gas conductance): pore diameter, pore length and total number of pores in an egg (Ar et al. 1974). A change in one or more of these pore characteristics could have consequences on the hatchability of that egg (Fox 1976). For example, if an egg has too few pores, or pores that are narrower than normal, embryo development could be stunted or the risk of hypoxia may increase and result in higher rates of mortality before hatching. Changes in the density of pores and the thickness of an eggshell are known to alter the properties of gas conductance across the eggshell (Ar et al. 1974, Fox 1976), and as the thickness of an eggshell determines the length of the pores through it and therefore the level of resistance gas will encounter during passive diffusion (Ar et al. 1974), changes in eggshell structure have the potential to disrupt

embryo development. Although there have been no in depth studies on the effect of pore shape on the ability of gas to diffuse through an eggshell, it has been suggested though that the shape of a pore may play more of an important role in gas transfer than initially thought (Toien et al. 1987). The models and techniques currently used to calculate gas conductance all assume that pores are round. However, if a pore is not entirely round, or even more irregular in shape, this could change the level of H_20, O_2 and CO_2 that is able to diffuse across the eggshell, affecting the development of the embryo.

Previous studies of hatching failure in birds have mostly focussed on the effects of environmental contaminants. For example, acid rain, heavy metal toxins, poisons and fertilizers have all been shown to have a negative impact on the ability of females to produce viable eggs (Enderson and Berger 1970, Nyholm and Myhrberg 1977, Drent and Woldendorp 1989, St Louis and Barlow 1993, Eeva and Lehikoinen 1995, Schwarzbach et al. 2001). Eggs with deformed shells or no shells, thinning of eggshells and high rates of unhatched eggs are among the main factors that appear to lead to increased problems with hatchability in species subject to excessive contaminants (Eeva and Lehikoinen 1995). Thinning of eggshells and changes in porosity of eggshells are two traits of particular concern in areas with heavy metal contamination and acidification of water supplies (Dauwe et al. 2004).

One group of pollutants that have well documented effects on eggshell structure are organochlorides such as DDT (Olsen et al. 1991, Schwarzbach et al. 2001). DDT causes both a thinning of eggshells and the reduction in the pore area (Fox 1976). The two potential consequences of a reduction of eggshell thickness

include the reduction of calcium available to developing embryos, and weaker layer of protection to the embryo. A normal shell thickness is important as developing chicks use the calcium in eggshells for development of bones, and it reduces the risk of micro-organisms from infecting the egg (Narushin and Romanov 2002). A change in thickness or a change in pore area in an eggshell may also change the degree of gas conductance, which could prevent the embryo from developing (Enderson and Berger 1970, Olsen et al. 1991, Schwarzbach et al. 2001).

Although environmental pollutants such as DDT, acid rain and heavy metals have been widely reported to affect hatchability (Glooschenko et al. 1986, Ormerod et al. 1988, Olsen et al. 1991), the role of "genetic stresses" have not attracted the same level of attention. Genetic stresses are likely to be common in species that pass through a severe population bottleneck, as such a demographic process is likely to lead to increased levels of inbreeding depression (Frankham et al. 1999). Inbreeding depression in turn can have negative consequences on populations such as reduced survivability (Jimenez et al. 1994, Brown and Brown 1997, Keller 1998) and increased levels of hatching failure (Bensch et al. 1994, Kempenaers et al. 1996, Briskie and Mackintosh 2004). Briskie and Mackintosh (2004) found a clear inverse relationship between the size of a bottleneck and the level of hatching failure. Hatching failure is known to increase in a variety of species with increased inbreeding (Bensch et al. 1994, Kempenaers et al. 1996, Briskie and Mackintosh 2004), though the actual mechanism by which this occurs has seldom been investigated.

New Zealand provides an excellent opportunity to determine whether severe bottlenecks (and presumably a subsequently highly level of inbreeding) lead to

changes in eggshell properties that might be linked with higher levels of hatching failure. During the late 1800's, acclimatisation societies introduced a variety of exotic species to New Zealand. As the number of individuals released differed across species, the size and severity of the bottleneck each species passed through also differed (Thomson 1922, Lever 1987). Due to the small number of founder individuals in some populations (termed genetic bottleneck), individuals are more likely to mate with close relatives. When this occurs, a high degree of inbreeding depression is observed (Charlesworth and Charlesworth 1987).

In Chapter 2 I suggested that severe population bottlenecks may cause lower than normal numbers of sperm to reach the site of fertilisation. However, this may not be the only reason why many eggs in highly inbred populations fail to hatch. It is also possible that a high genetic load from inbreeding may cause deformities in an eggshell or limit the ability of the females to obtain the right nutrients to produce normal eggshells. In this chapter I examine changes in pore number, size and shape across a range of introduced birds in New Zealand. Using data from the literature I then compare these values to the size of bottleneck each species passed through during their establisment in New Zealand, and to what would be expected in populations that have not passed through a severe bottleneck.

Methods

I collected eggs from a total of 12 introduced species in New Zealand. These included: Blackbird (*Turdus merula*), Song Thrush (*T. philmelos*), Dunnock (*Prunella modularis*), Chaffinch (*Fingilla colebs*), Goldfinch (*Carduelis carduelis*), Greenfinch

(*C. chloris*), Redpoll (*C. flammea*), Cirl Bunting (*Emberiza cirlus*), Yellowhammer (*E. citronella*), Indian Myna (*Acridotheres tristis*) and European Starling (*Sturnus vulgaris*). Collection sites in the South Island were Kaikoura Christchurch (43°48'58"S, 172°53'54"E), Kaikoura (42°38'66"S, 173°62'54"E) and Ward (41°79'69"S, 174°11'62"E); in the North Island, I collected eggs from Limestone Downs (37°47'69"S, 174°75'94"E). I located nests during nest building by following adults and searching likely locations. For starlings and mynas, I set up a series of nest boxes to attract birds. Nests and nest boxes were monitored daily and eggs were collected on the day, or within 2 days, that they were laid. Clutch sizes typically ranged from two eggs to five eggs in the species I studied. The first three eggs in a clutch were switched on the day of laying with a model egg so that the nesting female would continue laying. After collecting, the length and breadth of each egg was measured to the nearest 0.1mm using callipers and then used to calculate the total surface area of the egg using the formula given in Hoyt (1976). Mass was measured to the nearest 0.001g using an electronic balance (Acculab, PP-2060D). The eggs were then opened and the yolk membrane collected for an analysis of sperm numbers (see chapter 2). The shells were washed to remove any remaining albumin and air-dried before being stored in labelled vials for further analysis later. The number of clutches collected averaged 4 and ranged from 1 in the cirl bunting to 6 in the European starling.

To measure eggshell thickness, I randomly selected five fragments from each egg. The thickness of these fragments was measured using an electronic micrometer (Mitutoyo, 395-371, Tokyo, Japan) to the nearest 0.001mm. The micrometer was fitted with rounded anvils to ensure the shell was not punctured and thickness was

measured when a maximum force of 5 Newtons pressure was applied. All eggs from a clutch were measured, but to avoid pseudo-replication, I averaged eggshell thickness within each clutch before calculating the mean value for each species.

The number of pores on each egg was estimated by counting the pores from at least three known-size fragments of each shell. I used a pore counting technique similar to the one developed by Tyler (Tyler 1953). I first selected a series of shell fragments from: (1) the "pointed" end of the egg (equivalent to top 20% of pointed end), (2) the "blunt" end of the egg (equivalent to bottom 20% of blunt end), and (3) the "middle" section of the egg (remainder between pointed and blunt end). For ease of handling, I only used fragments at least $5mm^2$ in size. Each fragment was prepared by gently boiling it in a 5% NaOH solution for five minutes. This removed the inner membrane layers of the egg shell and the cuticle on the outer surface of the egg. Washed fragments were then mounted on a 25 mm stub and sputter-coated with gold palladium for four minutes. I then examined each fragment under a Leica 440 Scanning Electron Microscope (SEM) at magnifications ranging from 200X – 1000X at an accelerated voltage of 15.00 kV. The numbers of pores from each fragment were counted and a photograph of the entire fragment was taken with a scale bar. The surface area of the fragments was then calculated using Image-pro plus v.4.5 using the photographed scale bar as a calibration. Finally, the total number of pores per egg was estimated by averaging the pore density on each fragment and multiplying it by the total surface area of the egg. Because of the expense of preparing and analysing shell fragments under the SEM, I only examined one egg of each clutch.

SEM was next used to record the approximate shape of each pore. Pores were classified as either round (circular or nearly so), oval or elongated (obviously not circular but with no irregular edges), or irregular (any other shape but circular or oval). The classification of pore shape was based on the shape of the innermost part of the pore cone when the surface of the eggshell was laying perpendicular with respect to the detector in the SEM. This area appears black on the SEM images (Fig. 4.1) and is equivalent to the shape of the pore at its narrowest point. The relative numbers of the three pore shape categories were then calculated by dividing the number of each type by the total number of pores surveyed. As with my estimation of pore number, I only surveyed pore shape in one egg from each clutch.

To determine if pore number varies with bottleneck size, I compared the density of pores from the introduced species I examined in New Zealand with data from Ar and Rahn (1985). These authors surveyed birds around the world, but I only included their data on passerine species in my comparison as pore characteristics are different amongst different orders of birds. Ar and Rahn (1985) included a total 14 passerine species and estimated the relationship between egg mass and number of pores per egg (for passerines) as:

$$\text{Log}(N) = 0.9157(\log(w)) + 2.3452$$

Where N is the number of pores per egg and w is the mass of the egg in grams (g). For each species I calculated the difference between my observed counts of pore number with that predicted from the equation of Ar and Rahn (1985). This is the residual value and equivalent to the number of pores corrected for egg size. I then

compared the residual number of pores with population bottleneck size by linear regression. If severe bottlenecks lead to problems in pore production by females (either producing too few or too many), then I expected to see a relationship between bottleneck size and residual pore number. Data on the bottleneck size was obtained from Thompson (1922) and Lever (1987). A one sample t-test of means was carried out on the absolute values of the residuals versus zero (the expected regression line) to determine whether there was a significant difference in the number of pores on the eggshell from the expected values.

I next carried out a similar comparison with shell thickness in New Zealand's introduced birds to outbred species using data from Ar et al. (1974). These authors provide an equation to calculate eggshell thickness based on egg mass. Unfortunately, I was unable to separate passerine from non-passerine species in this equation based on the information given in their paper (Ar et al. 1974). For all species combined, eggshell thickness shows the following relationship to mass (Ar et al. 1974):

$$\text{Log}(L) = 0.456(\log(w)) - 1.29$$

Where L is the length of the pores in an eggshell, which is equivalent to shell thickness in millimetres (mm), and w is the mass of the egg. As with pore number, I used this formula to calculate the difference between my observed measures of shell thickness and the predicted values. This residual shell thickness was then compared to population bottleneck size. If passing through a severe bottleneck has a detrimental effect on the formation of the shell (either thinner or thicker than normal), then I expected a significant relationship between residual shell thickness and bottleneck

size. As with pore number, I carried out a one-sample t-test to compare residual eggshell thickness with an expected value of zero.

Unlike pore number and shell thickness, there is no information on the frequency of different pore shapes and how they might vary with egg size, pore number or shell thickness. The consequences of changes in pore shape are also unknown, but I assumed that if severe bottlenecks led to problems in the production of eggs, then the relative frequenies of each pore type might change, especially if it turns out that irregular-shaped pores are detrimental to efficient gas exchange or increase the chances of pathogen transmission (see discussion).

I could not calculate expected frequencies of pore shape in non-bottlenecked species (this data does not currently exist). However, I was able to plot the frequency of round pores, and irregular pores against bottleneck size and perform a regression analysis. If bottleneck size affects pore shape, then a change in the relative frequencies of each type might be expected to vary with bottleneck size.
All statistical analyses were carried out in Statistica v. 7.0.

The collection of eggs was conducted under the approval of the University of Canterbury Animal Ethics Committee. Introduced birds are not protected in New Zealand and no Department of Conservation permit was required for the collection of eggs.

Results

Eggshell thickness

The mean eggshell thickness of introduced birds was almost identical to that predicted from the model in Ar et al. (1974) (Fig. 4.2). As found previously (Ar et al. 1974), I found the relationship between egg mass and eggshell thickness was highly significant (ANOVA: $F_{1,9} = 171.43$, $P < 0.001$, $r^2 = 0.95$). To determine whether the size of the population bottleneck each species passed through affected eggshell thickness, I calculated the residuals from the regression line (Fig. 4.4) and then tested them against bottleneck size. The variation in the residuals around zero was small (Fig. 4.3) and the relationship between eggshell thickness and bottleneck size is not significant (ANOVA: $F_{1,9} = 0.70$, $P < 0.42$, $r^2 = 0.07$). Thus, there was no evidence that more severe bottlnecks have led to either a thinning or thickening of egg shells in introduced birds in New Zealand.

Pore Counts

There was a positive relationship between the number of pores on an egg and egg mass (Fig. 4.4). The regression between pore number and egg mass is highly significant (ANOVA: $F_{1,9} = 316.03$, $P < 0.001$, $r^2 = 0.97$), and the slope of this line (0.9489) is very similar to Ar and Rahn's (1985) regression line for passerine birds (0.9157). However, there is a significant difference in the intercept of these two lines (Fig. 4.4). The intercept for New Zealand's introduced species was 2.1202 compared with 2.3452 from Ar and Rahn (1985). This suggests that in general, the New Zealand

species had fewer pores than would be expected based on non-bottlenecked species elsewhere in the world. A single sample t-test of the residual number of pores (Fig. 4.5) shows a highly significant difference between New Zealand species and their expected values ($t = -14.7$, $df = 10$, $p < 0.001$). This difference could be explained by the level of inbreeding depression, if all species in my study were equally affected by bottleneck size, but if pore number was negatively affected by inbreeding, one might also expect a relationship between the severity of bottleneck size and the number of pores corrected for egg size. However, a regression of residual pore number versus bottleneck was not significant (ANOVA: $F_{1,9} = 1.17$, $P < 0.307$, $r^2 = 0.12$; Fig. 4.5). This suggests that the difference between my sample and that analysed by previous workers may not be due to inbreeding depression, but to some other cause such as a difference in techniques in counting pores. This possibility is addressed in the discussion.

Pore Shape

I compared the proportion of round (presumably normal) pores versus the size of population bottleneck within my sample of species. I found a significant and positive relationship between the frequency of round pores and bottleneck size (ANOVA: $F_{1,8} = 9.97$, $P < 0.013$, $r^2 = 0.55$). In other words, species that passed through the most severe bottlenecks had fewer round pores than those species passing through larger bottlenecks. Note that this result is dependent on my interpretation of a round pore. It can be difficult to distinguish between a round pore and an oval (or elongated) pore (Fig. 4.1) as the progression from one to the other is continuous. However, irregular shaped pores were more consistently categorised than differences

between an oval or a round pore (Fig. 4.1). A regression analysis of the proportion of irregular pores versus bottleneck size was not significant (ANOVA: $F_{1,8} = 3.06$, $P < 0.118$, $r^2 = 0.28$).

Discussion

I found no significant difference in the thickness of eggshells between populations of introduced passerines in New Zealand and that found in non-bottlenecked populations elsewhere. All species, regardless of the size of the bottleneck that the species went through, had shell thicknesses that were not significantly different from what would have been predicted given the mass of the egg. I did find a significant reduction in the number of pores on the eggshell in every species of introduced bird I examined. However, there was no relationship between the residual number of pores and the size of the bottleneck experienced by that species. Finally, species that went through more severe bottlenecks had fewer round pores (and more irregular pores) than species that went through larger bottlenecks. Pore shape across the species in my sample varied from nearly perfectly round, through to asymmetrical shapes with irregular boundaries (Figure 1). There is no data on the frequency of pore shapes from birds elsewhere, so I was unable to compare my samples of New Zealand birds with those that have not passed through a bottleneck.

E. cirlus, C. chloris and *A. tristis* are three sprecies introduced into New Zealand that went through relatively severe bottlenecks, having been founded by populations of 7, 66 and 70, respectively (Thomson 1922, Lever 1987). Although there is likely to be an increased level of inbreeding in these species, there was no

effect on either the thickness of eggshells or the number of pores on eggshells. Although I did not have data on eggshells for the same species I studied in their source populations, I was able to compare my data with what would be expected from non-bottlenecked species in general, by using the values in the literature. Extensive studies have been done overseas on the thickness of eggshells with relation to the mass of the egg (Ar et al. 1974), and eggshell thickness can be predicted with high degree of certainty for an egg of known mass. Bottlenecked species in New Zealand did not show any deviation from these expected values (Fig. 4.3). It would still be useful to collect and directly measure eggshells from populations in the source country (primarily the U.K.) but such an effort was beyond the scope of the present study.

I found a positive and significant relationship between the size of genetic bottleneck and the proportion of round pores (Fig. 4.6). The smaller the bottleneck the fewer the round pores. There is no significant relationship between the number of irregular pores and the size of genetic bottleneck though (Fig. 4.6). It is unknown if different shapes of pores have different fitness consequences as there have been no studies done on the effect on shape on gas conductance. Toien et al. (1987) does suggest that the shape of a pore may have more of an impact on the ability of gas to diffuse across an eggshell than previously thought. The presence of increased numbers of irregular shapes in the pore structure could alter the gas diffusion properties of those eggs. However, there is no data from non-bottlenecked species with which I can compare my results, but the change in relative ratios between round and irregular pore shapes with bottleneck size among populations of introduced birds

in New Zealand does suggest that severe bottlenecks may lead to differences in this structure.

The effects of inbreeding on populations are widely studied (Keller and Waller 2002). It is hypothesised that matings with close relatives over successive generations lead to increased levels of homozygosity, which consequently lead to increased expression of deleterious alleles (Charlesworth and Charlesworth 1987). Certain traits such as survivorship (Jimenez et al. 1994) and reproductive success (Kempenaers et al. 1996) seem to particularly susceptible to inbreeding depression. Environmental stresses cause problems with some reproductive traits including fertility of eggs (Dauwe et al. 2004) and particularly changes in the avian eggshell (Fox 1976, Nyholm and Myhrberg 1977, Eeva and Lehikoinen 1995). Pollution also causes severe damage to eggshells, clutch sizes, and egg sizes and shapes (Nyholm and Myhrberg 1977). In this study the, presence of soft eggshells, or eggs being laid without an eggshell were not observed. Clutch sizes and egg sizes have been observed to change with introduced birds in New Zealand (Niethhammer 1970), but this is probably due to behavioural changes with relation to seasonality differences (Evans 2005). Egg shape has changed too for introduced birds into New Zealand (Congdon 2005). Extreme changes of shapes are more variable with smaller genetic bottlenecks, but the consequences of this are unknown. Changes in the shape of eggs do not appear to change the respiratory ability of an egg. Gas is diffused passively across the eggshell, so diffusion is more a function of the density of pores and surface area, not shape (Hoyt 1976). However, changes in shape could still lead to increased hatching failure as eggs of a normal shape are more likely to hatch. This is probably related to the fact that the embryo changes its axial rotation in the later stages of development.

Any egg that is too narrow or too round could impede this behaviour (Narushin and Romanov 2002).

The presence of organochlorides such as DDT, acid rain and air pollution in industrialised areas has been the cause of thinning eggshells and changes in pore density in European populations of *Turdus spp.* in Britain (Green 1998), Common Terns in Canada (*Sterna hirundo*) (Fox 1976), Prairie Falcons (*Falco peregrinus*) in North America (Enderson and Berger 1970), and Clapper rails (*Rallus longirostris* obsoletus) in California (Schwarzbach et al. 2001). This pattern of thinning eggshells has not been seen in New Zealand's population of introduced birds. The eggshell thicknesses of New Zealand's introduced birds were almost identical to the expected values from Ar et. al. (1974). This suggests that birds in New Zealand are able to find sources of calcium, and they can use that calcium effectively to produce viable eggshells. It is likely that large changes in eggshell thickness would be strongly selected against and would no longer exist in the population. In shells that are too thick, chicks would be unable to hatch; on the other hand shells that are too thin would provide a little structural protection and a very limited barrier to infection (Narushin and Romanov 2002). It is possible that problems in eggshell thickness as a consequence of severe bottlenecks could have already been "purged" from the species I studied, although it would be difficult to test this idea.

There was a significant difference in the number of pores in all species from the values expected from non-bottlenecked species. The regression line of egg mass versus pore counts from Ar and Rahn (1985) and my observed values had very similar slopes (Fig. 4.4). The difference in the two lines was the intercept on the Y-axis.

However, this difference is probably not a function of inbreeding depression but due to differences in analysing technique. All my counts were carried out under a S.E.M, whereas the majority of studies done previously were acid etched to enlarge pores. This probably led to an overestimation in pore counts for those species (B. Burrows and J. Mackenzie, pers. obs.). This was further backed up when the analysis of residuals from the observed regression line was plotted against bottleneck size and it showed no significant relationship (Fig. 4.5). A reduction in pore counts constricts the amount of oxygen that can be diffused into the egg and embryo's ultimately die of hypoxia (Fox 1976). This pattern has not been seen in bottlenecked species of birds in New Zealand, so it cannot explain why there is increased hatching failure in these species.

No detailed study has shown any relationship between the shape of a pore and its ability to allow gas to diffuse through it. When gas is passing through a pore the area which faces the most resistance is at the narrowest point in the cone. It is in this area that the shape was described. On the S.E.M this showed up as a black shape at the bottom of the cone (Fig. 4.1). It is difficult to assess what is normal or not, but round pores would provide the least resistance to gas conductance (Toien et al. 1987). The frequency of round, oval or irregular pores could change the amount of gas that the developing embryo is able to receive. I could not determine the normal frequency of round pores or irregular pores in non-bottlenecked species because no research has been conducted on this subject. However, there was a significant relationship between bottleneck size and frequency of round pores (Fig. 4.6). It is difficult to determine what this means exactly, but this does suggest that the frequency of the shape of pores maybe one of the reasons for hatching failure. This only can be explained if gas

conductance is altered by the shape of the pores. From this analysis, I am unable to determine what the size of bottleneck at which a change in the shape of pores becomes significant. Briskie and Mackintosh (2004) found that species that went through bottlenecks of 150 individuals or less had higher than expected hatching failure rates. There is no clear indication that species that went through these bottlenecks have a lower proportion of round pores than the rest of the species.

Changes in eggshell characteristics are a known cause of hatching failure from work on pollutants such as acid rain, organochlorides and air and water pollution (Enderson and Berger 1970, Nyholm and Myhrberg 1977, Drent and Woldendorp 1989, St Louis and Barlow 1993, Eeva and Lehikoinen 1995, Schwarzbach et al. 2001). Increased levels of inbreeding bought on by populations going through severe genetic bottlenecks can also suffer fitness consequences such as reduced reproductive success (Bensch et al. 1994, Kempenaers et al. 1996, Briskie and Mackintosh 2004). However, it appears that this increased genetic stress does not affect the quality of the eggshell. Eggshell thickness and pore numbers did not change from expected values obtained from previous work on non-bottlenecked species elsewhere. There appears to be some relationship between the frequency of round pores and level of genetic bottleneck, but the consequences of these changes in proportion of pore shape are unknown at this stage. More work needs to be done on the consequences of pore shape on hatchability before any solid conclusions can be made. Direct comparisons between species in New Zealand and the respective source populations should be done as well as more replicates of all species particularly, *E. cirlus, Ca. chloris* and *A. tristis* as they went through severe bottlenecks of less than 100 individuals (Thomson 1922, Lever 1987). It would also be useful to determine if native species of birds that

have passed through severe bottlenecks and currently experience high levels of hatching failure (e.g. kakapo, takahe) have any obvious abnormalities in the structure of their eggshells that might explain their poor reproductive success.

Figures

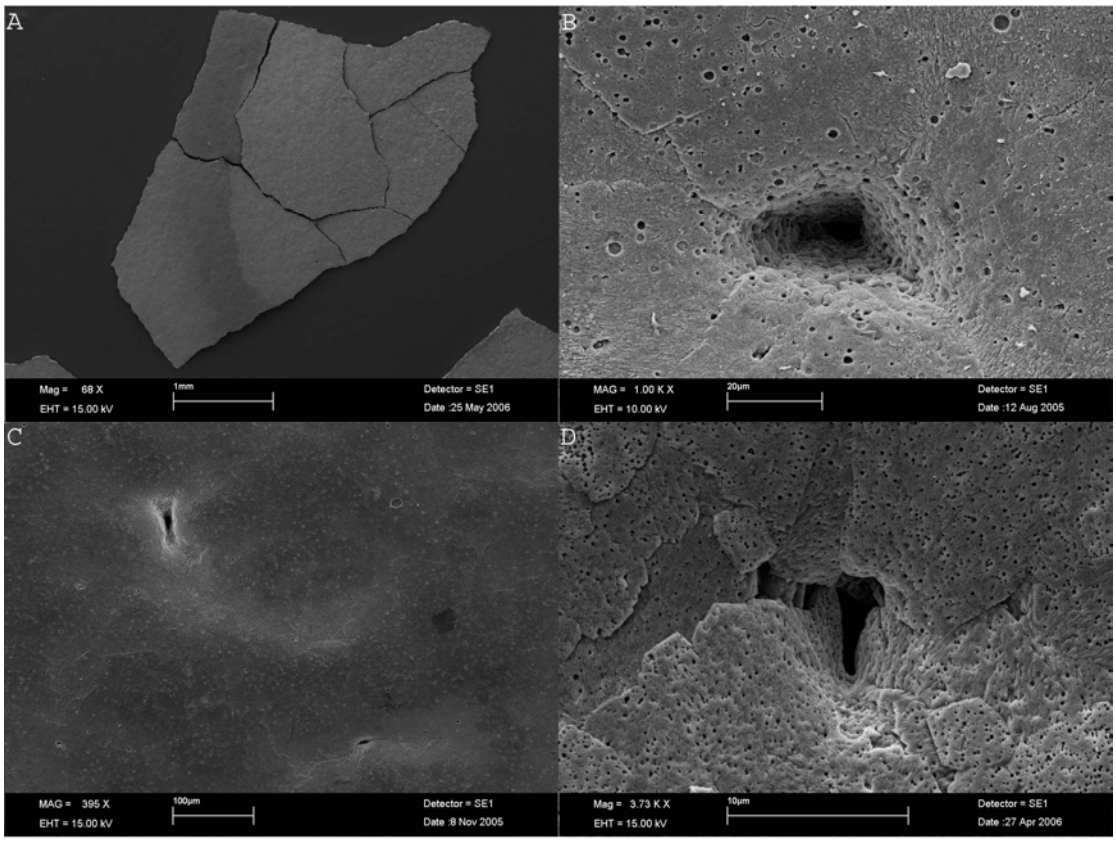

Figure 4.1 (A) Image of a fragment of a *Prunella modularis* eggshell at 68 x magnification. (B) Example of a round pore. Image from a *Sturnus vulgaris* eggshell at 1000 x magnification. (C) Examples of elongated oval pores from *S. vulgaris* at 395 x magnification. Pore at bottom right of image is an extreme example of oval pore. (D) An example of an irregular shaped pore from *Emberiza citrinella*. Image taken at 3730 x magnification.

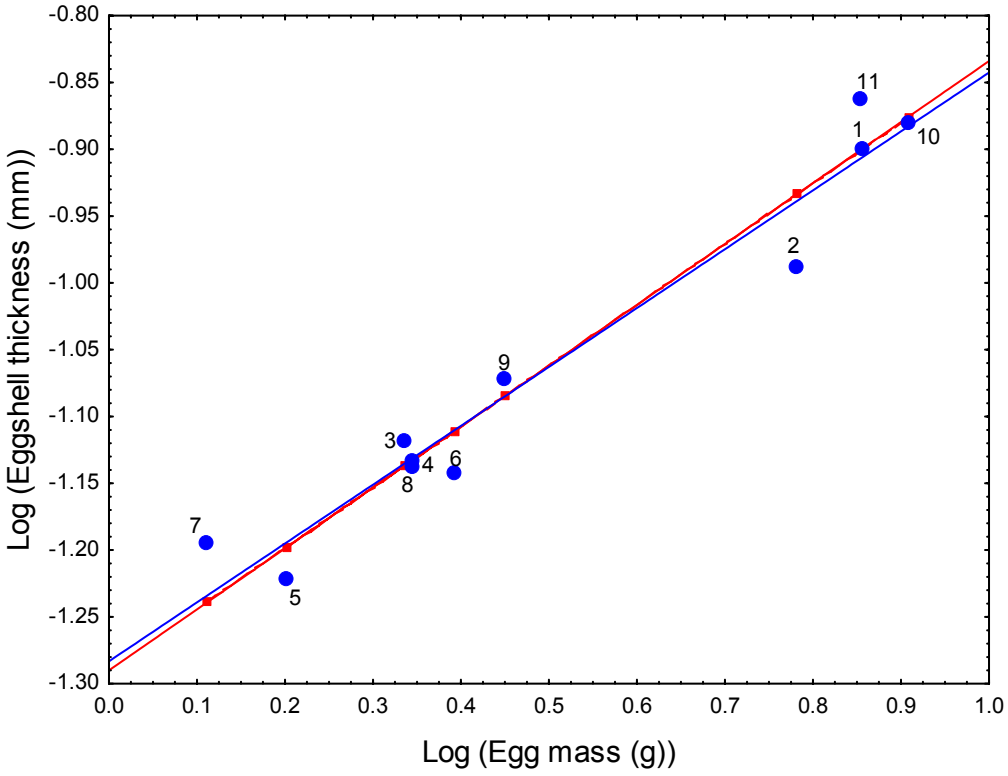

Figure 4.2 Linear regression of observed eggshell thickness from New Zealand introduced birds ( ) in relation to egg size; Log (eggshell thickness (mm)) = 0.4407 Log (Egg mass (g)) – 1.2832. This line is significant ($F_{1,9} = 171.43$, $P < 0.001$). Expected eggshell thickness for my study species ( ) as predicted from a linearregression of non-bottlenecked species obtained from Ar et al. (1974). **S**pecies are (number corresponds to label on graph) (Sample size): 1. *Turdus merula* (16); 2. *T. philomelos* (25); 3. *Prunella modularis* (18); 4. *Fringella coelebs* (24); 5. *Carduelis carduelis* (21); 6. *Ca. chloris* (16); 7. *Ca. flammea* (16); 8. *Emberiza cirlus* (1); 9. *E. citrinella* (10); 10. *Acridotheres tristis* (5); 11. *Sturnus vulgaris* (27).

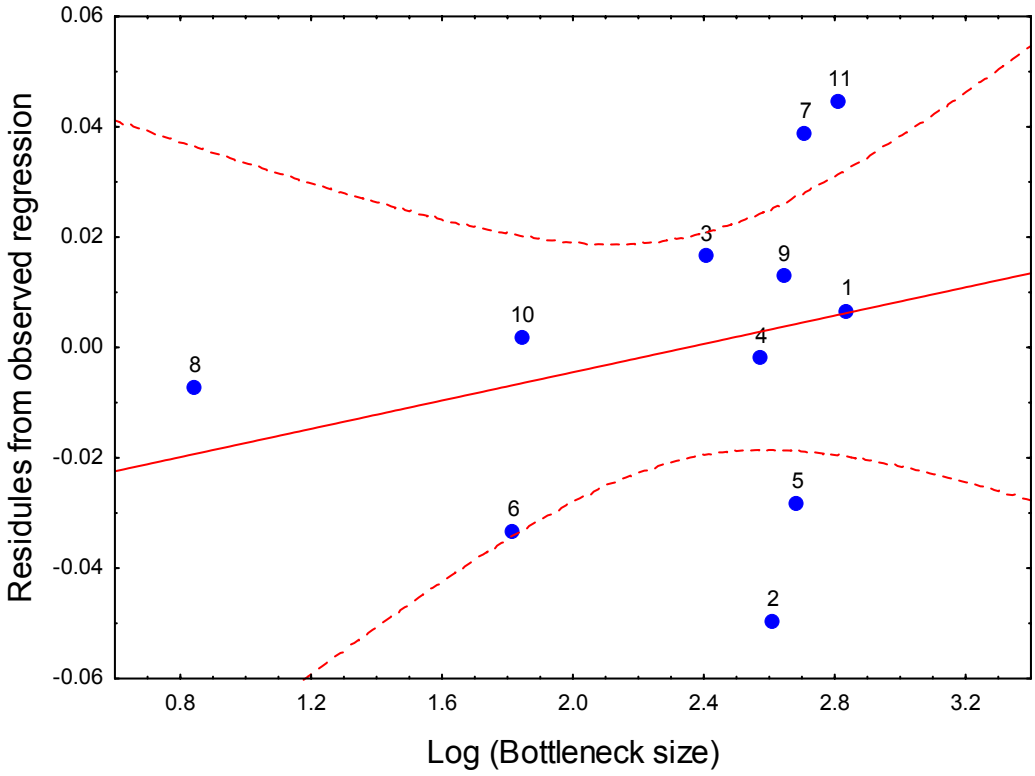

Figure 4.3 Linear regression of the residual eggshell thickness versus bottleneck size for introduced species of birds in New Zealand; Residuals = 0.0128 Log (Bottleneck size) -0.0301. This line is not significant ($F_{1,9} = 0.70$, $P < 0.423$). Species represented by the same number as in Figure 4.2.

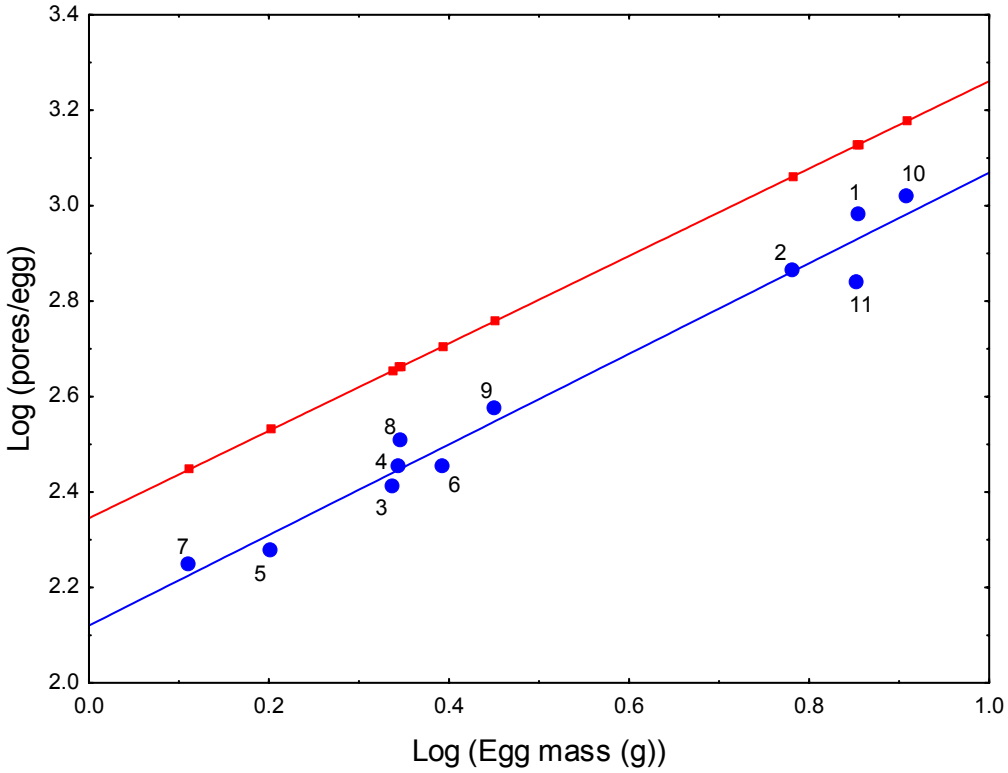

Figure 4.4 Linear regression of observed number of pores from New Zealand introduced birds ( ); Log (Pores/egg) = 0.9489 Log (Egg mass (g)) + 2.1202. This line is significant ($F_{1,9} = 316.03$, $P < 0.001$). Expected number of pores for my study species ( ) based on a linear regression obtained from Ar and Rahn (1985) for non-bottlenecked birds. **S**pecies are (number corresponds to label on graph) (Sample size): 1. *Turdus merula* (4); 2. *T. philomelos* (4); 3. *Prunella modularis* (4); 4. *Fringella coelebs* (5); 5. *Carduelis carduelis* (4); 6. *C. chloris* (4); 7. *C. flammea* (4); 8. *Emberiza cirlus* (1); 9. *E. citrinella* (5); 10. *Acridotheres tristis* (3); 11. *Sturnus vulgaris* (6).

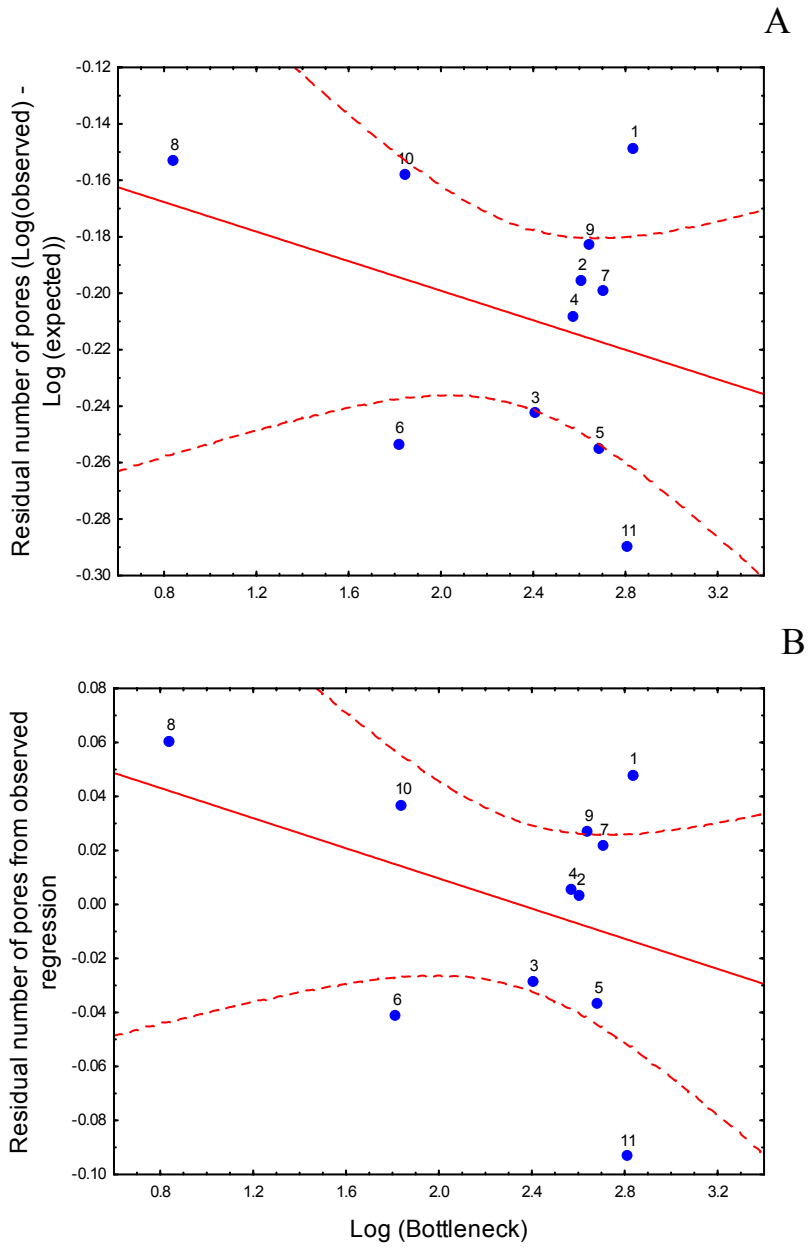

Figure 4.5 (A) Linear regression of the difference in observed number of pores from New Zealand introduced birds from the expected values from (Ar and Rahn 1985); Log (Log (observed pore counts) – Log (expected pore counts)) = -0.0262 Log (Bottleneck size) -0.1468, this line is not significant ($F_{1,9} = 1.17$, $P < 0.307$). (B) Linear regression of the residuals in observed number of pores from New Zealand introduced birds against bottleneck size from the regression of New Zealand soecies ; Log (Residuals) = -0.0279 Log (Bottleneck size) -0.0655, this line is not significant ($F_{1,9} = 1.43$, $P < 0.263$). Dotted lines represent 95% confidence intervals. Species represented by the same number as in Figure 4.4.

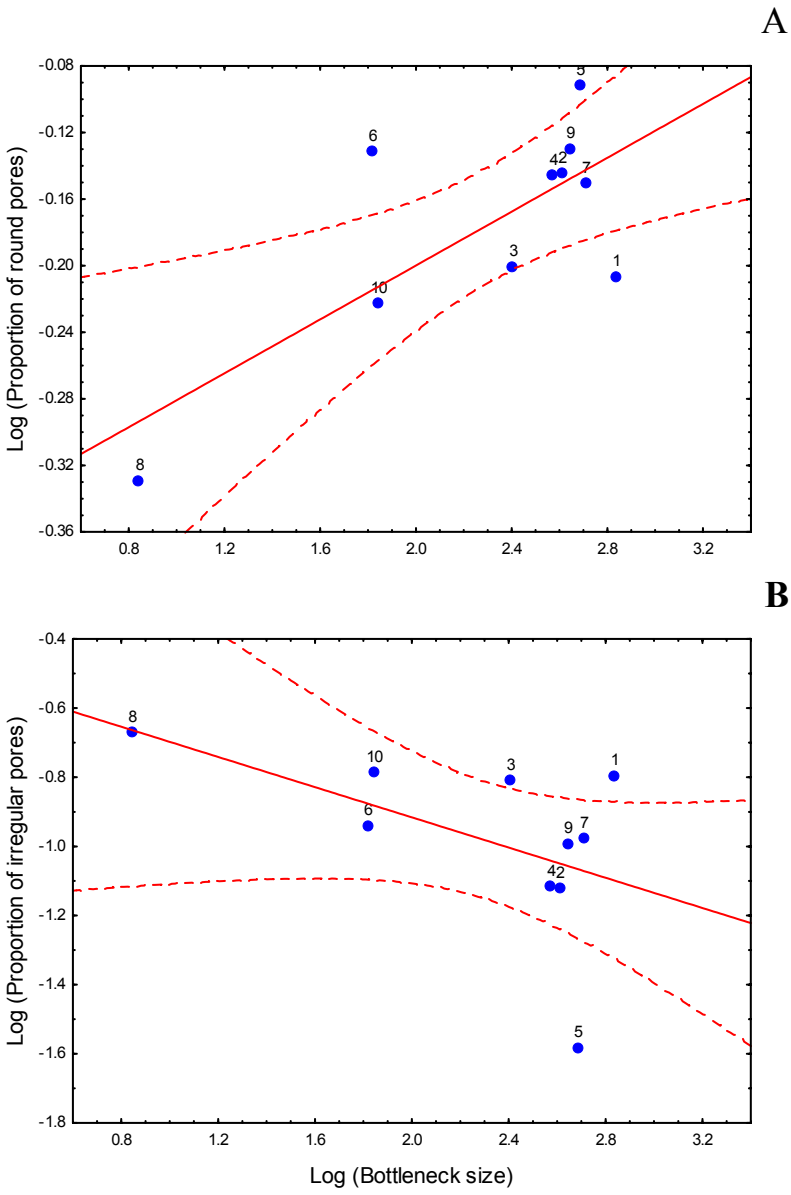

Figure 4.6 (A) Linear regression of the proportion of round pores versus bottleneck size from New Zealand introduced birds; Log (proportion round pores) = 0.0809 Log (Bottleneck size) – 0.3618, this line is significant ($F_{1,8} = 9.97$, $P < 0.013$). (B) Linear regression of the proportion of irregular pores versus bottleneck size from New Zealand introduced birds; Log (proportion irregular pores) = -0.2185 Log (Bottleneck size) – 0.4791, this line is not significant ($F_{1,8} = 3.06$, $P < 0.118$). Dotted lines represent 95% confidence intervals. Species represented by the same number as in Figure 4.4.

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Chapter 5:

General discussion

Summary of results

I found that introduced species in New Zealand that have been through a population bottleneck appear to suffer a range of reduced fitness traits. All species examined in this study had fewer sperm on the perivitelline membrane than would be expected when adjusted for both egg size and body mass. In some cases, sperm numbers were approximately what you would expect. For example, chaffinches (*Fringella coelebs*), greenfinches (*Carduelis* chloris) and redpolls (*C. flammea*) had sperm numbers below that expected on the perivitelline membrane, but appeared to have similar counts to other species of about the same body mass and egg size from Europe. However, all other species had substantially fewer sperm than expected.

Abnormalities associated with the midpiece in sperm from introduced birds was detected only twice, both in blackbirds. No other obvious abnormalities were evident. There was no correlation between the variation in midpiece length and the size of the bottleneck, although it appears that there was a trend towards species passing through more severe bottlenecks showing higher variability in the length of the midpiece and lower sperm motility. Sperm motility in the introduced birds from New Zealand appeared the same as a captive zebra finch population from England. However, dunnocks (*Prunella modularis*) had a mean sperm motility that was lower than other species (approximately 63% sperm motility compared with the 85% motility in zebra finches).

Finally, I found that the thickness of eggshells in introduced species did not deviate from what would be expected based on data in the literature. Pore density was significantly lower across all species introduced to New Zealand from the expected values, but this likely is a result of over-estimates of pore number in the literature. However, there was no relationship between the size of the bottleneck and the difference between the expected and observed values in pore number. I found a significantly lower proportion of round pores on species that had been through smaller bottlenecks. The relationship between proportion of round pores and the size of the bottleneck those species went through was significant.

Conclusions

Hatching failure is a common problem for bird species that have been through a genetic bottleneck. This has been shown in both native and introduced birds in New Zealand (Briskie and Mackintosh 2004) as well as inbred populations in Europe (Bensch et al. 1994, Kempenaers et al. 1996, Spottiswoode and Moller 2003, Van de Casteele et al. 2003). Not all hatching failure is necessarily explained by the onset of inbreeding depression, but it does appear to be one explanation. Nevertheless, other factors must be taken into consideration such as the effects of pollutants, the climate and the inevitable change in environment when a population is translocated from one area to another. Historically, pollution has been the cause of hatching failure in a range of species including game birds (Schwarzbach et al. 2001), raptors (Enderson and Berger 1970), sea birds (Fox 1976) and passerines (Green 1998). Global warming

and changing climate has also been responsible for changes in the breeding behaviour and ecology of some species which could lead to hatching failure if species fail to adapt (Coppack and Both 2002).

The fitness cost of hatching failure due to populations experiencing bottlenecks can be high, with more than half of all eggs failing to hatch in severely bottlenecked species (Briskie and Mackintosh 2004). The mechanism behind this phenomenon is still not fully understood. Reduced sperm numbers reaching the site of fertilisation could explain why some eggs are failing to hatch. In chapter 2, I investigated whether reduced numbers of sperm on the perivitelline membrane were lower than what would be expected based on work carried out in European birds. As all species had lower than expected counts I cannot conclusively say that this is the reason eggs fail to hatch. Presumably there is a threshold as to how many sperm are required on the perivitelline before fertilisation is guaranteed. Approximately 32 sperm are required in zebra finches to ensure fertilisation (Birkhead and Fletcher 1998). However, the number is not known for any of the species studied in this thesis. Some of the species I studied, including goldfinch (*C. carduelis*), greenfinch, dunnock, redpoll and yellowhammer (*Emberiza citrinella*) are all about the same size as a zebra finch, so a comparable number of sperm at the site of fertilisation might be needed. It is unknown how many sperm should be on the perivitelline membrane to guarantee fertilisation for species with larger eggs, such as song thrush (*Turdus philomelos*), blackbird (*T. merula*), European starling (*Sturnus vulgaris*) and Indian myna (*Acridotheres tristis*). For these species with larger eggs, the mean number of sperm on the perivitelline membrane fell well below what was expected so there is a chance that some of them were not fertilised. The range of sperm number on the

smaller species all fell above or around the threshold of 32 sperm. At this stage it is difficult to say whether membranes with low numbers of sperm on the membrane were fertilised as the threshold for all species concerned is unknown. Nevertheless, sperm numbers in general were substantially less than expected and it is likely this increases the likelihood of hatching failure due to fertilisation failure.

Some of the possible reasons behind there being lower than expected numbers of sperm on the perivitelline membranes were addressed in chapter 3. There are two possible reasons why sperm numbers are lower than expected when an egg is fertilised: (a) the ability of the female to store sperm is compromised, or (b) the quality of the sperm has been impaired. No evidence exists to suggest that females from a population that has gone through a bottleneck are the sole cause for infertility. This is not to suggest that females have not lost fitness through inbreeding depression brought on by population bottlenecks. However, the evidence from research on other species largely suggests that male fertility could be reduced in bottlenecked populations. Studies on big cats and ungulates have shown that inbreeding depression lowers male fertility with increased abnormalities and reduced motility (Wildt et al. 1987, Lindburg et al. 1993, Roldan et al. 1998). I could not conclusively say that this is the case in the introduced populations of New Zealand birds. Abnormalities in the midpiece were low in the species studied and motility was low in some species, but not at levels typical of other bottlenecked species. Variation in the midpiece was examined because this section of the spermatozoa is where the energy for movement and longevity is created. A single mitochondria is wrapped around the top section of the flagellum giving it a helical shape. An extremely short midpiece was only observed twice in one species, the blackbird. This species went through the largest

bottleneck in my sample and so presumably inbreeding depression would be less of a factor in this species compared to other introduced birds. Abnormalities may not have been observed in other species because they could potentially be lethally deleterious and therefore have been purged during the bottleneck when they were introduced. There is no direct evidence for this, and as sample sizes were small in this chapter, caution must be taken when interpreting the results.

In chapter 4, I found that eggshell thickness was no different to what would be expected based on work from non-bottlenecked populations. The difference in the counts of pores from the expected values is probably best explained by differences in the methodology employed by myself and that by Ar and Rahn (1985). Ar and Rahn (1985) based their pore counts on eggshells that had been etched in acid, while I examined the eggshells using a SEM. Etching the eggshells alters the external structure of the eggshell to enlarge the pores which makes it easier to see them. This technique has the potential to create pores that were not there and therefore overestimate the frequency of pores (pers. obs.). As I found no difference in the thickness of eggshells and the density of pores on the eggshell in relation to bottleneck size, changes in the properties of the eggshell cannot explain why hatching failure occurs in bottlenecked populations of birds in New Zealand. However, this does mean that a reduced fitness cost (hatchability of eggs) is not attributed to the females being able to find enough nutrients such as calcium from the environment. There is still the possibility that introduced birds would struggle to find the right nutrients in New Zealand as many of the plants and invertebrates are different from their source populations and this could cause an increase in hatching failure (Graveland and Drent 1997). This is unlikely the case with New Zealand introduced

birds as populations that could be under extreme genetic and environmental stress still appear able to find enough calcium (and probably other minerals) to lay eggs. In Europe, birds that are under environmental pressure from pollution lay eggs with thinner shells and reduced pore density (Fox 1976) but the lack of changes in shell thickness in New Zealand suggests the populations here are not currently under stress from pollutants.

There was a positive relationship between the frequency of round pores and the size of the bottleneck across my study species. The smaller the bottleneck, the smaller the proportion of round pores I observed. However, the fitness costs of having variability in the shape of the pores are unknown. A round pore is likely to have less resistance to gas exchange than an irregular pore which could alter the development rate of an embryo (Toien et al. 1987). Presumably the number of pores on an eggshell is optimised to ensure that the embryo does not dehydrate, as some water is lost over incubation due to evaporation. An increase in the frequency of irregular shaped pores may either slow down the rate of oxygen getting to the embryo causing it to die of hypoxia, or speed up the rate of water evaporation, causing the embryo to dehydrate. Both of these ideas are speculative at present and the level of change required for this to happen is unknown as little work has been done on the consequences of altering the shape of pores.

Implications for conservation

Conservation practices in New Zealand in the last few decades have focused primarily on transferring endangered populations of species to isolated predator-free islands (Armstrong and McLean 1995). This has primarily been undertaken to prevent introduced mammalian predators from decimating already declining populations. However, as a consequence, this has forced many species such as the saddleback through various sized bottlenecks, many of them smaller than 40 individuals (Taylor et al. 2005). Other species have had their entire population reduced to levels as low as 50 individuals such as in the kakapo (*Strigops habroptilus*) (Briskie and Mackintosh 2004), or only 5 birds in the black robin (*Petroica traversi*) (Ardern and Lambert 1997). In some instances these low numbers were unavoidable and without immediate intervention, the species simply would not have survived.

The introduction of predatory mammals and continueing habitat loss has forced conservation managers into using translocation practices with few founders but the genetic consequences were usually not considered. It has been argued that birds in New Zealand might be "pre-adapted" for existing in small populations and inbreeding depression is not an important issue (Craig 1991). The theory behind this is that any deleterious alleles present in a population would be purged. Recent studies contest this claim. Briskie and Mackintosh (2004) have shown that reproductive success is impaired due to inbreeding depression bought upon through bottlenecks. Other studies on birds also suggest that inbreeding have negative consequences on the reproductive success of individuals (Bensch et al. 1994, Kempenaers et al. 1996, Kruuk et al. 2002, Spottiswoode and Moller 2003), and that inbreeding should be avoided for this reason. Craig (1991) claims that New Zealand birds have existed in small isolated

populations for generations and would have naturally high levels of inbreeding anyway. A low level of minisatellite variation (a method for testing genetic diversity) in the black robin was used as evidence for this (Ardern and Lambert 1997). This species went through a bottleneck of 5 individuals (one was removed from the breeding population), and today the species suffers a hatching failure rate of about 33%, which is one of the highest in the world (Briskie and Mackintosh 2004).

Craig (1991) argued that the advantages of translocating a population to a predator-free island outweigh any potential costs of inbreeding. This may be true in the short term, but does not take into consideration any problems in the future which may arise from inbreeding depression. Environmental change, disease and rare climatic disasters are potential stressors that severely inbred populations may not be able to deal with. Reduced genetic diversity means lowered evolutionary potential (Frankham et al. 1999). Studies on inbred laboratory *Drosophila* showed they were less able to cope with higher concentrations of NaCl (an environmental stress) than outbred lines. Similarly, an isolated island population of song sparrows (*Melospiza melodia*) were decimated in a winter storm in 1989. Only 11% of the adult population survived, and as expected, inbred lines suffered higher mortality rates than outbred lines (Keller 1998). The endemic takahe (*Porphyrio* mantelli) of New Zealand has also passed through a severe bottleneck and is only naturally found in the high country in Fiordland. They show a reduced ability to successfully breed in new environments (Jamieson and Ryan 2000). Takahe that live on predator-free islands have impaired hatching success. This suggests that New Zealand birds that have passed through a severe bottleneck are not equipped to deal with large levels of change such as a new environment, disease or catastrophic events. This could be

important particularly as global warming is changing climates around the world, and is likely to change conditions for native endangered birds in New Zealand even further.

Many of New Zealand's bird species have passed through severe bottlenecks. Conservation policy should take into consideration bottleneck effects when managing populations. I was unable to determine the size of genetic bottleneck where reproductive performance would be impaired seriously as Briskie and Mackintosh (2004) did in their study of hatching failure rates. However, my findings on the reduced numbers of sperm on the perivitelline membrane suggest that species experiencing bottlenecks may be suffering fitness consequences at higher levels than previously suggested. Franklin and Frankham (Franklin and Frankham 1998) suggest that populations of at least 500 individuals are required to eliminate the negative effects of inbreeding. These numbers are impossible to reach in the case of many of New Zealand's endangered species. However, this does not mean that genetic considerations should not be taken into account. It may not always be possible to use high numbers of individuals when founding new populations, but by carefully managing different populations as a single population, some genetic recovery might take place, or at least the most severe effects of bottlenecks minimised. For example, it may only require that a few individuals are transferred between different islands every generation to restore higher levels of genetic variation as seen in a population of Scandinavian grey wolves (Vila et al. 2003). Even a single immigrant per generation could be enough to allow increased genetic variation (Mills and Allendorf 1996). Different inbred populations of the same species have a tendency to fix different deleterious alleles, so it may only take a few genetically distinct individuals to

increase genetic diversity (Charlesworth and Charlesworth 1987). Introducing a single individual may be bet-hedging, so when possible introducing several individuals might be more appropriate. This is an area that clearly needs more research in New Zealand, both on the theoretical side and the practical side.

The analysis in this thesis did not take into consideration differences in mating systems and reproductive behaviour. Some species are socially monogamous such as the thrushes, whereas dunnocks have a more complex polygynandrous mating system (Briskie 1993). These mating strategies affect behavioural patterns such as the level of sperm competition (Briskie et al. 1997), while at the same time some monogamous species are generally more faithful to their partners than others. As a consequence different reproductive strategies put different selective pressure on those species. This could alter the rate of population recovery between species even though they have experienced similar initial effects of a population bottleneck.

The kakapo for example, has a lek mating system where males set up a series of bowls to "boom", and females choose from a group of males who to mate with based on this display (Merton et al. 1984). The complexities of their unusual mating system make it difficult to set up a successful recovery programme (Trewick 1997). Kakapo have been through a severe bottleneck (approximately 50 individuals). However, because of the lek mating system, N_e (effective breeding population) is significantly lower than N (actual population size) (Robertson 2006). This has probably increased the severity of the bottleneck beyond the actual population size as only a fraction of the male likely breed successfully. Not surprisingly, only about 40% of the eggs laid hatch (Briskie and Mackintosh 2004). None of the introduced

species I studied is known to have a lek mating system, so it is unlikely that such a skew in male reproductive success has affected the N_e for introduced species in the same way as for kakapo. However, at present, the actual relationship between N_e and N for the introduced species is unknown.

Future research

My research has highlighted some areas in which further work might prove productive in determining the effects of bottleneck severity on reproductive fitness. For example, my finding that sperm numbers on the perivitelline membrane in all species are below what would be expected would be worthwhile extending to other species that went through more severe bottlenecks, such as cirl bunting (bottleneck of < 10 birds) and to native species. Although I only examined first-laid eggs, it is known that the number of sperm on the perivitelline membrane changes across the clutch, i.e., from the first laid egg to the last (Birkhead et al. 1994). By examining all the eggs in a clutch it would be possible to detect if there are higher instances of infertile eggs in the later laid eggs. Testing for the presence of fungal and bacterial infections in eggs could explain hatching failure too. Bacterial infections (addling) have been seen as a cause of hatching failure before, and I observed a couple of instances of membranes that had fungal hyphae growing on them (pers. obs.) Not knowing the reason why these membranes had fungus growing on them, but their presence does suggest further investigation is worthwhile. The methods I used for examining the membrane and testing for fungal and bacterial infection are invasive and destructive to the eggs. Accordingly it would not be appropriate to use

endangered native birds such as the takahe, black robin or kakapo as study species. However, more detailed study on introduced birds could further reveal patterns between the size of a population's bottleneck and its reproductive traits. The results from these tests could help management plans for native birds if it was found that more inbred birds were more susceptible to disease in the eggs or reduced fertility eggs.

Testing the level of sperm abnormalities and motility of sperm has large implications for examining reproductive fitness. Sperm counts, sperm motility and the level of sperm abnormalities are used as common indexes for the health of many endangered species, particularly mammals. The potential for research on New Zealand's introduced and native bird species is large, yet very little work has been done here. The methods I used were non-invasive as sperm could be collected from males with very little stress. If it is found that large abnormalities exist in native birds then perhaps recovery programmes could be designed to remove particularly infertile males. These techniques for studying sperm are commonly used in across the world for domesticated, captive and wild populations, so there is no reason why this could not be carried out on New Zealand's endangered species to assess their reproductive health.

Examining properties in avian eggshells has been commonly used to determine why eggs fail to hatch in polluted areas. However, there appeared to be no large fitness cost to eggshells in New Zealand introduced species that went through a population bottleneck except perhaps changes in the shape of pores. Research now needs to be done to determine whether there is a cost to having irregular pore shapes

and whether this is related to inbreeding depression. Eggshells could still be examined in endangered species, as it is possible to do this without interfering with the development of chicks. Once a chick hatches from the egg, the eggshell is deposited away from the nest by one of the parents. It is still in a condition that allows it to be examined for pore counts and density. Although eggshell thickness is difficult to measure in discarded shells, as the developing embryo uses calcium from the eggshell for bone development, other parameters can be tested such as calcium density and pore shape.

In conclusion, I believe that my research has provided some insight into the effects of bottlenecks but this is only the beginning and there is much value in continueing to use reproductive traits as markers for conservation purposes. None of the techniques I employed are common practice in the field of conservation in New Zealand and the genetics of the native species are only starting to be understood. The effects of bottlenecks are thought to be short term and current management of New Zealand's avifauna is assuming this. Research on other species such as cheetahs would suggest otherwise, and there is no reason to believe it would be any different in New Zealand's avian populations. I have highlighted the need for future research into the reproductive failure for critically endangered species. If managers do not take into account the breeding biology of the species they are trying to rescue it could result in loss of fitness potential for future generations. Although monitoring and management might solve the problems of population decline in short term, reduced hatching success bough upon by inbreeding depression could make recovery of those populations more difficult for those species in the long term. Without taking into account the effects of bottlenecks on traits such as reduced sperm quality, fewer

sperm reaching the site of fertilisation, or poorer eggshell quality the reproductive success of these species may never recover.

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Appendices

Appendix I: Data for predicting the number of sperm that should be present on the perivitelline membrane of New Zealand's introduced birds in Chapter 2.

Species	Body mass (g)	Ovum diameter (mm)	Number sperm + holes
Remiz pendulinus	9.3	8.6	1455
Parsus caeruleus	13.3	8.38	249
Panurus biarmicus	15.7	10.13	129
Erithacus rubecula	18.2	11.54	137
Emberiza schoeniclus	18.3	10.1	791
Prunella modularis	19.7	10.14	49
Sturnus vulgaris	82.3	13.6	594
Turdus merula	113	15.64	1178
Pica pica	177.5	16.75	1903
Columba palumbus	490	22.15	11661
Uria aalge	992.5	43.7	23084

Data adapted from Birkhead, T.R., B.C. Sheldon and F. Fletcher. 1994. A comparative study of sperm-egg interactions in birds. Journal of Reproduction and Fertility **101**:353-361.

Appendix II: Calculation of co-efficient of variation (C.V) for variation of midpiece length in sperm from different species of New Zealand's introduced birds in Chapter 3.

$$C.V. = \frac{s.d.}{\bar{x}} \cdot 100\%$$

Adapted from Zar, J. H. 1999. Biostatistical Analysis, 4 edition; C.V. = co-efficient of variation; s.d. = standard deviation; $\bar{x}$ = mean.

Appendix III: Calculation for the surface area of an egg and the volume of an egg.

$$S = 4.393 + \frac{0.394 \times \text{length}}{\text{breadth}} \left(V^{0.667}\right)$$

Hoyt, D.F. 1976. The effect of shape on the surface-volume relationships of birds' eggs. The Condor 78:343-349; S = surface area; V = volume.

$$V = 0.512 \text{ length} \times (\text{breadth})^2$$

Stonehouse, B. 1966. Egg volumes from linear dimensions. Emu **65**:227-228; V = volume.

Appendix IV: Data for predicting the number of pores that should be present the eggshell of New Zealand's introduced birds in Chapter 4.

Species	Egg weight (g)	Number of pores
Corvus corone	15.00	1992
Turdus merula	6.36	1418
Pycnonotus capensis	3.05	486
Galerida cristata	2.93	917
Passer domesticus	2.76	440
Lanius nubicus	2.47	481
Erythropygia galactotes	2.30	591
Carduelis chloris	1.94	343
Muscicapa striata	1.86	699
Passer moabiticus	1.50	347
Carduelis carduelis	1.23	368
Prinia gracilis	1.12	176
Hippolais olivetorum	1.11	230
Nectarinia osea	0.86	126

Data adapted from Ar. A., Rahn, H. 1985. Pores in avian eggshells: Gas conductance, gas exchange and embryonic growth rate. Respiration Physiology **61**:1-20.