THE EFFECT OF POPULATION BOTTLENECK SIZE ON PARASITIC LOAD AND IMMUNOCOMPETENCE OF INTRODUCED BIRDS IN NEW ZEALAND

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GENERAL ABSTRACT

I investigated parasitic infection and immunocompetence in populations of introduced bird species in New Zealand (NZ) that had experienced a range of population bottlenecks (11-808 individuals), and compared these parameters to non-bottlenecked conspecifics in the United Kingdom (UK). My aims were two-fold; firstly to assess if population bottlenecks are linked to increased parasite loads and/or decreased immunocompetence, and secondly, to assess at what severity of bottleneck these effects become evident. I found that ectoparasite load (chewing lice, Order: Phthiraptera, Sub-Orders: Amblycera & Ischnocera) was significantly higher in the more severely bottlenecked species in NZ than in the UK, whilst this difference became non-significant at more moderate bottlenecks. The difference was mainly driven by the Sub-Order Amblycera. The prevalence of avian malaria (*Plasmodium* spp.) was significantly negatively correlated to bottleneck size within NZ, after controlling for body mass. Total leucocyte and differential lymphocyte counts were elevated in the less bottlenecked species that were infected with malaria, whilst the populations at the more severe end of the bottleneck spectrum did not exhibit such a response. Furthermore, heterophil/lymphocyte (HL) ratio (a parameter used as an indicator of environmental and/or immunological stress), was significantly raised in the more bottlenecked species when compared to their UK counterparts, and this difference was correlated with the size of the bottleneck. Immunocompetence was further assessed by the experimental challenge of six introduced birds species in NZ with the mitogen phytohaemagglutinin (PHA). Immune response to PHA was significantly correlated to bottleneck size, but in the opposite direction to that predicted; immune response was greater in the more bottlenecked species. However, this may be an indication of increased investment in immunity, due to increased parasite and pathogen pressure or differential investment in varying components of the immune system. Finally, the immune response to PHA was compared in nestlings of two species that had experienced very different bottlenecks (70 vs. 653). After controlling for ectoparasitic infestation, I found no difference between the two species; however, this finding may be confounded by interspecific competition. Overall, my findings suggest that more severe population bottlenecks may result in increased susceptibility to pathogens, and impact on the immune system. This has a number of implications for the development of conservation protocols, and future avenues of research are suggested.

INTRODUCTION

Population bottlenecks occur when a population undergoes a temporary, large reduction in number (Keller et al., 2001) and can cause a reduction in genetic diversity, both in terms of heterozygosity and allelic diversity (England et al., 2003). This has now been demonstrated in a number of wild populations (Ardern et al., 1997; Bodkin et al., 1999; Bouzat et al., 1998; Bradshaw et al., 2007; Hajji et al., 2007; Hoelzel, 1999; Hudson et al., 2000; Keller et al., 2001; Nyström et al., 2006; Packer et al., 1991; Zhang et al., 2004). Decreased genetic diversity can be associated with reduced fitness (Reed and Frankham, 2003), and has been linked to extinction risk (Frankham, 1998).

In addition to bottleneck effects, the resulting small populations are often prone to high levels of inbreeding, since mate choice is reduced and individuals are more likely to mate with kin (Hedrick and Kalinowski, 2000). The negative impact of inbreeding on a population, termed inbreeding depression, is well known and encompasses a suite of interrelated fitness effects, including a reduction in reproductive success, an increase in physical defects, and greater susceptibility to parasites and pathogens (Crnokrak and Roff, 1999; Keller and Waller, 2002).

Endangered and fragmented populations, by definition, experience a population bottleneck, and hence understanding the fitness implications of bottlenecks is essential for conservationists. Although the aim of conservation measures will be to increase the population size of such species, even populations that recover to their pre-bottleneck size may continue to be affected by the genetic consequences of passing through an earlier bottleneck. In some situations, conservation projects may even intentionally create

population bottlenecks through translocation schemes and in the captive breeding of threatened species. The incidence with which bottlenecks are encountered by conservation biologists is set to increase, in large part due to the predicted rise in the number of threatened and endangered species, but also as habitat restoration schemes come to fruition, and the potential for translocations increase.

Ideally, to investigate the consequences of population bottlenecks, one would compare populations of the same species that have and have not experienced (or before and after) a bottleneck. Unfortunately, the existence of pre- and post-bottleneck populations of the same species is a rare occurrence in the wild, especially in the case of endangered species. A striking exception to this is the numerous human-introduced species that have established in areas outside their native range (Briskie, 2006). The introduced bird species of New Zealand are a good example of this system as during their establishment each species passed through a bottleneck. In the late 19th century acclimatisation societies in New Zealand (NZ) introduced at least 137 exotic bird species, of which 28 species are extant today (Veltman et al., 1996). The majority of these species were imported from the United Kingdom (UK), where they are still extant, and careful records were kept of the numbers that survived the voyage and were subsequently introduced. The number introduced varied per species, and so introduced birds in New Zealand today are represented by populations that have experienced a range of bottlenecks. The elegance of this system is that it allows comparison between pre- and post-bottlenecked populations (i.e., between UK & NZ) for a range of bottleneck sizes. This enables investigations to be made, not only of how bottlenecks may affect fitness

traits, but additionally, at what size bottleneck these fitness effects cease to be evident (or at least of lesser concern) in comparison to the source population.

GENETIC CONSEQUENCES OF BOTTLENECKS

A key assumption in this model system, is that the introduced bird populations of New Zealand exhibit the genetic consequences of experiencing a bottleneck (i.e., decreased heterozygosity and/or a reduction in allelic diversity). There are two major factors to consider when making this assumption. Firstly, the introduction of exotic bird species to New Zealand did not occur in a single introduction event, but multiple times for each species, with many introductions occurring at several different geographic locations across New Zealand, and with the releases of birds taking place over a number of years between the early 1860s to the late 1890s (Lever, 1987). The details of these introductions are summarised in Table 1. Secondly, most of the species were imported by the Acclimatisation Societies in New Zealand from southern ports in England, UK with the exception of the common myna, *Acridotheres tristis,* which originated from an introduced Australian population (Baker and Moeed, 1987; Lever, 1987). Whilst the assumption is that these birds were caught within a reasonable distance of the south coast of the UK, if trapping took place in the autumn or winter (when large flocks form), then birds may represent a mixture of over-wintering European and resident British populations.

The spatial and temporal complexity of the bird introductions to New Zealand may mean that genetic effects of passing through a bottleneck during their establishment may not be a simple linear relationship with the demographic size of that bottleneck. This is because the speed with which a population experiences a reduction in number, the

length of time the population remains bottlenecked, and the rapidity of recovery of population size all influences the genetic consequences to that population (Beebee and Rowe, 2008; Frankham et al., 2002). For example, a rapid fall in population size is expected to have more serious long-term genetic consequences than a gradual decline (Beebee and Rowe, 2008), whilst a short term bottleneck, followed by a fast recovery may have less impact (or at least a different effect) than a more extended bottleneck, with a gradual increase (Beebee and Rowe, 2008; England et al., 2003).

Although it is beyond the scope of this study to model how differences in introduction effort, geographic spread, and temporal patterns of each bottleneck may have affected the genetic consequences to the post-bottlenecked populations, it is clear from Table 1 that the introduction history was broadly similar for the majority of the species. In other words, most of the species in my study were introduced at multiple locations in New Zealand, in varying numbers, and over a span of 10-20 years. In lieu of a more direct measure of a genetic bottleneck, I have assumed that the consequences of this temporal and geographic pattern of introduction will have had similar consequences of the genetic outcomes for the majority of species. Thus for the purposes of this study, I have assumed the number of birds released in total, at all location and times (excepting those known to have failed) is a reasonable estimate of the demographic bottleneck size.

In addition to the 'shape' of the demographic bottleneck, the effective population size (N_e) also effects the genetic consequences of a bottleneck (Beebee and Rowe, 2008; Frankham et al., 2002). N_e represents the number of individuals contributing to the breeding population, and is influenced by a number of factors including sex ratios and reproductive success (Beebee and Rowe, 2008). The effective population size is

generally significantly lower than the census numbers (N_c) of a population (Beebee and Rowe, 2008). Frankham (1995), in a meta-analysis of almost 200 studies (from 102 species), looked at the estimated ratio of N_c:N_e and found that the average was around 0.1 (although it ranged hugely from 0.0009 to 1.04). This means that, on average, the effective population size is an order of magnitude less than the census size for a population. In the context of my study, the implication is that the genetic bottleneck the introduced species experienced may be an order of magnitude greater in severity than it appears from the founding population size, and therefore that even the species introduced in the greatest numbers (e.g. blackbird, 808 birds), may have effective population sizes of only 80 breeding adults, and a species such as the cirl bunting (11 individuals introduced) may well derive from only one breeding pair.

Ideally, detailed molecular studies would help resolve the effects of the complex demographic bottlenecks on current levels of genetic diversity in introduced species in New Zealand, however, the evidence is equivocal. A number of studies were conducted in the 1980's and 1990's, which examined the genetic differentiation of introduced bird species in New Zealand (Baker, 1992; Baker and Moeed, 1987; Baker et al., 1990; Merilä et al., 1996; Parkin and Cole, 1985; Ross, 1983), but the results from these studies were somewhat mixed. Merilä et al. (1996) conducted a review of studies on genetic differentiation in introduced bird species (globally, not limited to New Zealand), and found that average heterozygosity and the number of polymorphic loci was positively correlated with the number of birds introduced. All of these studies employed allozymes to estimate levels of genetic diversity between native and introduced populations however, given the low resolving power of allozymes (particularly in birds; Crochet

(2000)), further studies employing higher resolution molecular markers (e.g., microsatellites) are warranted, before any conclusions can be drawn. Nevertheless, it seems a logical expectation that the more severe a bottleneck experienced by a population, the less genetic diversity it is likely to retain, with all else being equal (Beebee and Rowe, 2008).

FITNESS CONSEQUENCES OF BOTTLENECKS

The value of introduced birds as a model system for the study of the fitness consequences of bottlenecks is well illustrated by Briskie & Mackintosh (2004), who examined reproductive success in bottlenecked populations. They compared hatching success of introduced birds within New Zealand and found it was significantly lower in species that had been founded by less than 150 individuals, and was only equal to pre-bottleneck populations in species founded by more than 600 individuals. Thus, these authors were able to use a study of introduced species to highlight the need to limit the severity of bottlenecks in the management of endangered native species.

Parasites are ubiquitous in nature, constituting more than half of all animal species (Loye and Zuk, 1991; Price, 1980). By definition, parasites exist by utilising the finite resources of their host for all, or at least part, of their lifespan (Szep and Møller, 2000). Such exploitation is predicted to incur a negative effect on host fitness, and these costs have been comprehensively studied in birds. A variety of negative impacts of parasitism on host fitness have been found, including reductions in survivorship, fecundity and growth rates (for reviews see Lehmann, 1993; Møller, 1997). Evidence is mounting that decreased genetic diversity renders individuals even more prone to disease and parasitic infection (Arkush et al., 2002; Hawley et al., 2005; Hedrick et al., 2001;

O'Brien and Evermann, 1988; Pearman and Garner, 2005), as does increased inbreeding (Acevedo-Whitehouse et al., 2003; Cassinello et al., 2001; Coltman et al., 1999).

Given the negative effect of parasites it is not surprising that hosts have developed an array of defences against parasitic attack (behavioural, mechanical and physiological) and a key component in this armoury is their immune system. Much work has been done on the role of immune defences in relation to avian life history and sexual selection (e.g. Zuk and Johnsen, 1998; Zuk and Stoehr, 2002), and studies have found a link between individual survivorship and the strength of immune response when challenged with a novel antigen (Møller and Saino, 2004). A reduction in genetic diversity has also been linked to decreased immunocompetence (Hawley et al., 2005; Sanjayan et al., 1996), and inbreeding has been shown to have detrimental effects on immune function (Reid et al., 2003).

Whilst links have been made between immunocompetence, inbreeding, and genetic diversity, there is a paucity of studies investigating the relationship between population bottlenecks and immunocompetence in wild populations. One exception is work by Hale and Briskie (2007), comparing a severely bottlenecked population (founded by 5 individuals) of the endemic New Zealand robin (*Petroica australis*) with its source population. The birds in the bottlenecked population displayed significantly weaker responses to an immune challenge (in the autumn) than the source population. However, whether this result is typical of other birds, and whether a similar response occurs in populations that have been subject to less severe bottleneck sizes, is unknown.

Parasites and pathogens have been implicated in the population decline of a number of endangered species (Dobson and McCallum, 1997) and emerging infectious

diseases are an acknowledged conservation issue (Dobson and Foufopoulos, 2001; Wikelski et al., 2004). Hence, whether population bottlenecks have a negative impact on immunity, or cause increased susceptibility to parasitic attack, and at what size of bottleneck this impact starts to compromise the long-term viability of populations, are questions of crucial relevance to conservation.

OUTLINE OF THESIS

In this study I examine the relationship between population bottlenecks and both level of parasitic infection and immunocompetence. Specifically, I examine whether parasite load increases and immunocompetence decreases with severity of bottleneck size, and if so how large a bottleneck is required to limit these negative effects when founding a population. I address these questions using the introduced bird species of New Zealand as a study system, comparing across a range of different bottlenecks (founding population size) in species within New Zealand, and comparing them directly to their non-bottlenecked conspecifics in the UK.

In Chapter 1, I examine the relationship between ectoparasite burden of chewing lice (order: Phthiraptera, suborders: Amblycera and Ischnocera) and population bottleneck size in ten introduced NZ bird species in comparison to their conspecifics in the UK. I quantify parasite load of chewing lice on these species in both countries using a dust-ruffling technique (Walther and Clayton, 1997), and relate differences in burden to the bottleneck size the species have experienced in NZ. I test the hypothesis that parasite burden is higher in NZ populations that have experienced the more severe bottlenecks, and that this difference will cease to exist for populations founded from larger numbers.

In Chapter 2, I continue my exploration of the role population bottlenecks play in parasitic infection by relating the prevalence of avian malaria (a protozoan blood parasite) in introduced NZ bird species, to the bottleneck size experienced during their establishment. I extend this investigation by examining the immunological responses (measured by leucocyte profiles) of these populations to malarial infection, and predict that the more severely bottlenecked populations may be less able to mount an immune response to the infection. In addition, I compare haematological profiles between the NZ populations and their source populations in the UK.

The effect of population bottlenecks on immunocompetence is further investigated in Chapter 3, by experimentally challenging adult birds in six introduced bird species in New Zealand with a novel antigen (phytohaemagglutinin, PHA). This foreign protein induces an immune reaction in the host that is expressed as an epidermal swelling and can be readily measured as an index to immunocompetence strength. I predict that a positive relationship will exist between the strength of the immune response and the size of the bottleneck (i.e., the number introduced).

In Chapter 4, I utilise the same immunological assay as Chapter 3 (PHA challenge) to compare nestling immunocompetence in two closely related bird species (the myna, *Acridotheres tristis*, and the starling, *Sturnus vulgaris*) that were introduced to New Zealand in differing numbers (and hence experienced different bottlenecks). I investigate immune response in the context of ectoparasitic infection and growth rates, and predict that starling nestlings (the less bottlenecked species) will exhibit stronger immune responses (after controlling for ectoparasitic infection) and be less growth restricted following immune challenge than myna nestlings.

Finally, in the General Discussion, I summarise my findings, and contextualise them in terms of conservation implications and beneficial future research.

Each chapter was written as a stand alone paper, in preparation for publication, and hence there is an inevitable degree of repetition.

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Table 1. Details of the introduction history of bird species introduced into New Zealand used in this study. All data is taken from Lever (1987), and excludes introductions noted as unsuccessful.

Species	Introduction number				Number of Introduction events [1]	Locations [2]	Years Introduced	Period introduced (in years)
	Total	Mean	Max	Min				
Blackbird *Turdus merula*	808	54	152	2	17 (2)	Ak, C, Nl, Ot, SI	1862-1889	27
Chaffinch *Fringella coelebs*	377	34	70	5	16 (5)	Ak, C, Nl, Ot, Wl	1862-1877	15
Cirl Bunting *Emberiza cirlus*	11	6	7	4	2 (0)	Ot, Wl	1871-1881	10
Common Myna *Acridotheres tristis*	70	35	40	30	3 (1)	Np, Wl	1875-1877	2
Dunnock *Prunella modularis*	284	18	80	1	17 (1)	Ak, C, Np, Ot, Wl	1867-1882	15
Goldfinch *Carduelis carduelis*	519	40	103	1	15 (2)	Ak, C, Nl, Ot, Wl	1862-1883	21
Greenfinch *Carduelis chloris*	66	13	33	2	7 (2)	Ak, C, Nl, Ot	1862-1875	13
House Sparrow *Passer Domesticus*	111	14	47	1	9 (0)	Ak, C, Nl, Ot, Wn	1862-1871	9
Redpoll *Carduelis flammea*	599	60	209	1	10 (0)	Ak, C, Nl, Ot, Wl	1862-1875	13
Song thrush *Turdus philomelos*	474	40	96	2	14 (2)	Ak, C, Ot, Wl	1865-1880	15
Starling *Sturnus vulgaris*	653	47	100	3	15(1)	Ak, C, Nl, Ot, Wl	1865-1883	18
Yellowhammer *Emberiza citrinella*	462	42	312	1	12 (1)	Ak, C, Nl, Ot, SI	1862-1879	17
Mean Total	369.5	33.6	104.08	4.42	11.42 (1.42)	n/a	n/a	14.58

[1] Numbers in brackets are number of introduction events mentioned in text, where no introduction numbers are known.
[2]. Location codes: Ak – Auckland, C- Christchurch/Canterbury, Nl – Nelson, Np – Napier, Ot – Otago, SI – Stewart Island, Wl – Wellington, Wn - Wanganui

CHAPTER 1

POPULATION BOTTLENECK SIZE AND ECTOPARASITIC LOADS IN INTRODUCED NEW ZEALAND BIRDS

ABSTRACT

Population bottlenecks are a serious concern for conservation biologists as they may lead to a reduction in genetic diversity and increased inbreeding which may cause increased susceptibility to parasitic infection. I examined the relationship between ectoparasite burden (chewing lice; order: Phthiraptera, suborders: Amblycera and Ischnocera) and population bottleneck size in ten introduced bird species of New Zealand (NZ) in comparison to their conspecifics in the United Kingdom (UK). Species in NZ that were introduced in low numbers (i.e., had experienced more severe bottlenecks) had higher ectoparasite loads (measured by prevalence and abundance) than their source populations in the UK, a relationship that was no longer evident at moderate bottleneck sizes. Intensity of infection tended to be higher in NZ, but was not correlated with bottleneck size. When the two suborders of lice (Amblycera and Ischnocera) were examined separately the relationship between bottleneck and parasite burden was stronger in Amblycera. Species that had experienced severe bottlenecks had higher genera richness of parasites in NZ, whilst the less bottlenecked species had greater diversity of genera in the UK. Further work is required to investigate if these relationships are present in endangered species, which may experience stronger fitness reductions due to increased environmental stresses.

INTRODUCTION

RELEVANCE OF POPULATION BOTTLENECKS TO CONSERVATION

It is an inevitable truth that many conservation schemes must ultimately deal with the consequences of population bottlenecks. When endangered species are brought 'back from the brink' by conservation initiatives, the process is one of increasing populations that have been reduced drastically in size (i.e., they have experienced a bottleneck), usually as a consequence of human activities. Captive breeding and translocation projects are another source of bottleneck events within the conservation arena. However, in these instances, the severity of the bottleneck (i.e., the number of individuals founding a population) is to some extent under the control of the conservation practitioner.

Population bottlenecks are a serious concern for conservation biologists because they can lead to a reduction in genetic diversity, both in terms of heterozygosity and allelic diversity (England et al., 2003). This has now been demonstrated in a number of wild populations (Ardern et al., 1997; Bodkin et al., 1999; Bouzat et al., 1998; Bradshaw et al., 2007; Hajji et al., 2007; Hoelzel, 1999; Hudson et al., 2000; Keller et al., 2001; Nyström et al., 2006; Packer et al., 1991; Zhang et al., 2004). Decreased genetic diversity can be associated with reduced fitness (Reed and Frankham, 2003). Indeed, a reduction in genetic diversity has been linked to extinction risk (Frankham, 1998). A recent study conducted by Spielman et al. (2004) comparing heterozygosity of threatened and non-threatened related taxa confirmed that the heterozygosity of threatened species was on average 35% lower than in the non-threatened counterparts.

When population size is very small (as in the case of a bottleneck), mate choice is reduced and individuals are more likely to mate with kin, thereby increasing levels of inbreeding. Inbreeding leads to a reduction in heterozygosity (Keller and Waller, 2002) and hence may be a main driver of the loss of genetic variation in populations that have experienced a bottleneck. The negative impact of inbreeding on a population, termed inbreeding depression, is well known and encompasses a suite of interrelated fitness effects, including a reduction in reproductive success, an increase in both physical defects and susceptibility to parasites and pathogens (Crnokrak and Roff, 1999; Keller and Waller, 2002).

While it is clear that population bottlenecks may have severe consequences for a population's fitness, and ultimately to its survival, the questions for conservation managers are: 'What are the exact fitness effects of bottlenecks?' and 'When is a bottleneck severe enough to cause negative effects?' To address these questions we need to determine which fitness traits are affected by population bottlenecks, and at what severity of bottleneck reductions in fitness are observed in those traits that might pose a significant threat to the health and survival of the post-bottlenecked population.

INTRODUCED BIRDS AS A MODEL STUDY SYSTEM

The most informative way to answer questions about the fitness effects of population bottlenecks is by comparing populations that have or have not been through bottlenecks. However, the existence of pre- and post-bottleneck populations of the same species is a rare occurrence in the wild, especially in the case of endangered species which, by definition, have already passed through severe bottlenecks.

A striking exception to this is the numerous human-introduced species that have established in areas outside their native range. Such species offer unique opportunities for a study of pre- and post-bottlenecked populations of the same species (Briskie, 2006), with the introduced bird species of New Zealand being a good example. In the late 1800's, Acclimatisation Societies introduced numerous bird species to New Zealand (Lever, 1987; Thompson, 1922), a number of which are successfully established today. The majority of these species were imported from the United Kingdom (UK), where they are still extant, and careful records were kept of the numbers that survived the voyage and were subsequently introduced. The number introduced varied per species, and so introduced birds in New Zealand today are represented by populations that have experienced a range of bottlenecks. The elegance of this system is that it allows comparison between pre- and post bottlenecked populations (i.e., between UK & New Zealand) for a range of bottlenecks. This enables investigations to be made, not only of how bottlenecks may affect fitness traits, but additionally, at what size bottleneck these fitness effects cease to be evident (or at least of lesser concern) in comparison to the source population.

The usefulness of introduced birds as a model system for the study of bottlenecks is well illustrated by Briskie & Mackintosh (2004), who examined reproductive success in bottlenecked populations. They compared hatching success of introduced birds within New Zealand and found it was significantly lower in species that had been founded by less than 150 individuals. Moreover, when they compared hatching success in introduced birds to their UK counterparts they discovered that hatching success was only equal to pre-bottleneck populations in species founded by more than 600 individuals. Thus, these

authors were able to use a study of introduced species to highlight the need to limit the severity of bottlenecks in the management of endangered native species.

PARASITES AND FITNESS EFFECTS

Parasites are ubiquitous in nature, constituting more than half of all animal species (Loye and Zuk, 1991; Price, 1980). By definition, parasites exist by utilising the finite resources of their host for all, or at least part, of their lifespan (Szep and Møller, 2000). Such exploitation is predicted to incur a negative effect on host fitness, and these costs have been comprehensively studied in birds. A variety of negative impacts of parasitism on host fitness have been found, including reductions in survivorship, fecundity, and growth rates (for reviews see Lehmann, 1993; Møller, 1997).

Populations that experience a bottleneck, and hence a decrease in genetic diversity and increased inbreeding, may be more susceptible to parasitic infection, leading to an associated reduction in fitness. A recent comparative study of three species of captive gazelle (*Gazella* spp.) confirmed that the species founded from the smallest population (i.e., the most severely bottlenecked), had the highest level of inbreeding and exhibited the highest level of gastrointestinal parasitic infection (Cassinello et al., 2001). This effect has also been observed at the individual scale; a study of an isolated population of Soay sheep (*Ovis aries*) found that more inbred individuals had higher levels of nematode infection (Coltman et al., 1999). In addition, Acevedo-Whitehouse et al. (2003), working on Californian sea lions (*Zalophus californianus*), observed that individuals exhibiting helminth and bacterial infections were more inbred than non-infected individuals. Furthermore, a recent study in which several frog (*Rana latastea*) populations of differing genetic diversity were exposed to a novel virus (Pearman and Garner, 2005), found that

populations with higher genetic diversity had significantly higher rates of survival following exposure.

In birds, parasites are as diverse as they are pervasive, ranging from ultramicroscopic viruses to (the often larger than host) brood parasites. Unsurprisingly, for a group of organisms with this breadth, effects on host fitness differ depending on the parasite in question (Loye and Zuk, 1991). In this study I focus on ectoparasite loads of birds that have passed through bottlenecks, specifically the prevalence and abundance of ectoparasitic chewing lice (Order: Phthiraptera) of the suborders Amblycera and Ischnocera. Both suborders are obligate parasites, completing their entire life cycle on the host (Clayton and Tompkins, 1994; Møller and Rozsa, 2005); however, Ischnocera and Amblycera lice differ somewhat in their attributes. Ischnocerans spend their entire life cycle living and feeding on the host's feathers and are dependent on vertical transmission from parent to offspring (Clayton and Tompkins, 1994; Møller and Rozsa, 2005). In contrast, amblycerans are faster moving, sometimes leave the host when it is disturbed or dying (Lindell et al., 2002), feed on the dermis and blood of the host, and hence activate the immune system (Møller and Rozsa, 2005). These differences suggest that host defence mechanisms may differ between suborders (Møller and Rozsa, 2005), and that differential fitness costs may be incurred by the two taxonomic groups.

The negative impact of ectoparasitic infection is well documented (Barbosa et al., 2002; Booth et al., 1993; Lehmann, 1993; Møller, 1997). Furthermore, recent work has found links between decreased heterozygosity and increased prevalence of ectoparasitism in a wild population of lesser kestrels (*Falco naumanni*)(Ortego et al., 2007), and

between inbreeding and ectoparasitism in fruit flies (*Drosophila nigrospiracula*) in the laboratory (Luong et al., 2007).

STUDY AIMS

To assess the effects of population bottlenecks on parasite loads, I compared introduced species in New Zealand to their non-bottlenecked source populations in the UK. Ten species of bird were examined; all species are passerines and all have large populations in both countries. However, the populations of these species in New Zealand were established in the late 1800's from founding populations ranging from a low of 66 to a high of 808 individuals. Thus, the objectives of my study are to (i) investigate if population bottlenecks cause an increase in the prevalence and/or intensity of ecto-parasite load, and if so (ii) at what size bottleneck that difference is no longer detectable. My prediction is that if the genetic effects of bottlenecks adversely impact host fitness, parasite loads (both prevalence and abundance) will be higher in severely bottlenecked species, compared to their non-bottlenecked populations in the UK. Furthermore, I predict that this difference will cease to be evident at more moderate bottlenecks, where effects on heterozygosity and inbreeding are reduced, indicating the number of founders required to avoid future negative impacts. Because of the differences in life history of the different ectoparasite taxa, I analysed both groups separately (Ischnocera & Amblycera) and together (Phthiraptera). If population bottleneck effects on these groups are specifically via immune function, effects may be greater for Amblycera which are more likely to initiate an immune response from the host.

METHODS

STUDY POPULATIONS & GENERAL METHODOLOGY

The ten study species are all passerines that were successfully introduced into New Zealand in the late 1800's and are still extant today in their native range in the UK. The number of individuals of each species released in New Zealand (i.e., the introduction effort) was carefully recorded by the Acclimatisation Societies responsible for their introduction (Lever, 1987; Thompson, 1922). I used introduction effort, calculated as the total number released for each species, excluding any introductions that were specifically recorded as being unsuccessful, as a surrogate for population bottleneck size. Introduction effort was different for each species, and hence corresponds to a range of bottlenecks (see Table 1 for a full list of species and the bottlenecks they experienced).

Birds were caught by passive mist-netting or trapping in a number of locations in the South Island and the south of the North Island, New Zealand (Wellington, Martinborough, Blenheim, Ward, Kaikoura, Lincoln, Dunedin) and in various southern sites in the UK (sites in Bedfordshire, Cambridgeshire, Suffolk, Norfolk, Devon Hertfordshire and East Sussex). Ectoparasite populations are known to fluctuate seasonally (Chandra et al., 1990; Wilson et al., 2002). Hence, as it was not possible to sample all populations in all seasons, I restricted my sampling to the autumn months for each hemisphere (Southern hemisphere: March-May 2006; Northern hemisphere: September-November 2005) to control for seasonal effects.

The data set presented here is comprised of samples collected by two different researchers (myself and C. J. MacLeod). The collection methods by each researcher are described below.

ECTOPARASITE COLLECTION.

Ectoparasites were collected by myself using a modified dust-ruffling technique (Clayton and Drown, 2001; Walther and Clayton, 1997), which employs a pyrethrin-based insecticide to remove the parasites. Pyrethrin is a commonly used insecticide, and is safe for use on birds (Walther and Clayton, 1997). The insecticide (Johnsons Cat & Dog Flea Powder, Johnson's Veterinary Products Ltd, UK) contained a mixture of 0.1% pyrethrin and 0.8% piperonyl butoxide (a synergist that improves the efficacy of pyrethrin), and has been shown to have no effect on survival of Rock Doves (*Columbia livia*) (Clayton and Tompkins, 1995).

On capture, birds were immediately individually placed in a new paper bag which lined a cloth bird bag, and held until ready to be processed. On removal from the bag, the bird was held over a shallow collecting tray lined with clean paper, a metal identification ring was attached and basic biometrics taken. The bird was then dusted with sufficient insecticide powder to cover the plumage, whilst being held over the collecting tray (see Figure 1). The insecticidal powder was thoroughly distributed throughout the bird's feathers by hand, and the plumage 'ruffled' for a period of 3 min to dislodge the parasites. The bird was then returned to the paper bag for a period of 15 min, and the contents of the collecting tray tipped into a 1.5 ml screw-top vial (SARSTEDT Australia Pty. Ltd, Australia) containing 90% ethanol. After 15 min the ruffling process was repeated for another 3 min and the bird released. The content of the paper bag was

emptied onto the paper in the collecting tray, and everything on the paper emptied into the vial of 90% ethanol. A digital kitchen timer with an alarm was used throughout to ensure consistency of timing. To avoid contamination, a new paper bag and collecting tray paper was used for each individual. Before a new bird was processed, the collecting tray was swabbed with alcohol as were the latex gloves worn by the researcher.

Samples collected by C.J MacLeod (C.J.M.) were made at the same time as those collected by myself, in both New Zealand and the UK but at different locations within each country. This allowed us to combine our data and increase our survey effort and sample sizes above that what each could accomplish alone. The ectoparasite collection methods used were very similar; birds were held in paper bags, and the same protocol of dust-ruffling for 3 min, holding for 15 min and ruffling for a further 3 min was employed. The insecticide used in New Zealand by C.J.M. differed in the active ingredient (Vitapet Dog Flea Powder, 10g/kg Permethrin, Vitapet Corporation, Lower Hutt, NZ), however, permethrin is a synthetic derivative of pyrethrin that has the same insecticidal effects. There were no significant differences in prevalence (Fisher's exact test) or mean intensity (Bootstrap t-test) between the two data sets (S.E.A & C.J.M), in either country (S.E.Allen unpubl.). Birds were dust-ruffled over a large funnel, with a 1.5 ml vial attached to the base. After the dust ruffling procedure, the funnel contents were flushed into the vial with 90% ethanol.

IDENTIFICATION OF ECTOPARASITES.

Ectoparasite samples were identified and quantified by either Ricardo Palma (Te Papa Museum, Wellington), or Terry Galloway (University of Manitoba, Canada). Chewing lice (Phthiraptera) were identified to the genus level. There are difficulties

differentiating species of louse in some genera at certain life stages (R. Palma. pers. comm.), and some taxonomic ambiguities exist (MacLeod et al. unpubl. data), therefore all analyses took place at the genus level or higher (see Table 1 for details of the genera identified per host species). Ectoparasites other than chewing lice were identified to genus or order, and were present in very low numbers.

QUANTIFICATION OF ECTOPARASITES.

Parasitic infection is generally quantified in three ways: prevalence, intensity and abundance. According to Bush et al. (1997), prevalence is calculated as the number of hosts infected by one or more parasite of a taxonomic group, divided by the total number of hosts; intensity is the number of individual parasites found on a host, and abundance is the number of parasites on a host, irrespective of whether the host is infected or not. Thus mean intensity is therefore the total number of parasites found in a sample of birds, divided by the number of hosts infected by that parasite, and mean abundance is calculated as the total number of parasites (i.e., total number of phthirapterans, amblycerans, or ischnocerans) divided by the total number of hosts sampled and includes hosts with no parasites.

In this study, I calculated all three measures of parasite load but primarily used abundance and prevalence to quantify and analyse parasite loads across populations and species. The use of abundance is preferable to intensity when wishing to examine and compare whole host populations, including hosts that harbour no parasites (Bush et al., 1997). It should be noted that mean abundance is the product of mean intensity and prevalence, and thus not an independent measure of parasite load from prevalence.

STATISTICAL ANALYSES

The number of parasites in Phthiraptera (PHTH), Amblycera (AMBL) and Ischnocera (ISCH) were analysed separately. My aim was to compare differences in prevalence and abundance of ectoparasites within host species, between the two countries, New Zealand (NZ) and UK, and to investigate whether these differences are related to bottleneck size.

As age and sex of a host are known to influence parasite load (Wilson et al., 2002), the data were examined to see if these variables required inclusion in the final models. Generalized Linear Mixed Models (GLMMs) were used to investigate age effects, as GLMMs enable analysis of data that are structured into groups and are non-normally distributed (Paterson and Lello, 2003). A GLMM was fitted to each data set with a binomial error term fitted for prevalence, and a quasipoisson error term for abundance and intensity. Age ('hatch year' or 'after hatch year'), country (NZ or UK), and the interaction between age and country were fitted as fixed effects, and species as a random effect. Birds of unknown age were excluded. The term of interest in the model was the interaction between age and country which, if significant, would suggest that age effects differed between countries. This interaction term in the PHTH and AMBL data sets was non-significant (at $P < 0.05$) for both prevalence and abundance, indicating that age class can be excluded as a variable. Ischnocera lice were not found on enough species in both countries to conduct this analysis, and hence the ISCH data set was not examined. However, it seems fair to make the assumption that age classes would not differ between countries for Ischnocera parasite load anymore than they would for Amblycera.

A different approach was necessary to investigate the influence of sex class on parasite load, as unfortunately, sex was unknown for a subset of the UK data. Therefore, only the NZ data was checked to see if prevalence, intensity or abundance was affected by sex class. GLMMs were run with sex (male or female) as the fixed effect, and species as the random effect (with a binomial error term fitted for prevalence, and a quasipoisson error term for abundance and intensity), after excluding birds of unknown sex. Sex was not found to have a significant effect on abundance, intensity or prevalence ($P < 0.05$) in any of the three data sets (PHTH, AMBL or ISCH). Hence, for all subsequent analyses sample populations were not subdivided into age or sex categories, and birds of unknown age and/or sex were included in the data sets.

Prevalence, mean intensity and mean abundance were calculated for each species in both NZ and UK for all three data sets (PHTH, AMBL, ISCH; see Table 2). For completeness, the variance-to-mean ratio (a measure of aggregation) is also reported. The difference in prevalence between the UK and NZ was calculated for each bird species, for all three data sets. Linear regressions were then fitted to these differences, with bottleneck (log-transformed) as the explanatory variable and difference in prevalence (UK – NZ) as the response variable.

Mean intensities were compared between countries by calculating the percentage change from the UK value to the NZ value. Intensity comparisons were conducted on a reduced number of species, as comparison was only possible when populations in both countries were infected with the louse sub-order in question. Linear regressions were fitted between percentage change in mean intensity against bottleneck (log transformed).

The difference in mean abundance (which is a composite of mean intensity and prevalence) between the two countries was also calculated (UK – NZ per species) and linear regressions fitted against bottleneck (log transformed).

Comparisons of prevalence, mean intensity, and mean abundance were also made on a species-by-species basis. Prevalence was compared using Fisher's exact test, and mean abundance and intensity compared using a bootstrap test (see Rosza et al. (2000) for details), using the software Quantitative Parasitology 3.0.

To investigate differences in richness of parasites at the genus level, the difference in genera richness between the two countries was regressed against bottleneck size (log transformed).

All statistics, other than the individual species comparisons (using Quantitative Parasitology 3.0) were carried out using R v2.6.2 (R Development Core Team, 2008).

RESULTS

Difference in prevalence between UK and NZ:

The difference in Phthiraptera prevalence on birds in the UK and NZ was positively correlated with the population bottleneck the NZ birds had experienced ($F = 6.54$, $df = 8$, $r^2 = 0.45$, $P = 0.03$). At severe bottlenecks, prevalence tended to be higher in NZ, whilst at more moderate bottlenecks the reverse was true (Figure 2a). The difference in prevalence of amblyceran lice between the UK and NZ exhibited the same relationship, and this correlation was also significant ($F = 6.23$, $df = 8$, $r^2 = 0.44$, $P = 0.04$, Figure 2b). Prevalence differences in ischnoceran lice again exhibited a similar trend as

phthirapterans and amblycerans, but this trend was not significant ($F = 2.51$, $df = 8$, $r^2 = 0.24$, $P = 0.15$, Figure 2c).

Difference in intensity between UK and NZ:

The proportional change in parasite intensity between the two countries was regressed against bottleneck size. No correlation was found between bottleneck size and change in intensity for either the phthirapteran or amblyceran data set, ($P = 0.30$ & $P = 0.29$, respectively); however, the majority of species experienced higher intensities in NZ (see Figures 3a and b). The correlation between change in intensity and bottleneck size for ischnoceran lice was found to be positive ($P = 0.03$), indicating that the difference in intensity increased significantly at larger bottlenecks. However, the regression was only conducted on three species, and lacks statistical rigor. All three species had higher intensities in NZ (see Figure 3c).

Difference in abundance between UK and NZ

A positive correlation was found between the differences in mean abundance of Phthiraptera lice and bottleneck size (see Figure 4a). At severe bottlenecks, mean abundance was greater in New Zealand than the UK, while at less severe bottlenecks, the differences in mean abundance became less. This correlation was highly significant ($F = 11.96$, $df = 8$, $r^2 = 0.60$, $P = 0.009$). The same highly significant correlation was found if mean abundance of amblyceran lice was regressed against bottleneck size ($F = 11.88$, $df = 8$, $r^2 = 0.60$, $P = 0.009$, Figure 4b). The regression of difference in mean abundance of Ischnocera lice against bottleneck, was non-significant ($P = 0.13$), although the trend was in the same direction as the amblyceran data (see Figure 4c.)

Within-species comparisons.

The prevalence, mean intensity, and mean abundance of Amblycera and Ischnocera were compared between the UK and New Zealand on a species-by-species basis. Prevalence of Amblycera was found to be significantly higher in New Zealand in the two species that had experienced the most severe bottlenecks – greenfinch ($P < 0.05$), house sparrow ($P < 0.05$), and tended towards significance in third most severely bottlenecked species, the dunnock ($0.05 < P < 0.1$). Mean abundance of Amblycera was also significantly higher in house sparrows ($P < 0.05$) in New Zealand. No other species exhibited significant differences in prevalence, abundance, or intensity for Amblycera lice. No significant differences existed for Ischnocera load for any species,

Genera richness

The difference in the number of genera of chewing lice (Phthiraptera) found on host populations in the UK and NZ (see Table 1) was regressed against bottleneck size, there was a positive, non-significant trend with populations in New Zealand that had experienced more severe bottlenecks having greater genera richness than in the UK ($F = 4.49$, $df = 8$, $r^2 = 0.36$, $P = 0.067$). The reverse was true for species that had gone through more moderate bottlenecks (Figure 5).

DISCUSSION

In this study I found that the ectoparasite load of chewing lice (Order: Phthiraptera) on introduced bird species in New Zealand was significantly affected by the severity of the bottleneck that each species had experienced, when compared to their

source populations in the UK. Species in NZ that were introduced in low numbers (and therefore had experienced more severe bottlenecks), had a significantly higher ectoparasite load when compared to their non-bottlenecked counterparts in the UK, while loads were similar between the two countries at more moderate bottlenecks.

Ectoparasite load was quantified using three measures: (1) prevalence, which is the number of birds in a population that were infested with ectoparasites; (2) mean intensity, defined as the average number of parasites on infested individuals (i.e., excluding zero-class individuals); and (3) mean abundance, which is the average number of parasites per bird, including un-infested individuals. Both prevalence and mean abundance (which accounts for intensity of infection) were correlated with the size of the population bottleneck. Although intensity did not demonstrate the same correlation, it also tended to be higher in NZ birds. The implication of these correlations is that ectoparasitic infection increases with severity of bottleneck, and in general, species that passed through the most severe bottlenecks were found to have more individuals infested with lice (higher prevalence). As the analyses were conducted on intraspecific differences, this pattern is independent of any species specific levels of infestations.

The two suborders of chewing lice, Amblycera and Ischnocera, were also examined individually. I found that prevalence, mean intensity and mean abundance of Amblycera exhibited the same relationship with population bottleneck size as Phthiraptera. The regression between bottleneck size and change in mean abundance was particularly strong, with almost 60% of the variance in differences between mean amblyceran abundance in the two countries being explained by the bottleneck size the NZ populations experienced. Intensity was not correlated with bottleneck size: in 4 out of 6

species, intensity was higher in NZ (although these differences were not significant in single species comparisons). When species were analysed individually, it was found that significant differences in prevalence existed for the two species that had experienced the most severe bottlenecks – greenfinches, house sparrows (bottlenecks of 66 and 111 respectively). and tended towards significance in the third most bottlenecked species the dunnock (bottleneck of 284).

Whilst the differences in prevalence and mean abundance of Ischnocera between the two countries exhibited the same trend as for Amblyceran lice, the relationship was not significant. Change in mean intensity was correlated with bottleneck size, and increased as bottleneck size increased, but a comparison was only possible between three species, and so the statistical rigor of this regression is weak, although in all three instances intensity was higher in NZ. It would appear that ischnoceran load is less affected by population bottlenecks in the host populations studied than Amblyceran load. However, this result may be due to lower numbers of ischnocerans being collected overall, and therefore a lack of statistical rigor, rather than a biologically significant difference.

When differences in genera richness were investigated, I found a trend indicating that species that had experienced severe bottlenecks had higher genera richness in New Zealand, whilst the less bottlenecked species had greater diversity of genera in the UK. To some extent this appears to contradict the hypothesis that introduced species experience a 'release' from their native parasites (MacLeod et al., 2008b; Torchin et al., 2003). However, my results may be more an indication of the abundance of different genera, as opposed to their existence *per se*. The fact that avian lice 'miss the boat' and

fail to establish on introduced bird populations in New Zealand has been well documented (Paterson et al., 1999), but whilst bird species introduced in low numbers have a lower diversity of lice genera than their source populations (as predicted by the 'missing the boat' theory), this reduction may be tempered by a concomitant increase in the abundance of surviving lice genera, meaning they are more often detected in New Zealand. Additionally, host switching events, whereby parasites colonise new hosts, may also have occurred following bird introductions in New Zealand, especially as a number of the bird species were close relatives (Torchin and Mitchell, 2004). Introduced bird species that experienced severe bottlenecks may be more prone to being colonised (or re-colonised) by lice genera originating from populations of related bird species, that were introduced in higher numbers, with subsequently higher parasite richness, or indeed by native parasites. Such potential occurrences have fascinating and significant implications, both for enemy release theories (Torchin et al., 2003), and for conservation management at a community level.

Birds host an extensive parasite fauna, ranging from microscopic viruses to the larger than host brood parasites, and ectoparasites represent only one component of this parasitic fauna. Ectoparasites however are a good candidate for study, as they can be readily sampled from wild host populations with minimum disturbance to the bird (e.g., the dust-ruffling procedure takes less than 30 mins). Whilst phthirapterans were not the only external parasites I collected, they were the only group to be reliably found on enough hosts (and host species) for comparisons to be made. The other ectoparasites collected (e.g., Diptera, Siphonaptera**)** were found in such low numbers, that the sampling effort would require amplification to unrealistic levels to detect any trends and accurate

quantification would be complicated by the increased mobility of these taxa. Feather mites (Astigmata) were the only other class of arthropod found in any significant number on the birds sampled, however current evidence suggests they form a commensalistic relationship with the host (Walter and Proctor, 1999), and whether they exert fitness costs is ambiguous.

Introduced populations of birds in New Zealand that had experienced severe population bottlenecks had higher louse loads than their source population, and whilst the fitness consequences of this increase is beyond the scope of this study, it seems reasonable to assume that costs are incurred. Chewing lice (Order: Phthiraptera) are obligate parasites, that complete their entire life cycle on a bird's body surface, and have a number effects on host fitness. For example, they cause feather damage (Barbosa et al., 2002; Pap et al., 2005), which in turn impacts on flight behaviour (Barbosa et al. 2002), and has increased energetic costs (Booth et al., 1993). Chewing lice load has also been negatively linked to body condition and territory ownership (Whiteman and Parker, 2004) and to over-winter survival (Clayton et al., 1999). Furthermore, there are 'hidden' costs to ectoparasitism, as behavioural adaptations designed to reduce parasite load (e.g., preening) are both energetically costly, and decrease time available for other important functions such as foraging and predator vigilance (Lehmann, 1993).

The two suborders of chewing lice, Amblycera and Ischnocera, may induce differing fitness costs. Ischnocera spend the entirety of their life cycle on a bird's feathers, feeding on the non-living parts of the feather barbules, and rarely coming into direct contact with the host's living tissues (Clayton et al., 1999; Clayton and Tompkins, 1994; Møller and Rozsa, 2005). Consequently, the main fitness effects they impose on a

host are believed to be due to feather damage, and the energetic costs associated with this (Barbosa et al., 2002; Booth et al., 1993; Clayton, 1990). Amblyceran lice, on the other hand, feed on the skin and blood of hosts (Møller and Rozsa, 2005), and also act as vectors for several endoparasites (Clayton, 1990). Amblycera would thus be expected to stimulate both the immunological and behavioural defences of a host. Indeed, amblyceran infestation is known to stimulate scratching and dermatitis (Clayton and Tompkins, 1995) in hosts, an expected response to ectoparasitic stimulation of the immune system (McLaren, 1990). Furthermore, in an interspecific comparison, Møller & Rozsa (2005) found that amblyceran, but not ischnoceran, taxonomic richness was predicted by immune response, suggesting an interaction between Amblycera lice and host immunity that is absent for Ischnocera. It would be predicated then, that amblyceran lice are more virulent than ischnocerans, stimulating costly immunological and behavioural responses, whilst introducing further infection and directly feeding on host nutrients (blood). In a study of chewing lice load on Galapagos hawks (*Buteo galapagoenisis*), Whiteman and Parker (2004) found that whilst correlations between louse abundance and body condition existed for both suborders, it was stronger for amblycerans.

In my study, I found that amblyceran prevalence and abundance was much more strongly correlated to population bottleneck size than ischnoceran load. In fact, when species were analysed individually, the three species that had experienced the most severe bottlenecks (greenfinches, house sparrows and dunnocks, with bottlenecks of 66, 111 and 284, respectively) all exhibited significantly (or in the case of dunnocks, tending towards significantly) higher prevalence of amblyceran lice in NZ, whilst significant differences

did not exist for Ischnocera. It seems then, that populations that have experienced a severe bottleneck are more prone to infestation by ectoparasites that incur higher fitness costs, activating immunological and behavioural defences and directly competing for host resources.

Whilst my study strongly suggests that a negative correlation exists between ectoparasite load and population bottleneck size, it is unknown whether bottlenecked populations experienced a reduction in fitness which subsequently led to an increase in parasite load, or vice versa. Population bottlenecks may reduce genetic diversity and increase levels of inbreeding, and a number of studies have found evidence that these factors lead to increased parasitism (Acevedo-Whitehouse et al., 2003; Cassinello et al., 2001; Coltman et al., 1999; Ortego et al., 2007). A study of the effects of inbreeding on susceptibly to ectoparasitism in fruit fly (*Drosphila nigrospiracula*) – mite (*Macrocheles subbadius*) system, (Luong et al., 2007) found that inbreeding increased susceptibility and that this appeared to mediated by energetic constraint. These authors also found that more inbred lines of flies exhibited lower stamina, and that exhausted flies were more likely to suffer parasitism, suggesting that more inbred flies were less able to mount suitable, energetically costly defensive behaviour (Luong et al., 2007).

Immunocompetence has been linked to genetic diversity (Hawley et al., 2005; Sanjayan et al., 1996), and some studies have demonstrated a decrease in immunological responses in more inbred or bottlenecked populations (Hale and Briskie, 2007; Reid et al., 2003; Reid et al., 2007). Effects on immunity could be mediated directly through genetic impoverishment, or, as immune defence is itself costly (Lochmiller and Deerenberg, 2000), through more general energetic constraint processes.

In my study system, it would seem likely that defence against amblyceran lice (but not ischnoceran) is partly dependent on mounting suitable immunological defences (Møller and Rozsa, 2005), and hence if bottlenecked populations of birds are immunocompromised it may explain why significantly higher amblyceran, but not ischnoceran loads exist on these populations. However, in a concurrent study on immunity (measured via the PHA stimulated immune response) in the same study species (Chapter 3), I found that species that had experienced more severe bottlenecks had stronger immune responses. This suggests that birds that are experiencing higher parasite loads are investing more in immunological defence than less bottlenecked species, presumably to the detriment of other energetically costly processes.

Factors other than the bottleneck a population experiences could account for variation in parasitic load. Previous studies have found links between host life history (e.g., polygyny, group-living) and risk of parasitism (see Møller (1997) for review). However, to a large extent, the design of this study avoids these confounding factors, as comparisons are made within species. Potentially, species could change their life history characteristics when introduced to a novel environment, and so introduced bird species in New Zealand may differ from their source populations in a trait that influences parasite load. The species examined in this study have similar life histories and are found in the same locations (indeed several species form mixed flocks in the autumn and winter), so if such a change did occur following introduction, then one would expect it to take place in all species equally, and therefore the trend with bottleneck would not be evident. Parasite abundance may also be linked to host population density (Arneberg et al., 1998), and recent work has found that densities of introduced bird species are considerably higher in

New Zealand than in the UK (MacLeod et al., 2008a). Whilst these densities do not correlate to bottleneck size (S.E.Allen unpubl. data), it is of note that two severely bottlenecked species (greenfinch and house sparrow) are found at very high densities in New Zealand, and this avenue of study requires further investigation.

The findings of this study suggest that species that experience severe population bottlenecks will be more likely to suffer higher parasite loads, even though many species in my study passed through bottlenecks of several hundred birds. It should be remembered that the species these data were collected from are introduced birds that have successfully established in a new country. These species are in fact more successful in New Zealand, their introduced range, than in the UK, their source country (MacLeod et al., 2008a), suggesting that conditions are more favourable in their introduced range. However, one implication of the work here is that despite birds being introduced to perhaps a relatively benign environment, the negative effects of population bottlenecks are still exhibited, suggesting that for species under greater environmental stress (i.e., endangered species) the effects might be stronger and expressed at even larger bottleneck sizes. Studies investigating the effect of bottlenecks on endangered and threatened species are required to investigate this hypothesis.

Taken literally, my observations on high parasite loads in species passing through relatively large bottlenecks would suggest that conservation practitioners should aim to conduct translocations with numbers in the hundreds, rather than the tens of individuals, to avoid potential deleterious impacts linked to increased parasite burdens. In most instances this will be unrealistic (in some cases the total global population of an endangered species will be less than that), however the rule of thumb for translocation

should perhaps be 'the bigger the better'. In the early 90's the average number of individuals translocated was 75 individuals (Wolf et al., 1996), my findings suggest that at these numbers populations will experience increased parasitism. Whilst this may be unavoidable, it should not be discounted, and management plans would benefit from incorporating these considerations. Furthermore, these findings may have community level implications. For example, commonly in New Zealand a number of different endangered bird species are translocated to the same predator-free offshore island (e.g., TiriTiri Matangi, Ulva), which will already have extant populations of bird species. As population bottlenecks may increase parasite abundance and potentially the likelihood of colonisation by new parasites, then interspecific interactions on crowded islands may exacerbate this.

In conclusion, the introduced bird species of New Zealand that experienced relatively severe bottlenecks (66-284) had higher parasite loads than their source populations, a relationship that was no longer evident at moderate bottleneck sizes. Further work is required to investigate if this pattern is also present in endangered species, which may experience stronger fitness reductions due to increased environmental stresses.

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TABLES

Table 1. List of bird species and the population bottleneck they experienced in New Zealand (calculated as the maximum number of successfully introduced individuals from Lever (1987)) and parasite genera found on host species in this study in the United Kingdom (UK) and New Zealand (NZ)

Species	Bottleneck	UK	NZ
Greenfinch *Carduelis chloris*	66	*Menacanthus* *Myrsidea*	*Menacanthus* *Myrsidea* *Brueelia*
House Sparrow *Passer Domesticus*	111	*Philopterus*	*Menacanthus* *Brueelia*
Dunnock *Prunella modularis*	284	*None*	*Menacanthus* *Myrsidea* *Philopterus*
Chaffinch *Fringella coelebs*	377	*Menacanthus* *Ricinus*	*Menacanthus*
Yellowhammer *Emberiza citronella*	462	*None*	*Brueelia*
Song Thrush *Turdus philomelos*	474	*Menacanthus* *Brueelia*	*Menacanthus* *Philopterus*
Goldfinch *Carduelis carduelis*	519	*Menacanthus* *Myrsidea*	*Menacanthus* *Myrsidea*
Redpoll *Carduelis flammea*	599	*Menacanthus* *Philopterus*	*None*
Starling *Sturnus vulgaris*	653	*Menacanthus* *Myrsidea* *Sturnidoecus*	*Menacanthus*
Blackbird *Turdus merula*	808	*Menacanthus* *Myrsidea* *Philopterus* *Brueelia*	*Menacanthus* *Philopterus*

Table 2. Summary statistics for ectoparasites collected on NZ and UK populations a) Phthiraptera, b) Amblycera and c) Ischnocera. See methods section for definations of the summary statistics. Intensity and aggregation can not be calculated for prevalances = 0%, dashed lines (---) indicate where this is the case.

a) Phthiraptera

Species	Sample size		Prevalence		Abundance		Intensity		Aggregation	
	UK	NZ	UK	NZ	UK	NZ	UK	NZ	UK	NZ
Carduelis chloris	61	49	3.28	22.45	0.07	1.84	2.00	8.18	2.48	20.11
Passer Domesticus	47	89	17.02	44.94	0.38	2.33	2.25	5.18	3.58	9.04
Prunella modularis	61	61	0.00	13.11	0.00	0.31	---	2.38	---	4.45
Fringella coelebs	35	50	5.71	2.00	0.09	0.02	1.50	1.00	1.63	1.00
Emberiza citrinella	5	21	0.00	9.52	0.00	0.10	---	1.00	---	0.95
Turdus philomelos	31	30	19.35	26.67	0.45	2.07	2.33	7.75	3.08	22.02
Carduelis carduelis	59	86	6.78	4.65	0.17	0.50	2.50	10.75	3.29	27.89
Carduelis flammea	16	17	18.75	0.00	0.19	0.00	1.00	---	0.87	---
Sturnus vulgaris	13	9	69.23	33.33	3.62	2.11	5.22	6.33	7.63	9.17
Turdus merula	62	74	34.43	40.54	3.97	3.12	11.52	7.70	102.05	21.27

b) Amblycera

Species	Sample size		Prevalence		Abundance		Intensity		Aggregation	
	UK	NZ	UK	NZ	UK	NZ	UK	NZ	UK	NZ
Carduelis chloris	61	49	3.28	20.41	0.07	1.80	2.00	8.80	2.48	20.60
Passer Domesticus	47	89	0.00	28.09	0.00	1.27	---	4.52	---	6.92
Prunella modularis	61	61	0.00	8.20	0.00	0.21	---	2.60	---	5.34
Fringella coelebs	35	50	5.71	2.00	0.09	0.02	1.50	1.00	1.63	1.00
Emberiza citrinella	5	21	0.00	0.00	0.00	0.00	---	---	---	---
Turdus philomelos	31	30	12.90	13.33	0.39	1.70	3.00	12.75	3.56	26.70
Carduelis carduelis	59	86	6.78	4.65	0.17	0.50	2.50	10.75	3.29	27.89
Carduelis flammea	16	17	6.25	0.00	0.06	0.00	1.00	---	1.00	---
Sturnus vulgaris	13	9	61.54	33.33	3.54	2.11	5.75	6.33	7.94	9.17
Turdus merula	62	74	24.59	37.84	3.79	2.99	15.40	7.89	107.10	22.27

c) Ischnocera

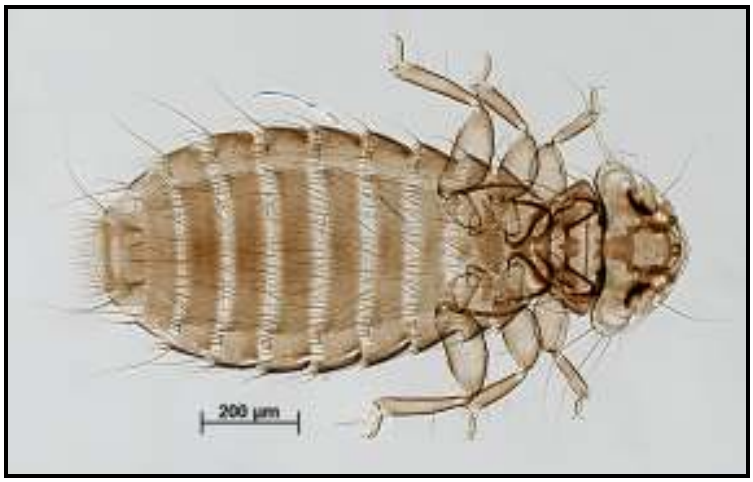
Species	Sample size		Prevalence		Abundance		Intensity		Aggregation	
	UK	NZ	UK	NZ	UK	NZ	UK	NZ	UK	NZ
Carduelis chloris	61	49	0.00	4.08	0.00	0.04	---	1.00	---	0.98
Passer Domesticus	47	89	17.02	24.72	0.38	1.06	2.25	4.27	3.58	12.29
Prunella modularis	61	61	0.00	6.56	0.00	0.10	---	1.50	---	1.59
Fringella coelebs	35	50	0.00	0.00	0.00	0.00	---	---	---	---
Emberiza citrinella	5	21	0.00	9.52	0.00	0.10	---	1.00	---	0.95
Turdus philomelos	31	30	6.45	16.67	0.06	0.37	1.00	2.20	0.97	3.66
Carduelis carduelis	59	86	0.00	0.00	0.00	0.00	---	---	---	---
Carduelis flammea	16	17	12.50	0.00	0.13	0.00	1.00	---	0.93	---
Sturnus vulgaris	13	9	7.69	0.00	0.08	0.00	1.00	---	1.00	---
Turdus merula	62	74	9.84	4.05	0.11	0.11	1.17	2.67	1.19	2.68

FIGURES

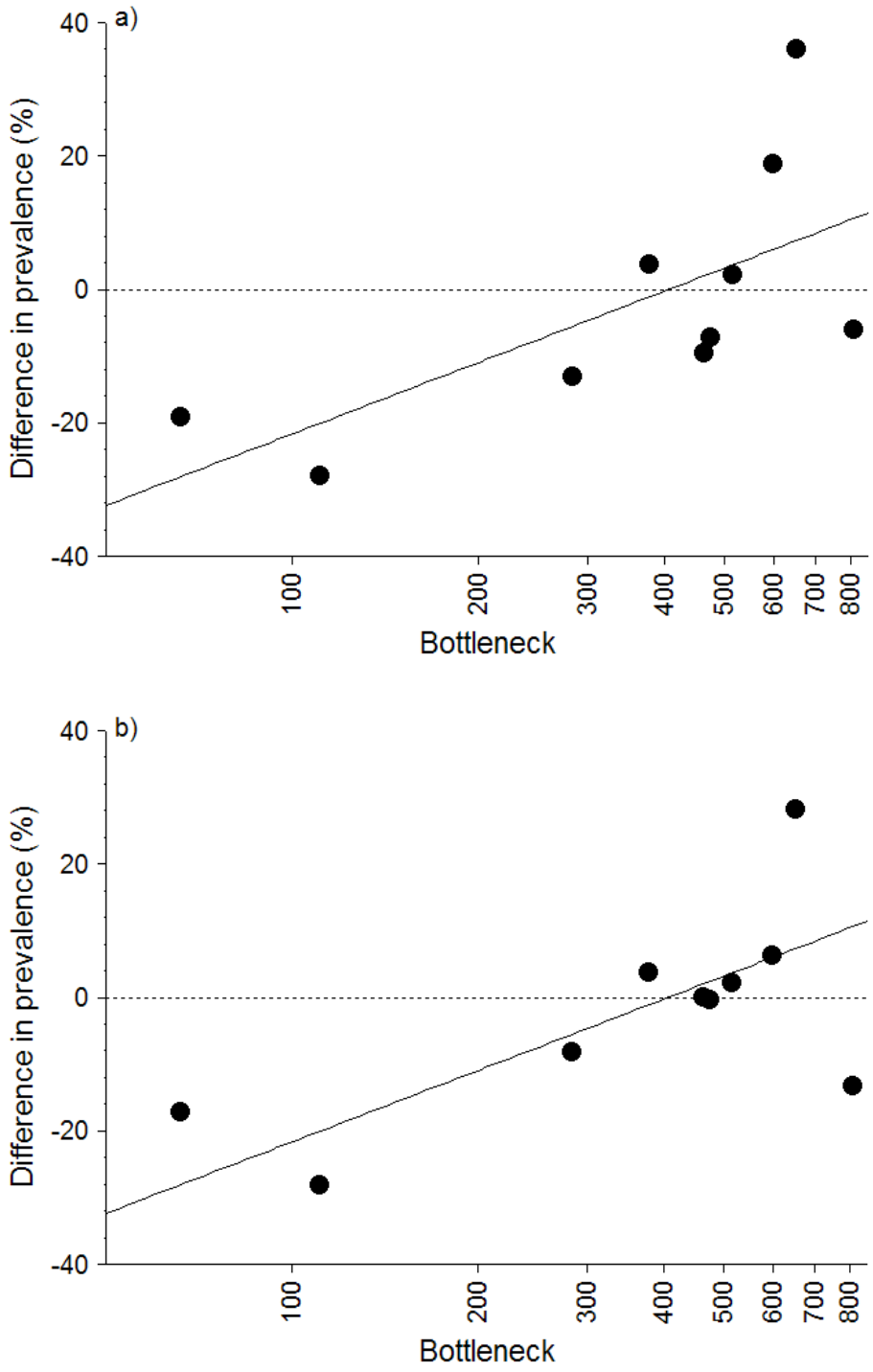
Figure 1.. Ectoparasite collection and an example of a chewing louse.

a) Goldfinch (*Carduelis carduelis*) being 'dust-ruffled' over a collecting tray to obtain ectoparasite samples.

b) *Menacanthus eurysternus*[a] (Phthiraptera: Amblycera: Menoponidae), a commonly found ectoparasite.



[a] Voucher specimen (T.Galloway, University of Manitoba, Canada) collected from a starling (*Sturnus vulgaris*) in Canada.


a)
40
20
0
-20
-40
Difference in prevalence (%)
100
200
300
400
500
600
700
800
Bottleneck
b)
40
20
0
-20
-40
Difference in prevalence (%)
100
200
300
400
500
600
700
800
Bottleneck

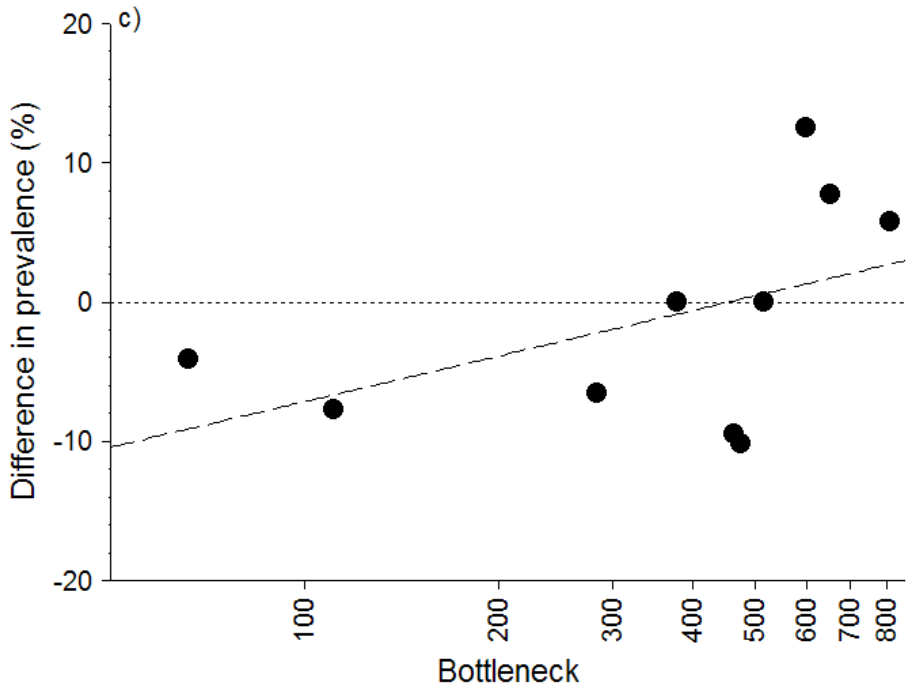
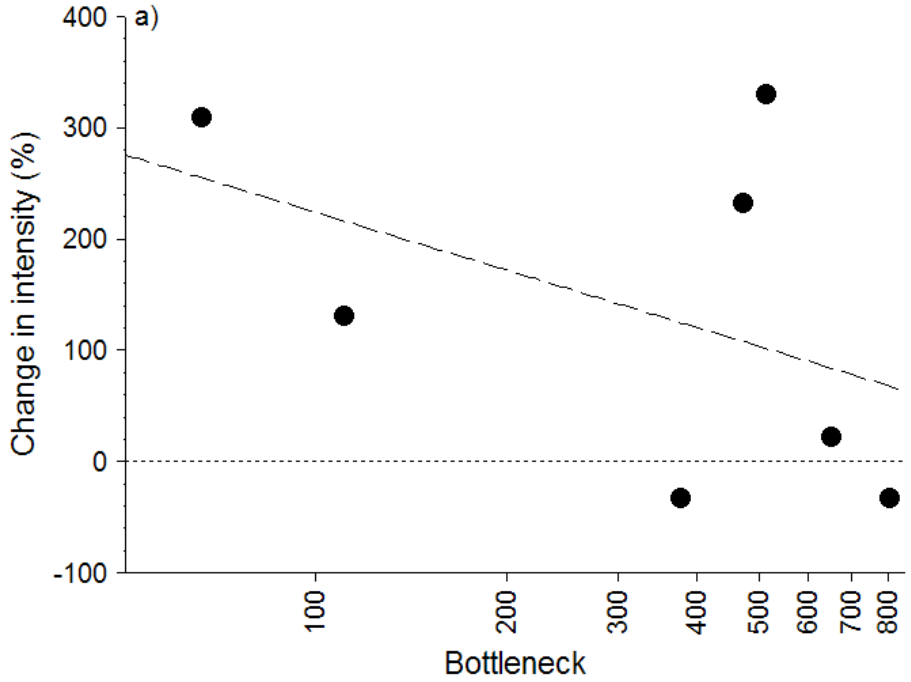


Figure 2. Difference in prevalence between the UK and New Zealand (UK-NZ) in relation to the population bottleneck (log scale) the NZ birds experienced of a) Phthiraptera, b) Amblycera and c) Ischnocera. Solid lines indicate a significant linear regression, dashed indicate non-significant.



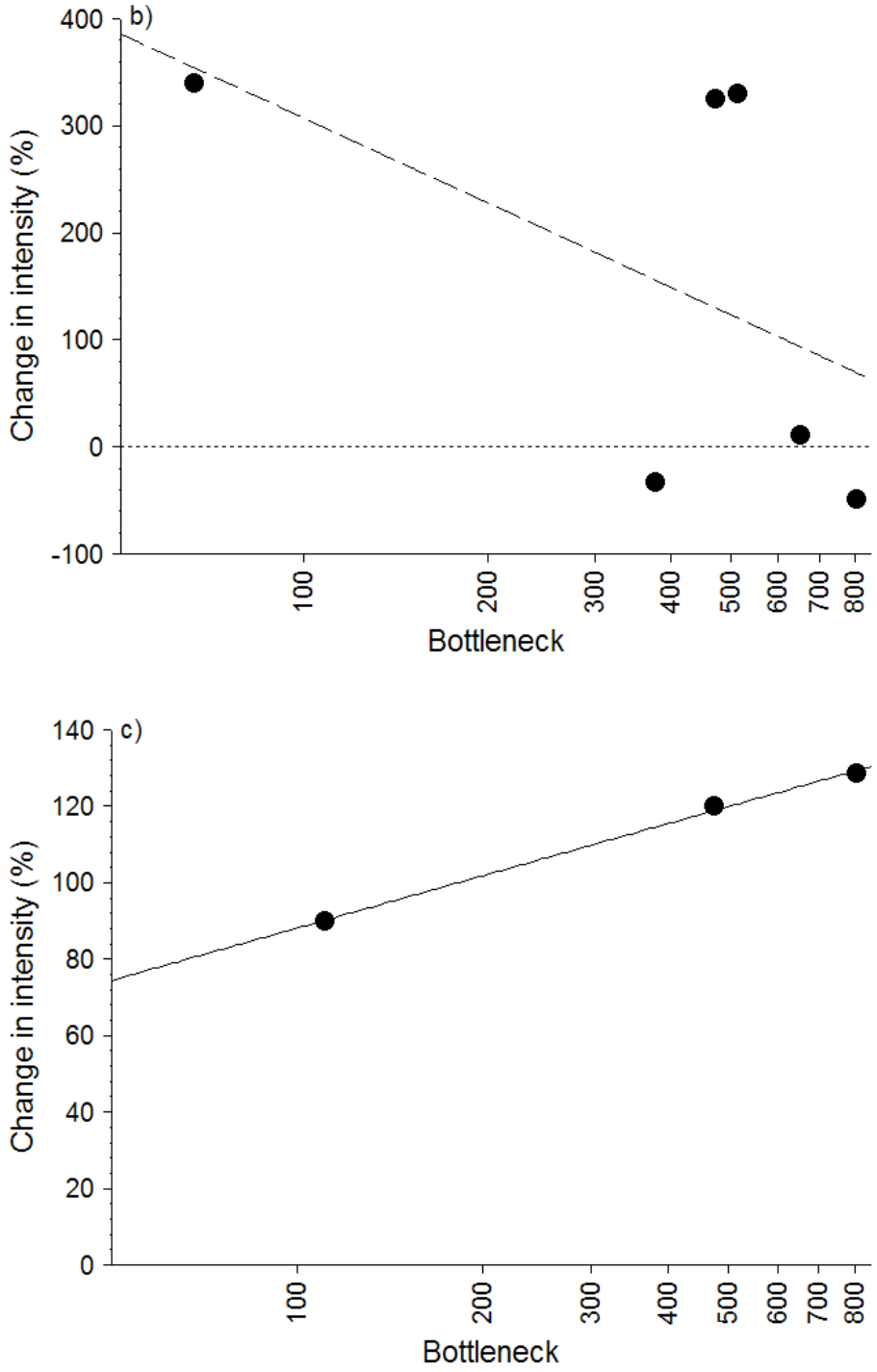
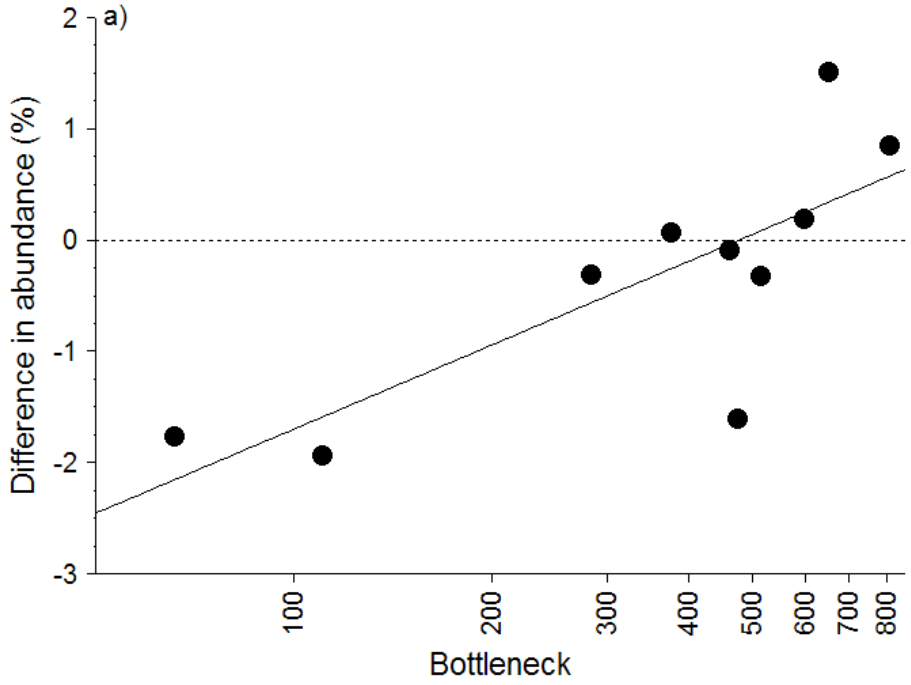
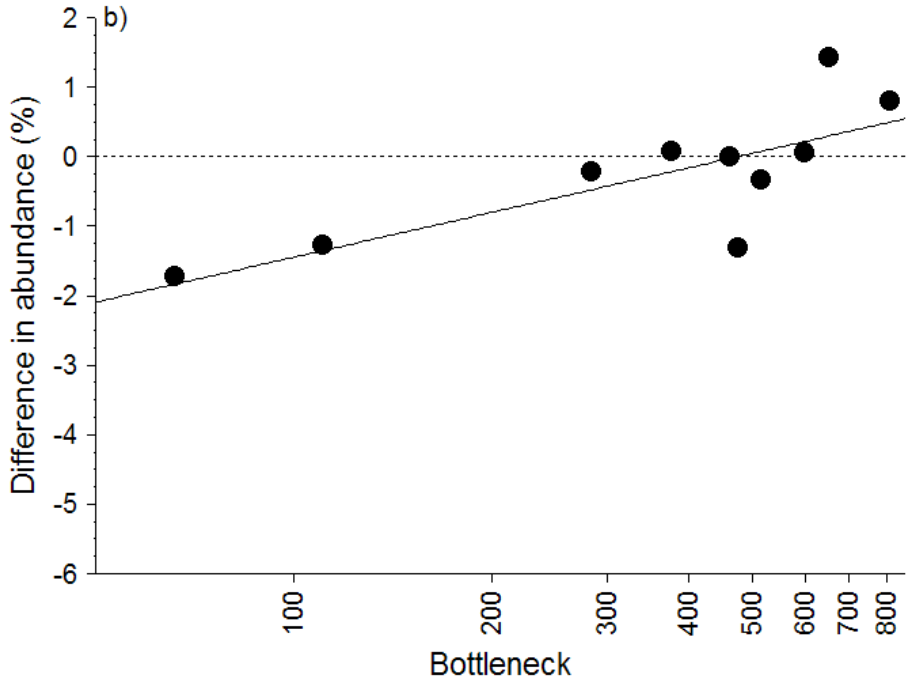


Figure 3. Difference in mean intensities between the UK and New Zealand populations (UK-NZ) in relation to the population bottleneck (log scale) NZ birds experienced of a) Phthiraptera, b) Amblycera and c) Ischnocera. Solid lines indicate a significant linear regression, dashed indicate non-significant.


a)
Difference in abundance (%)
2
1
0
-1
-2
-3
100
200
300
400
500
600
700
800
Bottleneck


b)
Difference in abundance (%)
2
1
0
-1
-2
-3
-4
-5
-6
100
200
300
400
500
600
700
800
Bottleneck

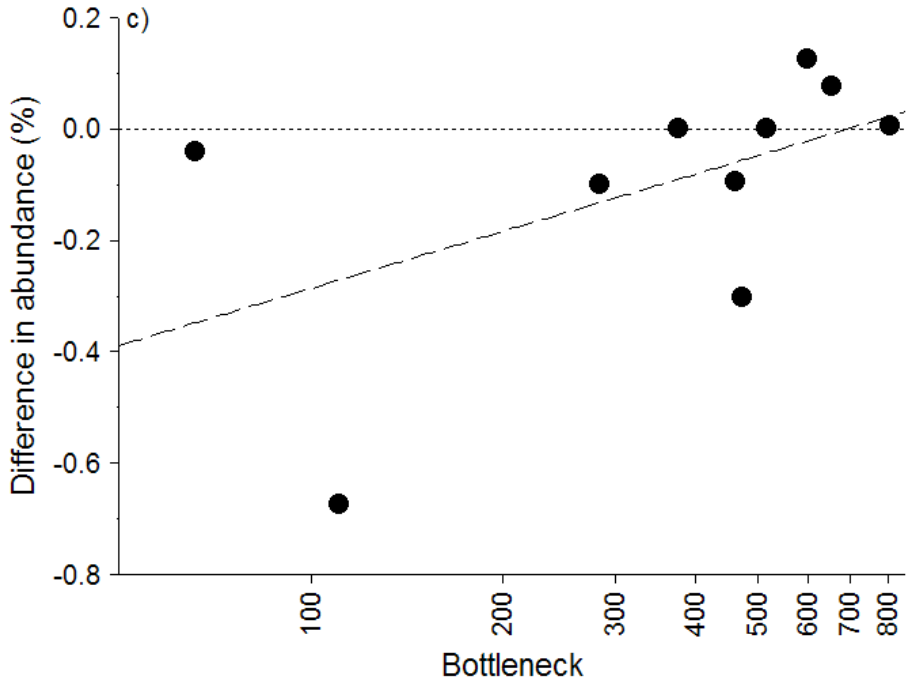


Figure 4. Difference in mean abundance between the UK and New Zealand populations (UK-NZ) in relation to the population bottleneck (log scale) NZ birds experienced of a) Phthiraptera, b) Amblycera and c) Ischnocera. Solid lines indicate a significant linear regression, dashed indicate non-significant.

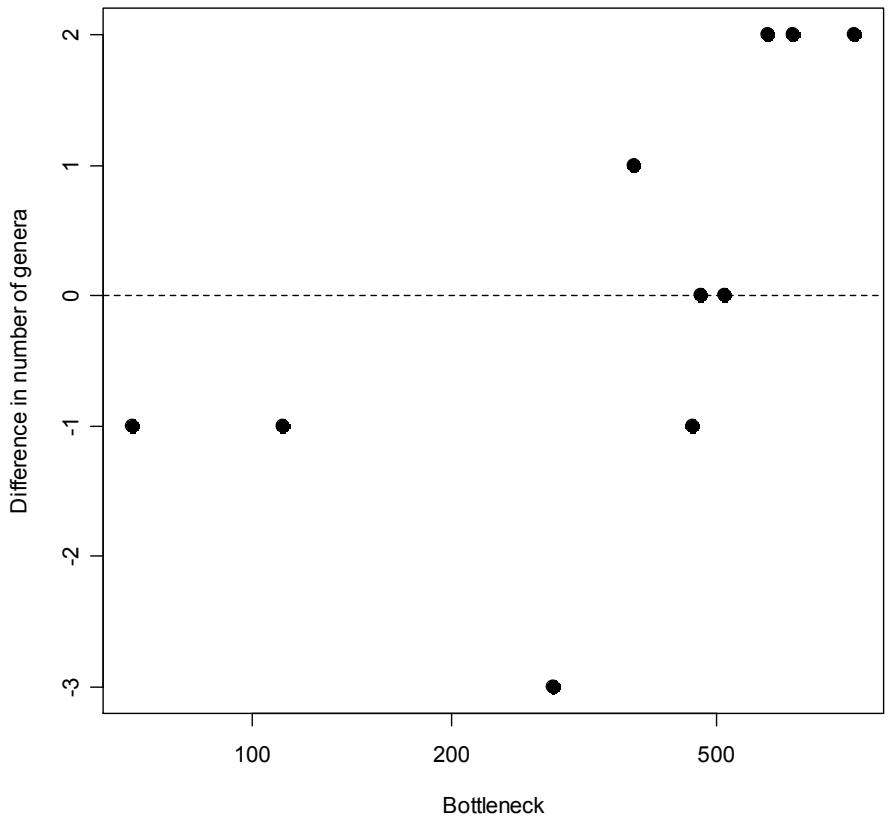


Figure 5. Difference in number of Phthiraptera Genera between UK and NZ populations in relation to population bottleneck (log scale).

CHAPTER 2

THE INFLUENCE OF POPULATION BOTTLENECK SIZE ON IMMUNOCOMPETENCE AND INFECTION BY AVIAN MALARIA IN INTRODUCED BIRDS OF NEW ZEALAND

ABSTRACT

Population bottlenecks may lead to a reduction in genetic diversity and an increase in inbreeding, which together can result in a decrease in fitness. One consequence of population bottlenecks may be a reduction in immunocompetence and an associated increase in susceptibly to parasites and pathogens. This is of conservation significance, as bottlenecks are frequently encountered in species under conservation management and disease has been implicated in the demise of a number of endangered species. In this study, I investigated haematological responses and prevalence of avian malaria (*Plasmodium & Haemoproteus spp.*) in introduced bird species of New Zealand that experienced a range of bottleneck sizes during their establishment. I found that heterophil/lymphocyte ratio (a measure of chronic stress or current infectious disease) was significantly higher in species that had experienced severe bottlenecks, when compared to their non-bottlenecked conspecifics in the United Kingdom. Prevalence of avian malaria was negatively correlated with bottleneck size (species experiencing more severe bottlenecks had higher prevalence) within New Zealand. Furthermore, less severely bottlenecked species exhibited elevated lymphocyte and total leucocyte counts in response to malarial infection, whilst more severely bottlenecked species did not. Overall, these results suggest that population bottlenecks may be linked to a decrease in immunocompetence and increased susceptibility to malaria. The implications for conservation are discussed, and recommendation for future study made.

INTRODUCTION

POPULATION BOTTLENECKS & CONSERVATION RELEVANCE

Parasites and pathogens have been implicated in the population decline of a number of endangered species (Dobson and McCallum, 1997) and emerging infectious diseases are an acknowledged conservation issue (Dobson and Foufopoulos, 2001; Wikelski et al., 2004). Any conservation project, therefore, must consider the immunological health of a population and guard against increased susceptibility to disease.

Population bottlenecks are commonplace in conservation, occurring whenever a population experiences a significant reduction in size. Whenever a captive breeding programme is implemented or when individuals of a threatened species are translocated to found a new population, a bottleneck occurs. The reduction of populations to small numbers through such bottlenecks is hypothesized to cause reductions in genetic diversity, both in terms of decreased allelic diversity, and decreased heterozygosity as a result of inbreeding (England et al., 2003; Keller and Waller, 2002). These predicted genetic effects have now been demonstrated in a number of wild populations (Ardern et al., 1997; Bodkin et al., 1999; Bouzat et al., 1998; Bradshaw et al., 2007; Hajji et al., 2007; Hoelzel, 1999; Hudson et al., 2000; Keller et al., 2001; Nyström et al., 2006; Packer et al., 1991; Zhang et al., 2004). Furthermore, a decrease in genetic diversity has been correlated with a reduction in fitness (Reed and Frankham, 2003), and increased extinction risk (Frankham, 2005).

Whilst it is becoming clear that population bottlenecks, via inbreeding and genetic impoverishment, may have severe consequences to a population's fitness, and ultimately to its survival, the question of relevance for conservation managers is how are these fitness costs manifested, and at what severity of bottleneck?

Evidence is mounting that decreased genetic diversity renders individuals more prone to disease and parasitic infection (Arkush et al., 2002; Hawley et al., 2005; Hedrick et al., 2001; O'Brien and Evermann, 1988; Pearman and Garner, 2005). For example, a recent study by MacDougall-Shackleton et al. (2005) found that increased heterozygosity decreased the individual risk of infection by avian malaria (*Haemproteus* spp.) in an out-bred population of white-crowned sparrows (*Zonotrichia leucophrys oriantha*).

A decrease in immunocompetence has also been linked to reduced genetic diversity (Hawley et al., 2005; Sanjayan et al., 1996), and inbreeding has been shown to have detrimental effects on immune function. For example, work on an insular, pedigreed population of song sparrows (*Melospiza melodia*) found that more inbred individuals had lower immune responses (Reid et al., 2003).

Despite the inference that population bottlenecks may render a population more prone to disease, due to the associated reduction in genetic diversity and increased inbreeding, few studies have explicitly examined this prediction. In a recent study, Hale and Briskie (2007) compared a severely bottlenecked population (founded by 5 individuals) of the endemic New Zealand robin (*Petroica australis*) with its source population and found that birds in the bottlenecked population were immunocompromised. However, little other work has been done on this subject and research on the interplay between genetic diversity and disease resistance in bottlenecked

populations is urgently required. For conservation biologists, questions of most urgent relevance are whether population bottlenecks decrease immunocompetence and increase susceptibility to disease and, if so, at what size bottlenecks are these fitness problems manifested?

INTRODUCED BIRDS AS STUDY SYSTEM

Introduced species offer unique opportunities for a study of pre- and post-bottlenecked populations of the same species (Briskie, 2006), and the introduced bird species of New Zealand are a good example. In the late 1800's Acclimatisation Societies introduced numerous bird species (mainly from the United Kingdom – UK) to New Zealand (Lever, 1987; Thompson, 1922), a number of which are successfully established today. The number introduced was carefully recorded and varied per species, and so introduced birds in New Zealand today are represented by populations that have experienced a range of bottlenecks. The elegance of this system is that it allows both interspecific comparisons across a range of bottlenecks, and intraspecifc comparisons between pre- and post- bottlenecked populations (i.e., between the U.K. & New Zealand). This enables investigations to be made, not only of how bottlenecks may affect fitness traits, but additionally, at what size bottleneck these fitness effects cease to be evident (or at least of lesser concern) in comparison to the source population.

Briskie & Mackintosh (2004) exploited this system to examine reproductive success in pre- and post- bottlenecked populations, and found that hatching success was significantly lower in populations that had been founded by less than 150 individuals; hatching success was only equal to pre- bottleneck populations in species founded by

more than 600 individuals. Whether other fitness traits, such as the functioning of the immune system and/or susceptibility to disease, might be similarly affected is unknown.

BLOOD PARAMETERS AS AN INDICATOR OF IMMUNITY AND HEALTH

There are a number of options open to ecologists wishing to assess the immunocompetence of an avian population (Norris and Evans, 2000; Salvante, 2006, and see Chapters 1 & 3). One such method is the identification and quantification of circulating leucocytes (white blood cells) in the peripheral blood (Ots et al., 1998). Leucocytes form the basis of both specific and non-specific immunity, as their main function is offering protection against pathogenic factors (Fairbrother et al., 2004). Hence the creation of a leucocyte profile, offers a snapshot of a bird's current immunological status.

Elevated leucocyte number (termed leucocytosis) is often associated with inflammatory diseases, stress, and trauma (Campbell, 1995), and is indicated by a raised total white blood cell count (WBC). Avian leucocytes fall into 6 classes – lymphocytes, heterophils, eosinophils, basophils, monocytes, and thrombocytes – and the proportional representation in the WBC of these leucocytes (termed leucocyte differential), particularly the heterophil and lymphocyte counts, may be indicative of the immunological challenge an individual is facing (Campbell, 1995). In addition, an index of the relative abundance of heterophils to lymphocytes – the heterophil/lymphocyte ratio (HL ratio) – has been demonstrated to be a reliable stress indicator in poultry (Maxwell, 1993), increasing in response to a number of environmental and immunological stressors. Static immunological monitoring techniques, such as leucocyte profiles, can be difficult to interpret (Norris and Evans, 2000). For example, raised lymphocyte numbers may

represent either a strong base-line immunity or the presence of an infection. However, assessing leucocyte profiles in tandem to assessing the prevalence of naturally occurring infections such as avian malaria can be informative (Ricklefs and Sheldon, 2007).

AVIAN MALARIA – PATHOGENICITY AND FITNESS CONSEQUENCES

Avian haemosporidian parasites (phylum: Apicomplexa, including *Plasmodium*, *Haemoproteus* and *Leucocytozoon* species) are protozoan blood parasites that include the avian malarial parasites. These have been well studied because of their use as models for human malaria (Valkiunas, 2005), and their well-documented disastrous effect on the Hawaiian avifauna (e.g., van Riper et al. 1986). Whilst traditionally only *Plasmodium* species were considered to be malarial parasites, recent phylogenetic developments suggest that *Haemoproteus* species should be included in this category (Perez-Tris et al., 2005), and hereafter avian malaria will refer to both genera.

Avian malarial infections are characterised by a having high prevalence (Valkiunas, 2005) and, whilst their pathogenicity varies, they have been clearly demonstrated to induce fitness costs in the host including lowered reproduction and survival (Dawson and Bortolotti, 2000; Marzal et al., 2005; Merino et al., 2000; Sanz et al., 2001) and increased predation (Møller and Nielsen, 2007). More dramatically, avian malaria (of the species *Plasmodium relictum*) has been responsible for the widespread decline of the endemic birds of Hawaii (van Riper et al., 1986). Furthermore, previous work has demonstrated that malarial infections cause activation of the immune system, including elevated WBC and lymphocytes counts (Atkinson et al., 2001; Figuerola et al., 1999; Ots and Hõrak, 1998; Ricklefs and Sheldon, 2007), and recent studies have

suggested a genetic component to malarial resistance (Bonneaud et al., 2006; Westerdahl et al., 2005).

Tompkins & Gleeson (2006), in a recent survey of avian malaria distribution in the introduced birds of New Zealand, found evidence of infection in all six species they sampled. They suggested that avian malaria in New Zealand could constitute an emerging infectious disease, and may be linked to recent spread of the exotic mosquito vector *Culex quinquefasciatus* (the same vector that was introduced into Hawaii). Malarial infection in the introduced birds of New Zealand offers an ideal study system to investigate the effects of population bottlenecks on disease susceptibility, whilst simultaneously monitoring the spread of this potential disease threat.

STUDY AIMS

The aims of this study were two fold; first to use haematological parameters to gain an insight into the immunocompetence of species that had experienced a range of bottlenecks, and second to examine the prevalence of avian malaria in these same species to determine if the haematological responses of infected individuals were affected by the bottleneck the species experienced.

The immunological status of the birds was assessed by creation of leucocyte profiles for six species of birds in New Zealand and compared to that of their non-bottlenecked counterparts in the UK. The prediction I tested was that more severely bottlenecked species (in New Zealand) exhibit lower immunological defences than compared to their non-bottlenecked conspecifics (in the UK). I also assessed the prevalence of avian malaria of eleven introduced species within New Zealand, and compared the haematological response to infection in each species. I expected species

that have experienced severe bottlenecks to exhibit a higher prevalence of malaria, and to be less immunologically able to respond to that infection.

METHODS

STUDY SPECIES & GENERAL METHODOLOGY

In total eleven species were studied in New Zealand (NZ), and six in the UK (see Tables 1-3 for details and sample sizes). All species are passerines that were successfully introduced into New Zealand in the late 1800's and are still extant today in their native range in the UK. The number of individuals of each species released in New Zealand (i.e., the introduction effort) was carefully recorded by the Acclimatisation Societies responsible for their introduction (Lever, 1987; Thompson, 1922). I used introduction effort, calculated as the total number released for each species, excluding any introductions that were specifically recorded as being unsuccessful, as a surrogate for population bottleneck size. Introduction effort was different for each species, and hence corresponds to a range of bottlenecks (see Table 2).

Birds were caught by passive mist-netting or trapping in four locations in the South Island of New Zealand (Lincoln, Kaikoura, Ward, Blenheim, 43°38' S 172°28'E, 42°23' S 173°37'E, 41°48' S 174°06' E, 41°28' S 173° 57' E, respectively), and in the UK at a single site in south-east England (Rye Bay Wetlands Trust Reserve, Icklesham, East Sussex, 50°54' N 0°41' E). All of my sampling was conducted in the autumn months for each hemisphere (Southern hemisphere: March-May 2006, Northern hemisphere: September-November 2005) to control for any seasonal effects. On capture, birds were

fitted with a metal identification ring, had an age and sex assigned according to plumage characteristics (Svensson, 1992), and basic biometrics taken.

Blood samples were collected by brachial veni-puncture. The left wing was extended and the brachial vein swabbed with alcohol to sterilise and expose the area, the vein was punctured by a sterile needle (27½ gauge), and no more than 100 µl of blood was collected passively in a micro-capillary tube. A drop of blood was used to make a blood slide, and the rest was stored in 1 ml of Queen's lysis buffer (Seutin et al., 1991) until molecular analysis. At the end of blood collection a clean swab was held against the puncture wound to ensure complete coagulation. Previous studies have demonstrated that blood sampling at this volume has no detrimental effects to birds (Ardern et al., 1994; Hoysak and Weatherhead, 1991; Lubjuhn et al., 1998).

BLOOD COUNTS

Blood slides were made to enable identification and quantification of leucocytes (white blood cells). A drop of blood was smeared on a microscope slide, air dried and subsequently fixed in absolute methanol and stained using a modified May–Grunwald Giemsa staining method (Lucas and Jamoz, 1961). Slides were examined to assess the total white blood cell count (hereafter WBC), and the proportion of different types of leucocyte. An area of the slide with an evenly distributed monolayer of cells was selected and estimates of WBC were obtained by counting all leucocytes in 10 fields of view under 400 x magnification, which approximates a total of 10 000 erythrocytes (S.E. Allen unpubl. data). The average of the 10 fields was then calculated, and hence WBC is expressed as total leucocyte count per 1000 erythrocytes. The proportion of different leucocytes was assessed by examining 100 consecutive leucocytes under 1000 x

magnification with oil immersion, and identifying them as either lymphocytes, heterophils, eosinophils, basophils, or monocytes (according to Campbell (1995)). Differential leucocyte counts of the two most common cells (lymphocytes and heterophils) were obtained by multiplying these proportions with WBC (Ots and Hõrak, 1998). In both the WBC and the differential counts, thrombocytes (a further type of avian leucocyte) were excluded, as they play a significant role in haemostasis, and hence tend to clump making counting difficult (Campbell, 1995). An increase in the number of circulating heterophils and a decrease in lymphocytes is a general avian stress response (Maxwell, 1993), which is sensitive to a variety of stressors including infectious disease and psychological disturbance (Ots et al., 1998). Hence the heterophil/lymphocyte (H:L) ratio was calculated as a measure of this.

The New Zealand slides were analysed by the same person (S.E. Allen), whilst a sub-sample of the UK slides were analysed by a different observer (K. Hale). A number of slides (n = 14) were analysed by both observers and repeatabilities (Lessells and Boag, 1987) were found to be acceptable (r^2 = 0.76, 0.76, 0.83 & 0.59, for WBC, HL, lymphocyte and heterophils respectively, all $P < 0.001$).

DETECTION AND IDENTIFICATION OF HAEMATOZOA

A polymerase chain reaction (PCR) assay, that detects both avian *Plasmodium* spp. and *Haemoproteus* spp., was employed to assess haematozoan infection. DNA from blood samples was extracted using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol. PCR was used to amplify a c. 355 base pair fragment of the mitochondrial cytochrome *b* gene, using specific malarial parasite primers. Primers and PCR protocols followed Massey et al (2007)

A sub-sample of 20 PCR products, positive for the blood parasite PCR marker and chosen to span the range of bird species, were sequenced to allow parasite identification. The samples were aligned to allow identification of unique haplotypes and these haplotypes were then identified using the BLAST search algorithm available on the NCBI GenBank nucleotide database.

STATISTICAL ANALYSES

The effect of population bottlenecks on blood parameters (WBC, differential counts and HL ratio) and malarial infection was investigated in a number of ways, as described below.

Intraspecifc comparison between UK and NZ.

To investigate whether the past bottleneck a population experienced affects current blood parameters an intraspecific comparison was conducted. The effect of country, representing a comparison between a pre-bottleneck population (UK) and a post bottleneck population (NZ), on each blood parameter was examined using ANOVAs. Country was included as the explanatory variable, sex and age were included as covariates, and the response variable (the blood parameter) was transformed according to Table 1.

Intraspecifc differences with relation to population bottleneck

To investigate whether differences in blood parameter values between the two countries (i.e., populations pre- and post bottleneck) was influenced by the severity of the bottleneck, the relative change in each blood parameter (log (mean NZ parameter/ mean UK parameter)) per species was regressed against bottleneck size (log transformed).

Malarial prevalence in relation to population bottleneck.

The relationship between malarial infection and population bottleneck size within New Zealand was investigated using a generalized linear mixed effect model. A binomial error structure and a logit link was specified, as the response variable (malaria) was binary (infected or not infected). The maximal model included log (bottleneck), log (body mass), sex and age as main effects, and species nested within year and location as random effects. The random effects in this instance control for any consistent year (2005 & 2006), species (as detailed) and geographic (4 locations) differences in the prevalence of malarial infection. The model was simplified by backwards elimination of variables, based on AIC scores.

Interspecific comparison within New Zealand of blood parameters and malarial infection

To investigate if the past bottleneck a population experienced affects haematological responses to malaria, interspecific comparisons were conducted within New Zealand on 9 species. General linear mixed effects models (GLMMs) were fitted (for an explanation of GLMMs please refer to Chapter 3), for WBC, HL ratio and differential lymphocyte and heterophil counts on the data set of birds that had been assayed for malarial infection (for sample sizes see Table 3). The maximal model contained bottleneck (log transformed) and malaria (2 levels – Yes or No) as the response variables. Sex (fitted as 3 levels – Male, Female and Unknown), age (3 levels – After Hatch Year, Hatch Year, and Unknown), and mass (mean per species, log transformed) were fitted as covariates and species was specified as a random effect. First and second order interaction terms between bottleneck, malaria and mass were also included. In each

case a maximal model was fitted and explanatory variables were removed one at a time, using a stepwise deletion method, based on Akaike information criteria (AIC). The backwards elimination of variables continued until a minimum adequate model was obtained.

Each model was initially fitted to a data set excluding birds assigned to the 'Unknown' sex category, as this category was confounded by species (the sex of dunnocks and song thrushes cannot be assigned in the autumn season). Sex was not retained in any of the minimum adequate models (MAM), and so the analysis was repeated on the full data set, including birds of unknown sex but excluding sex as a variable. All statistics were carried out using R v2.6.2 (R Development Core Team, 2008).

Any comparative analysis has the potential to be confounded by phylogenetic effects. I used the software package "CAIC" (Comparative Analyses by Independent Contrast) to control for phylogeny in my analyses (Purvis and Rambaut 1994). Note that at present it is not possible to control phylogenetic effects in the GLMM I used to assess the interactions between variables and thus I only present the results of phylogenetic analyses examining one variable at a time.

RESULTS

Intraspecifc comparison of blood parameters between UK and NZ.

Single species ANOVAs were conducted, comparing total WBC, HL ratio and differential lymphocyte and heterophil counts between the two countries. Age and Sex were included as covariates (sex was not determined for dunnocks or song thrushes, and

hence was not included for these species; Table 1). Greenfinches, the species that experienced the most severe bottleneck in this comparison (a bottleneck of 66 birds), exhibited significant differences (at $P < 0.05$) between countries in all blood parameters. New Zealand greenfinches had lower WBC and differential lymphocyte counts, and higher heterophil and HL ratios than UK birds. House sparrows and dunnocks (the second two most bottlenecked species), also exhibited significant differences in the same direction as greenfinches, in some of the parameters (see Table 1 and Figure 1), whilst song thrushes exhibited differences between the two countries in WBC and lymphocyte counts, but in the opposite direction (i.e., higher WBC and lymphocytes counts in NZ).

Intraspecifc differences in blood parameters with relation to population bottleneck size

A linear regression of change in HL ratio (log (NZ HL/UK HL)) against population bottleneck size (log transformed) demonstrated a significant negative relationship ($r^2 = 0.68$, $F_{1,4} = 8.3$, $P < 0.05$). This indicates that decreases in HL ratio between the two countries (pre- and post- bottleneck populations) were greater in more severely bottlenecked species (Figure 2). This result remained significant when controlled for possible phylogenetic constraints ($P < 0.04$). Linear regressions between the differences in other blood parameters and population bottleneck size were all non-significant and remained non-significant when controlled for phylogeny (all $P > 0.05$).

Prevalence and identification of avian malaria.

In total, 516 blood samples were tested for the presence of avian malaria in New Zealand, from 11 species. All 11 species were found to be infected with malaria, but the

prevalence ranged considerably from 9% (in redpolls) to 95% (in song thrushes). The prevalence results and samples sizes for each species are summarized in Table 2

Of the subsample of 20 PCR products that were sequenced (to allow identification of the parasite), 16 were of a quality to analyse. The sequences were aligned, and 4 potentially unique haplotypes were distinguished. These 4 haplotypes were compared to sequences deposited in NCBI GenBank (using the BLAST search algorithm). The PCR assay utilised primers sensitive to both *Haemoproteus* and *Plasmodium* spp., however all 16 subsamples sequenced were identified as *Plasmodium* spp.

Malarial prevalence in relation to population bottleneck size.

Bottleneck size (log transformed) and body mass (log transformed) were the only two variables retained in the final model. Prevalence of avian malaria decreased significantly with bottleneck size ($P < 0.05$), indicating populations that had experienced more severe bottlenecks had a higher prevalence of malaria than less severely bottlenecked species (Figure 3). This result remained significant after controlling for possible phylogenetic effects ($P = 0.041$). Malarial prevalence exhibited a positive relationship with body mass ($P < 0.001$), indicating that species of larger body mass were more likely to be infected by malaria. This analysis was repeated excluding cirl buntings, as they experienced the most severe bottleneck (11 birds), and exhibited very high prevalence (67%). The effect of bottleneck became non-significant on removal of this species, suggesting that they were driving this trend.

Interspecific comparison within New Zealand of blood parameters and malarial infection

The resulting minimum adequate models from GLMMs for WBC, HL ratio and lymphocyte counts are summarised in Table 4. The model for differential heterophil counts is not shown as none of the variables were retained in the final model.

The haematological response of birds infected by avian malaria only differed from their non-infected conspecifics in less bottlenecked species. Essentially, individuals from species that had experienced more severe bottlenecks did not appear to increase their WBC or lymphocyte count in response to infection by malarial parasites, whilst the less bottlenecked species did.

In the WBC model a positive interaction between bottleneck size and malaria tended towards significance ($P = 0.055$), whilst the main effects of malaria and bottleneck were non significant ($P > 0.1$), indicating that at severe bottlenecks the WBC count did not differ between infected and non-infected individuals, whilst at more moderate bottlenecks, infected individuals exhibited higher WBC (see Figure 4).

In the model investigating the response of HL ratio to malarial infection, a number of variables were retained in the final model as they improved the overall fit (see Table 4). Malarial infection status had a significant effect ($P = 0.03$), although somewhat counter-intuitively this suggested that birds infected with malaria had a lower HL ratio than non-infected birds. The interaction term between malaria status and bottleneck was not retained, indicating that the difference in HL ratio between infected and non-infected birds remained constant across the range of bottlenecks.

The minimum adequate model explaining the response of differential lymphocyte counts between infected and non-infected individuals is detailed in Table 4. The interaction term between malarial infection status and bottleneck size (log transformed) is significant ($P = 0.03$) and positive, indicating that difference in lymphocyte count between infected and non-infected individuals increases at larger bottlenecks (see Figure 5). The other terms retained in the final model improved model fit, but were not significant.

The final model for differential heterophil count, was a null model, indicating that none of the variables, including malarial status and bottleneck had a significant effect.

These analyses were repeated on a data set excluding cirl buntings (as they were found to be highly influential when investigating malarial prevalence). The exclusion of this species did not change the significance of any of the terms in the models. Note that I was unable to control for possible phylogenetic effects in these models as the statistical methods to do so are not available and the results should be interpreted with this possible confounding factor in mind.

DISCUSSION

Overall, I found that the size of a population bottleneck experienced by European species during their establishment in New Zealand had a significant effect on haematological responses, general stress responses and prevalence of avian malaria.

The single species comparisons suggest that individuals from the more severely bottlenecked species in NZ are less immunocompetent than individuals from their source populations in the UK. The most bottlenecked species in the analysis (the greenfinch, founded by 66 individuals) was significantly different in all categories. Total white blood

cell count (WBC) and lymphocyte differential was significantly lower in greenfinches in New Zealand, and the heterophil count and HL ratio significantly higher. HL ratio was also higher in house sparrows and dunnocks, (the next two most bottlenecked species founded by 111 and 284 birds respectively) but was driven by different processes (elevated heterophils in house sparrows and depressed lymphocytes in dunnocks). The least bottlenecked species (the blackbird) exhibited no differences in any of the categories. The song thrush (which experienced a moderate bottleneck of 474 birds) exhibited higher WBC and lymphocytes counts in NZ, however this species exhibited the highest prevalence of avian malaria (20 out of 21 birds), and so may be under exceptional immunological stress.

Interpretation of white blood cell counts can be problematic without knowledge of the individual's disease and parasite status; at a population level the same is true. In the case of greenfinches and dunnocks for example, whilst lowered lymphocytes in the New Zealand population may be indicative of less robust immunity, it may also be that the populations in the UK were experiencing an immunological challenge that had activated the immune system, and hence elevated WBC and lymphocyte levels.

However, the inference that more bottlenecked species are less immunocompetent than their UK conspecifics was strengthened by the finding that the difference in HL ratio between the countries (pre- & post- bottleneck) decreased as bottlenecks got bigger (i.e., less severe), and was highest for the more bottlenecked species in NZ. HL ratio has been demonstrated to be a reliable indicator of stress in poultry and wild birds (Maxwell, 1993). It would appear that, when compared to their source populations, more severely

bottlenecked species are under higher stress than species founded by more individuals (less bottlenecked).

A variety of environmental, psychological, and immunological stressors can cause a raised HL, and it has been demonstrated to be negatively correlated with survival (Kilgas et al., 2006). Whilst this study could not assess the variety of stressors that cause a raised HL ratio, the design of the study sought to minimise any differences. Handling birds causes a stress reaction (Maxwell, 1993) birds in both countries were caught in the same fashion (mist-nets or walk in traps) and the blood sampling protocol was identical and carried out by the same person (S.E. Allen). However, in the UK birds were caught at a large, permanent ringing site (with several hundred metres of mist nets, and a number of volunteers), whilst the NZ birds were caught at smaller field-sites (with generally only 2 people present). The expectation then, would be that the UK birds, experiencing increased handling times and higher noise levels would, if anything, experience greater handling stress. Other environmental stresses are very difficult to assess; however in general it would seem that New Zealand offers a more benign environment for these bird species than the UK, (e.g., lower predation pressures and greater food availability) (MacLeod et al., 2008). This leads to the tentative conclusion that the elevated HL ratios found in the more bottlenecked species may be related to immunological stress and not some other confounding variable. This lends support to the conclusion that the New Zealand populations are immunocompromised, rather than the converse scenario that the UK populations are experiencing greater immunological activation. This concept is further supported by the finding that species that had passed through more severe bottlenecks exhibited a higher prevalence of avian malaria, although this was primarily

driven by the high prevalence found in the most bottlenecked species (i.e., cirl bunting). Prevalence estimates of malaria also demonstrated a strong positive correlation with body mass, a finding that is supported by previous studies (Ricklefs et al., 2005; Scheuerlein and Ricklefs, 2004; Valkiunas, 2005), in which it is suggested that a larger body size makes birds more attractive to vectors.

The haematological response of birds that were infected with avian malaria was related to the population bottleneck the species had experienced. Birds from species that had been founded from a reasonably large number (e.g., over 400 birds) exhibited leucocytosis (increased WBC) and an elevated lymphocyte count in response to infection (when compared to their non-infected conspecifics), whilst this response was not as evident in the more severely bottlenecked species (bottlenecks of 11- 400). Ots and Horak (1998) studied the haematological response to malarial infection (*Haemaproteus* spp.) in great tits (*Parus major*) and found that birds infected with avian malaria had significantly elevated WBC and lymphocyte count. The birds were sampled from large (presumably out-bred) continental populations, and hence one would assume their haematological responses to infection were those of healthy, genetically robust individuals. Similar findings were reported by Figuerola et al. (1999), who studied cirl buntings in Europe (where they are common), and found elevated WBC in birds infected by *Plasmodium* spp. These findings would suggest that the immunological response of out-bred, non-bottlenecked birds to malarial infection is an increase in WBC, due (at least in part) to an elevated number of circulating lymphocytes.

In the context of my study, these findings suggest that less bottlenecked species in New Zealand, displaying elevated WBC and lymphocyte counts in response to malarial

infection were exhibiting the expected immunological response. Conversely, the species that went though more severe bottlenecks and failed to display leucocytosis (or increased circulating lymphocytes), may lack the ability to mount the appropriate immune response (i.e., are immunocompromised), and this in turn may explain the increased prevalence of avian malaria in these species. However, Ricklefs and Sheldon (2007) urge caution when interpreting the leucocyte response to malarial infection across species and locations. They conducted a study comparing leucocyte response to malaria between a temperate and a tropical thrush species (*Turdus migratorius* and *Turdus grayi* respectively), and whilst they found that lymphocytes were elevated in infected birds at both locations (although only significantly so in the temperate species), they also collected data from an additional 28 species (86 individuals) in the two regions, to see if the results could be generalised, and found no consistency in results. They concluded that leucocyte response to infection is highly idiosyncratic; however their results may be confounded, as they pooled data from all species without controlling for body mass (a variable shown to have influence on white blood cell counts in my study).

Birds infected with avian malaria experience the most severe fitness consequences on initial exposure (Atkinson and Van Riper III, 1991), but are less likely to be caught during this acute phase, due to decreased mobility and/or mortality (Valkiunas, 2005). Thus, when sampling from a wild population of birds, the majority that test positive for malaria will have survived the acute phase, and be in the chronic stage, whilst the individuals that have no malarial parasites have either never been exposed to the parasite, or have cleared the infection (Westerdahl et al., 2005). In this study, of 156 blood slides examined during the leucocyte counting procedure, blood parasites were only visible in 9

slides (S.E Allen unpubl. data) indicating the majority of infections were at the chronic stage (Ricklefs and Sheldon, 2007). Chronic stages are characterised by exhibiting very low intensities of infection, that can persist for years or even the lifetime of the host, and exhibit relapses during times of additional stress (e.g., during the breeding season) (Atkinson and Van Riper III, 1991). Potentially then, a population exhibiting a high prevalence of malaria indicates an ability to survive the acute phase, whilst a population with a low prevalence may be indicative of high mortality following exposure to the parasite (the non-infected class being naïve to exposure). In this scenario, where high prevalence indicates an improved ability to survive the acute phase of the infection (rather than a lack of resistance to the infection), the failure of infected individuals of the more bottlenecked species to exhibit elevated lymphocyte counts or leucocytosis may be a reflection of the relatively benign nature of the chronic infection in that individual. Conversely, the raised lymphocyte count and WBC in the less bottlenecked species may be an indication that the infection exerts greater costs on these individuals.

The key to clarifying these somewhat opposing interpretations is evaluating exposure to the avian malaria parasite. Whilst no solid inferences regarding an individual's exposure to avian malaria can be made from my data, it would seem likely the species tested had similar exposure levels, a cross section of species were caught at each location, and the inclusion of location as a random effect in the analysis controlled for differences in prevalence between locations. This, taken in conjunction with the current indication that more severely bottlenecked species are under more stress (higher HL ratio), suggests, that the observed difference in prevalence is due to the less bottlenecked species being more efficient at clearing the infection (resulting in more

'non-infected' individuals), as opposed to the more bottlenecked species resisting the acute phase infection better.

That being said, the correlation between prevalence and bottleneck in this study is highly influenced by the most bottlenecked species (cirl buntings) exhibiting a high prevalence. Whilst this may indeed indicate that severely bottlenecked species are less competent at clearing the infection, the analysis would benefit from the inclusion of more species at the severe end of the bottleneck scale.

An illuminating extension to this study, to elucidate the difference between exposure and prevalence, would be to employ serological techniques (Atkinson et al., 2001) in conjunction with a PCR assay. Serological techniques depend on detection of malarial antibodies, meaning both current and previous infections are detected, whilst PCR assays only detect infections active and present in the peripheral blood (Fallon et al., 2003). Birds testing negative in a PCR, but positive for antibodies in a serological assay have been previously exposed to malaria, and the infection has either been cleared or become latent in tissues other than peripheral blood (Fallon et al., 2003).

If the higher prevalence of malaria in more bottlenecked species is due to a decreased ability to clear the infection, what is the mechanism responsible for this? The lack of haematological response of the more bottlenecked species suggests they may be immunocompromised in comparison to the less bottlenecked species. The immune system is known to have a genetic component (Wakelin and Apanius, 1997), and hence the reduction in genetic diversity associated with population bottlenecks (England et al., 2003) may lead to a reduction in immunity.

Several human studies have demonstrated that both the resistance and outcome of malarial infections are influenced by genetic factors, and in particular by MHC (major histocompatibility complex) alleles (reviewed in Hill et al., 1997), a group of genes that have long been implicated in immunity (Acevedo-Whitehouse and Cunningham, 2006). Recently, links have been made between resistance to avian malaria and a particular MHC allele in the great reed warbler (*Acrocephalus arundinaceus*) and the house sparrow, (Bonneaud et al., 2006; Westerdahl et al., 2005, respectively), and resistance and the general level of heterozygosity of MHC alleles in the great reed warbler (Westerdahl et al., 2005). Furthermore, a recent study by Foster et al. (2007) of Hawaiian honeycreepers suggests that at least one species (Hawaii amakihi, *Hemignathus virens*) have evolved resistance to avian malaria. Overall, the evidence is mounting that genetic resistance to malaria exists in birds, and as population bottlenecks are hypothesised to cause a reduction in heterozygosity, and changes in allelic frequency (England et al., 2003), then this resistance may be severely impaired in bottlenecked populations. Furthermore, the genetic consequences of a bottleneck may have more general effects on metabolic pathways and cause energetic constraints (Luong et al., 2007); immune responses are energetically costly (Bonneaud et al., 2003; Lochmiller and Deerenberg, 2000) and hence may be adversely affected by such energetic constraint.

These findings have a number of conservation implications , it would appear that susceptibility to avian malaria increases with severity of bottleneck suggesting that species targeted for translocation schemes and captive rearing projects (i.e., that experience a population bottleneck) may be more susceptible to contracting the infection. This is particularly worrying, considering species targeted for such schemes – often

vulnerable, endemic species – may be immunologically naïve to many diseases, including malaria. Whilst this finding alone cannot be generalised to suggest that bottlenecks cause increased susceptibility to other pathogens, taken with the additional results suggesting reduced immunocompetence, this is a possibility. Furthermore, species that were founded from low numbers appear to be under greater stress (either in response to immunological or environmental stressors) than their source population conspecifics (as measured by HL ratio), and the difference only ceases to be significant above a bottleneck of 284 birds. It would appear then, that to avoid the detrimental effects of population bottlenecks when founding new populations, numbers in the hundreds maybe recommended. Whilst that will rarely be achievable in most conservation schemes, avoiding extremely low numbers (e.g., 10) should be standard practice.

This study was conducted in New Zealand, a biodiversity 'hotspot', home to one of the highest proportions of endemic bird species in the world (Myers et al., 2000) Many of these species now only exist on off-shore islands protected from mammalian predators by the physical barrier of the sea, however, no such barriers exist to the movement of introduced birds and the pathogens they carry between the mainland and these islands, and indeed introduced birds are found on the majority of island sanctuaries (Diamond and Veitch, 1981). The *Plasmodium* spp. identified in this current study matches samples taken from the endemic and threatened South Island saddleback (*Philesturnus carunculatus carunculatus)* and a number of other native birds (D. Gleeson pers. comm.*)*. At present, little is known about the pathogenicity or origins of this strain of *Plasmodium*, however it would seem likely from my current results that a considerable number of introduced birds are carrying it. Tompkins & Gleeson (2006) suggested that

introduced birds in New Zealand may act as reservoirs of malarial infection for native bird species, and the high prevalence I've found in some species in this current study (up to 95% in some species) would certainly support this. Furthermore, it seems that prevalence of avian malaria in New Zealand may be on the increase; at the beginning of 2005 Tompkins & Gleeson (2006) reported a prevalence of 9% (6/64) in non-native birds in Christchurch, whilst a year later in 2006 I found that 40% (10/25) of birds sampled in the same region (Lincoln) tested positive. Whilst the sample sizes and species composition and season (summer 2005 and autumn 2006) differ somewhat in the two studies, the PCR assay used in both studies was identical, and these results certainly warrant further investigation.

In light of the potential threat to native birds and the indication that avian malaria may indeed represent an emerging infectious disease, a better understanding of the fitness implication of chronic malarial infections would greatly benefit and inform research and conservation. The introduced birds of New Zealand seem to offer an ideal study system to investigate this in wild populations. The birds are populous, naturally exhibit reasonably high levels of malaria, are geographically isolated, and do not migrate, enabling measures of exposure to mosquitoes (the vector) to be estimated year round. Furthermore, the avian malaria species identified to date are mainly confined to *Plasmodium* spp., facilitating comparisons between species. Experimental manipulation of infection status using either medication or sub-inoculation of wild caught birds would be possible and could yield fascinating and urgently required information on this ubiquitous avian pathogen.

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Table 1. Single species comparisons of blood parameters between the UK and NZ populations of introduced birds. P values are derived from ANOVAs that included age and sex as covariates. The estimates (± SE) are linear parameter estimates. NS indicates non-significance at P<0.05

Species		Sample size	Bottleneck size[a]	WBC (log)	HL Ratio (log+1)	Lymphocyte (log)	Heterophil (log+0.5)
Greenfinch	NZ	19	66	-	-	-	-
Carduelis chloris	UK	21		0.25 ±0.15 P=0.099	-0.49 ±0.14 P=0.001	0.58 ±0.25 P=0.026	-0.30 ± 0.13 P= 0.031
House Sparrow	NZ	23	111	-	-	-	-
Passer Domesticus	UK	10		NS	-0.44 ±0.17 P=0.012	NS	-0.77 ± 0.29 P= 0.0115
Dunnock	NZ	25	284	-	-	-	-
Prunella modularis	UK	26		NS	-0.22 ±0.11 P=0.001	NS	NS
Chaffinch	NZ	18	377	-	-	-	-
Fringella coelebs	UK	16		NS	NS	NS	NS
Song Thrush	NZ	17	474	-	-	-	-
Turdus philomelos	UK	24		-0.62 ± 0.21 P=0.007	NS	-0.69 ±0.23 P=0.004	NS
Blackbird	NZ	21	808	-	-	-	-
Turdus merula	UK	33		NS	NS	NS	NS

[a] Data from Lever (1987)

Table 2. Prevalence of avian malaria in New Zealand birds detected by PCR assay.

Species	Bottleneck [a]	Number tested	Body mass[b] (g)	Positive for malaria	% Prevalence
Cirl Bunting *Emberiza cirlus*	11	15	24.4	10	67
Greenfinch *Carduelis chloris*	66	71	28.6	31	44
House Sparrow *Passer Domesticus*	111	80	28.3	21	26
Dunnock *Prunella modularis*	284	40	20.4	8	20
Chaffinch *Fringella coelebs*	377	70	21.3	12	17
Yellowhammer *Emberiza citrinella*	461	50	26.5	22	44
Song Thrush *Turdus philomelos*	474	21	70.9	20	95
Goldfinch *Carduelis carduelis*	516	64	15	7	11
Redpoll *Carduelis flammea*	599	44	11.4	4	9
Starling *Sturnus vulgaris*	653	6	78.7	2	33
Blackbird *Turdus merula*	808	58	93.4	48	83
Total	--	519	--	185	36

[a] Data from Lever (1987)
[b] Mean mass from NZ data set.

Table 3. Sample sizes for haematological comparisons

Species [a]	Bottleneck [b]	Blood slides (n) Positive for malaria	Blood Slides (n) Negative for malaria
Cirl Bunting	11	6	3
Greenfinch	66	10	13
House Sparrow	111	16	1
Dunnock	284	4	19
Chaffinch	377	1	17
Yellowhammer	461	12	10
Song Thrush	474	16	1
Starling	653	2	2
Blackbird	808	15	6

[a] Refer to Table 2 for scientific names
[b] Data from Lever (1987)

Table 4. The minimum adequate model (MAM) for the relationship between a) WBC, b) HL ratio, c) differential lymphocyte count, and bottleneck size, from a general linear mixed effects model. The MAM was derived by backward deletion using AIC scores, from a full model; non-significant terms were retained if they improved model fit. Species was included as a random effect. The estimates are linear parameter estimates.

a) WBC

Predictor		Estimate	SE	P-value
(Intercept)		9.40	5.5	0.091
Log (Bottleneck)		-1.32	0.86	0.184
Malaria –	No	0	-	
	Yes	-0.75	0.3	0.157
Log (Mass)		-2.23	1.66	0.236
Log(Bottleneck) x Malaria (Yes)		0.19	0.10	**0.055**
Log (Bottleneck) x Log (Mass)		0.40	0.26	0.182

b) HL ratio

Predictor		Estimate	SE	P-value
(Intercept)		-8.62	6.32	0.175
Log (Bottleneck)		1.56	0.98	0.173
Malaria –	No	0	-	
	Yes	-0.18	0.08	**0.031**
Log (Mass)		2.83	1.94	0.203
Log (Bottleneck) x Log (Mass)		-0.48	0.30	0.172

c) Differential Lymphocyte Count

Predictor		Estimate	SE	P-value
(Intercept)		21.63	12.05	0.075
Log (Bottleneck)		-3.57	1.88	0.116
Malaria –	No	0	-	
	Yes	-1.08	0.74	0.149
Log (Mass)		-6.21	3.67	0.151
Log(Bottleneck) x Malaria (Yes)		0.29	0.14	**0.034**
Log (Bottleneck) x Log (Mass)		1.07	0.57	0.120

FIGURES

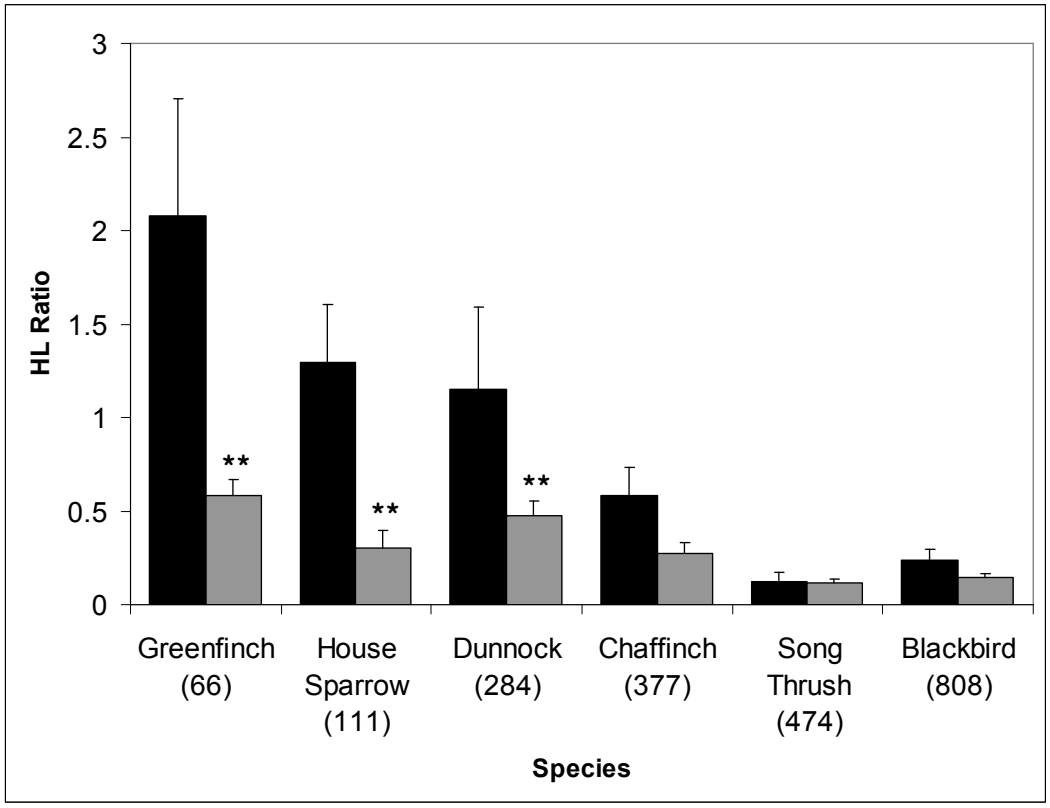

Figure 1. Mean (± SE) HL Ratios of introduced species in NZ (black bars) and their source populations in the UK (grey bars). The numbers underneath the species names are the size of bottleneck the NZ populations experienced. Significant differences (at P<0.05) between the countries are marked with **

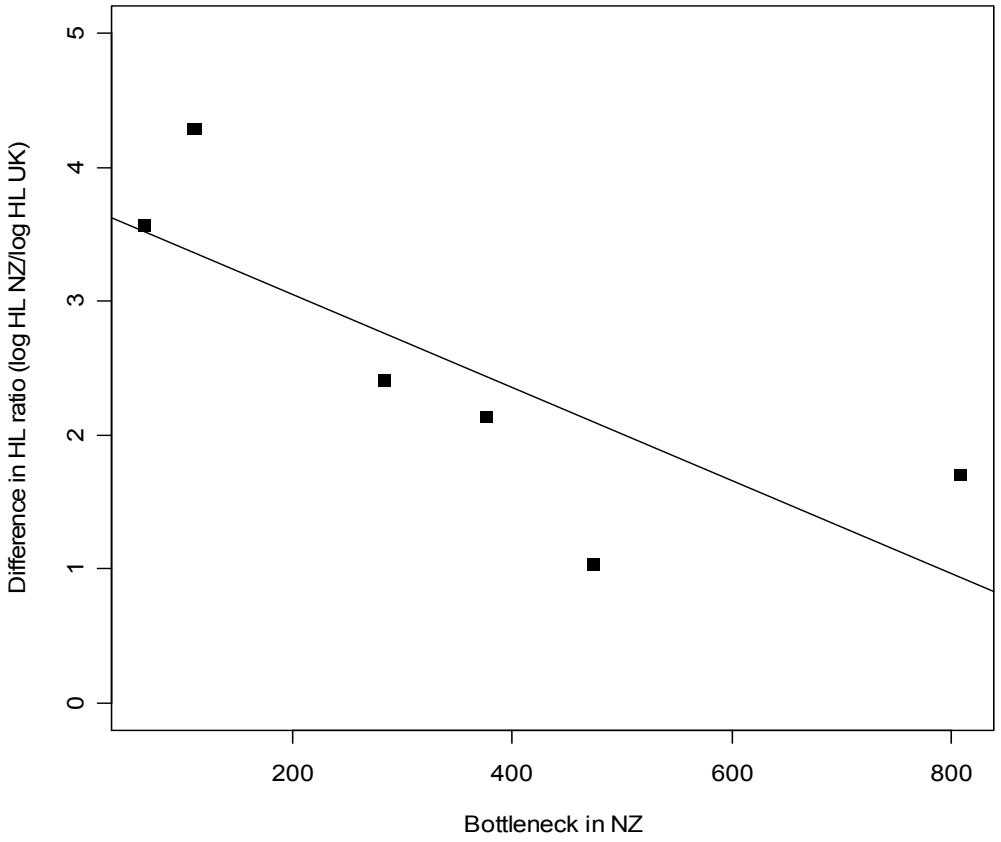

Figure 2. Linear regression of the difference in HL ratio between the UK and NZ (log HL ratio NZ/log HL ratio UK) against bottleneck size. In the statistical analysis bottleneck size was log transformed.

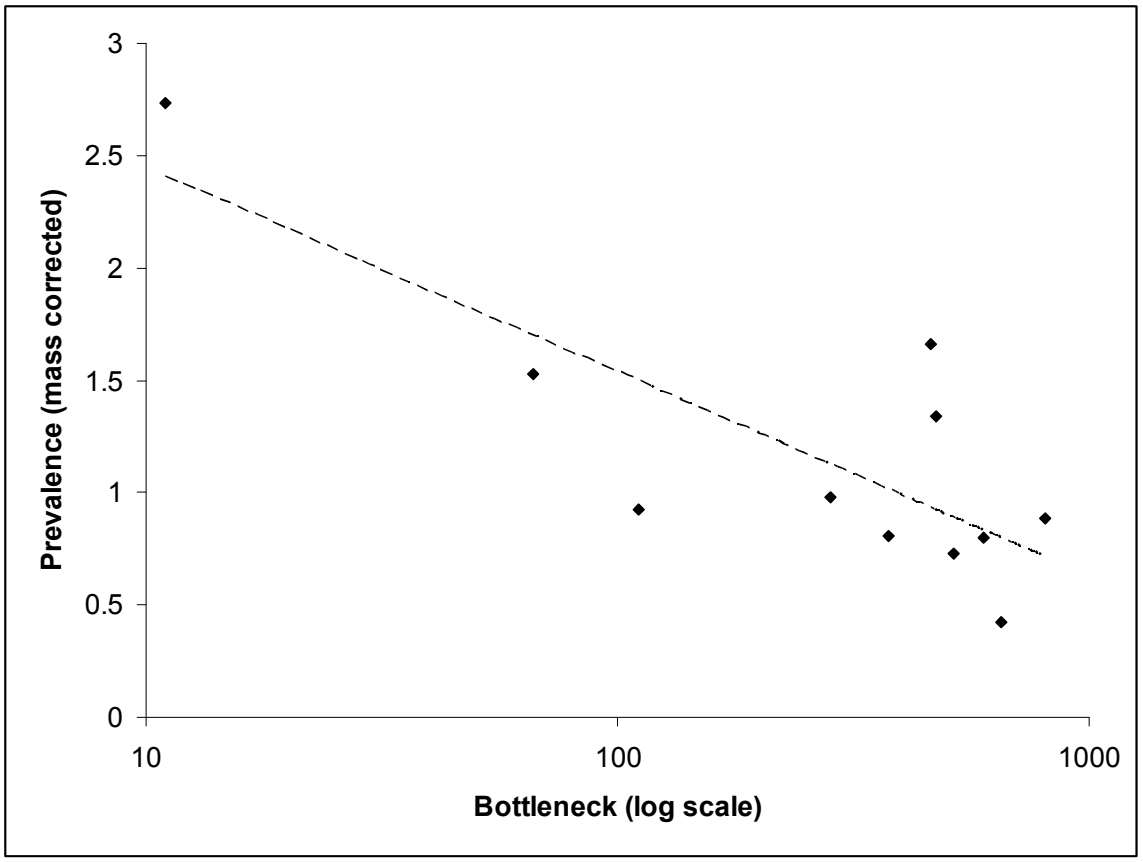

Figure 3. Prevalence of avian malaria in relation to bottleneck size (log transformed). Prevalence is corrected for mass (prevalence/mean mass) as mass was shown to have a significant and opposing affect on the prevalence in this data set.

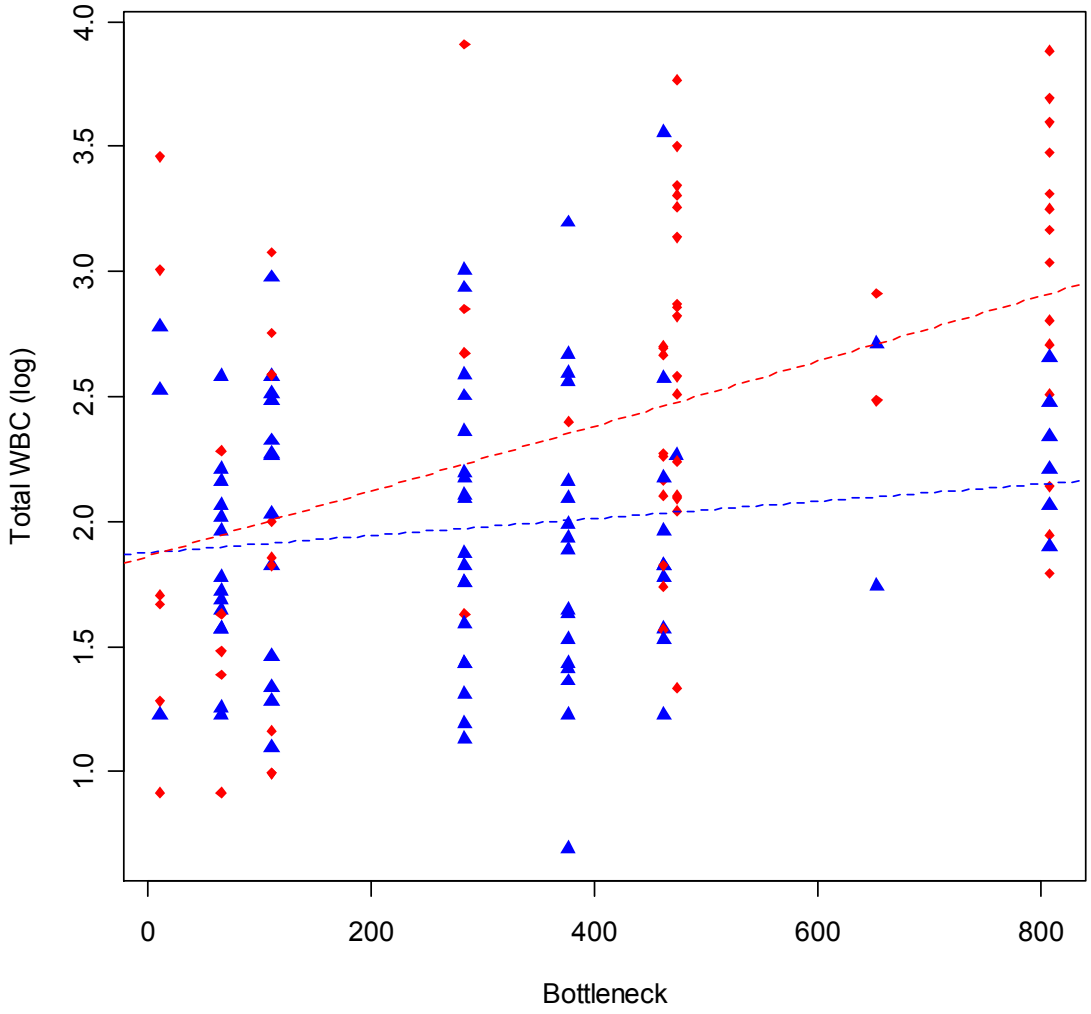

Figure 4. The effect of malarial infection on total WBC at different bottleneck sizes. Red diamonds (♦) are infected individuals and blue triangles (▲) are non-infected. The trend lines indicated are linear regressions of total WBC (log transformed) against bottleneck.

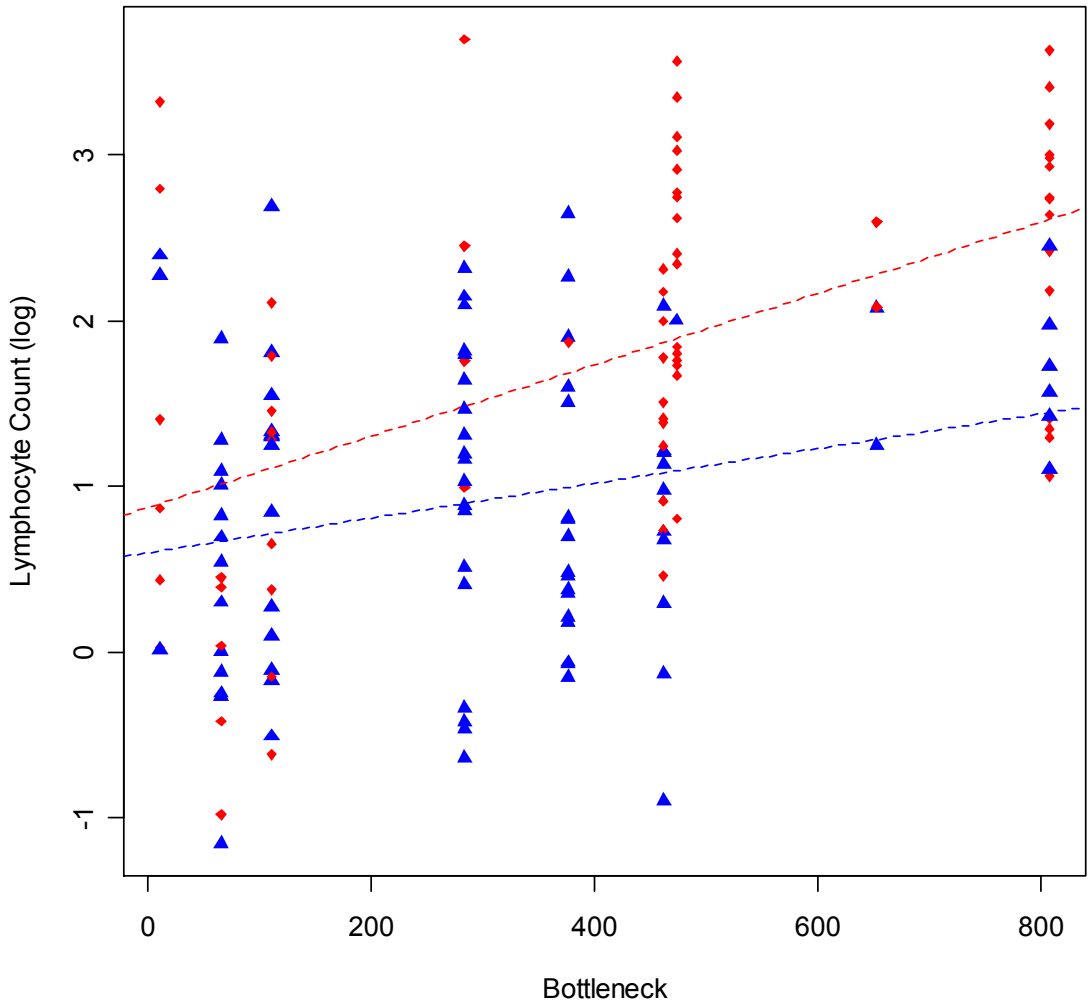

Figure 5. The effect of malarial infection on differential lymphocyte counts at different bottleneck sizes. Red diamonds (♦) are infected individuals and blue triangles (▲) are non-infected. The trend lines indicated are linear regressions of lymphocyte count (log transformed) against bottleneck

CHAPTER 3

THE RELATIONSHIP BETWEEN PHA-INDUCED IMMUNE RESPONSE AND POPULATION BOTTLENECK SIZE IN INTRODUCED BIRDS OF NEW ZEALAND.

ABSTRACT

Population bottlenecks may have negative fitness implications as they are hypothesised to cause a reduction in genetic diversity and increased inbreeding. A reduction in immunocompetence is one such potential fitness cost, which has significant relevance to conservation, as endangered species commonly experience population bottlenecks and may be more vulnerable to pathogenic and parasitic attack. I investigated the immune responses of 6 introduced bird species to immunological challenge by the mitogen phytohaemagglutinin (PHA). The 6 species were introduced to New Zealand in numbers varying from 66 to 599 individuals, and I predicted that immune responses would be lower in the species introduced in more restricted numbers (i.e. a more severe bottleneck). I found the reverse to be true; more severely bottlenecked species exhibited stronger immune responses. The larger immune response in the more bottlenecked populations may be an indication of increased investment in immunity, due to increased parasite and pathogen pressure or differential investment in varying components of the immune system. Simultaneously investigating several immunological components, whilst assessing a population's current parasite and pathogen challenge is recommended to further investigate the complex relationship between immunocompetence and population bottlenecks.

INTRODUCTION

POPULATION BOTTLENECKS AND THEIR IMPLICATIONS

Population bottlenecks (a significant reduction in a populations size) can cause a reduction in genetic diversity, both in terms of heterozygosity and allelic diversity (England et al., 2003). This has been demonstrated in a number of wild populations of birds such as the Crested Ibis (*Nipponia Nippon)* (Zhang et al., 2004), Song Sparrow (*Melospiza melodia)* (Keller et al., 2001), North Island Kokako (*Callaeas cinerea wilsoni)* (Hudson et al., 2000), Greater Prairie Chicken *(Tympanuchus cupido)* (Bouzat et al., 1998) and South Island Robin (*Petroica australis*) (Ardern et al., 1997), and in mammals such as the Banteng *(Bos javanicus)* (Bradshaw et al., 2007), Sea Otter *(Enhydra lutris)* (Bodkin et al., 1999), Lion *(Leo Panthera)* (Packer et al., 1991), Arctic Fox *(Alopex lagopus)* (Nyström et al., 2006), Northern elephant Seal *(Mirounga angustirostrus*) (Hoelzel, 1999), and Barbary Red Deer *(Cervus elaphus barbarus)* (Hajji et al., 2007). As genetic diversity can be correlated with fitness (Reed and Frankham, 2003), populations that have passed through a bottleneck and as a result have reduced genetic diversity may experience a reduction in population fitness.

In addition to bottleneck effects, the resulting small populations are often prone to high levels of inbreeding, since mate choice is reduced and individuals are more likely to mate with kin (Hedrick and Kalinowski, 2000). The negative impact of inbreeding on a population, termed inbreeding depression, is well known and encompasses a suite of interrelated fitness effects, including a reduction in reproductive success, an increase in

physical defects and susceptibility to parasites and pathogens (Crnokrak and Roff, 1999; Keller and Waller, 2002).

THE IMPORTANCE OF THE IMMUNE SYSTEM

The immune system is at the core of an animal's ability to survive and reproduce, as it provides a complex and dynamic protection against a wide array of parasites and pathogens. Under most circumstances, anything that detrimentally impacts on an organism's ability to mount a suitable immune response (i.e., its immunocompetence) will likely have significant fitness costs.

Evidence is mounting that decreased genetic diversity renders individuals more prone to disease and parasitic infection (Arkush et al., 2002; Hawley et al., 2005; Hedrick et al., 2001; O'Brien and Evermann, 1988; Pearman and Garner, 2005). Considering the role immunity plays in defence against pathogens, and the genetic basis for much of the immune system (Wakelin and Apanius, 1997), it is not surprising that a reduction in genetic diversity has been linked to decreased immunocompetence (Hawley et al., 2005; Sanjayan et al., 1996). Inbreeding has also been shown to have detrimental effects on immune function; for example, work on an insular, pedigreed population of song sparrows found that more inbred individuals had lower immune responses (Reid et al., 2003). Whilst links have been made between immunocompetence, inbreeding, and genetic diversity, few studies have directly investigated the relationship between population bottlenecks and immunocompetence in wild populations. One exception is work by Hale and Briskie (2007), comparing a severely bottlenecked population (founded by 5 individuals) of the endemic New Zealand robin (*Petroica australis*) with its source population. The birds in the bottlenecked population displayed significantly weaker

responses to an immune challenge (in the autumn) than the source population. However, whether this result is typical of other birds, and whether a similar response occurs in populations that have been subject to less severe bottleneck sizes, is unknown.

RELEVANCE TO CONSERVATION

Understanding the effects of population bottlenecks is fundamental to modern day conservation. Endangered and fragmented populations, by definition, experience a population bottleneck. Although the aim of conservation measures will be to increase the population size of such species, even populations that recover to their pre-bottleneck size may continue to be affected by the genetic consequences of passing through an earlier bottleneck. In some situations, conservation projects intentionally create population bottlenecks through translocation schemes and in the captive breeding of threatened species. The incidence with which bottlenecks are encountered by conservation biologists is set to increase, in large part due to the predicted rise in the number of threatened and endangered species, but also as habitat restoration schemes come to fruition, and the potential for translocations increase. In New Zealand alone, over 400 translocations have taken place, and the rate of occurrence is rapidly increasing (Armstrong and McLean, 1995). Each of these translocations creates a new population that has passed through a bottleneck.

Parasites and pathogens have been implicated in the population decline of a number of endangered species (Dobson and McCallum, 1997) and emerging infectious diseases are an acknowledged conservation issue (Dobson and Foufopoulos, 2001; Wikelski et al., 2004). Any conservation project, therefore, must consider the immunological health of populations. Hence, whether population bottlenecks have a

negative impact on immunity, and at what size of bottleneck this impact starts to compromise the long-term viability of population, are questions of crucial relevance to conservation.

INTRODUCED BIRDS AS A STUDY SYSTEM

The introduction of species to ecosystems outside their native range has, on the whole, been disastrous for those ecosystems (Blackburn et al., 2004). Nowhere is this more evident than in New Zealand, where the decimation of the endemic flora and fauna by introduced mammals is well documented (Craig et al., 2000). However, in some cases, the purposeful introduction of species to areas isolated from their source populations offers unique and fortuitous opportunities for study (Briskie, 2006). In the late 1800's numerous bird species were introduced to New Zealand, mostly from the UK, a number of which are successfully established today (Lever, 1987; Thompson, 1922). The Acclimatisation Societies responsible for their introduction kept careful records of the numbers introduced, which varied considerably from species to species (Thompson, 1922). Therefore, introduced birds in New Zealand are today represented by a series of wild populations that have each experienced bottlenecks. Moreover, different species of introduced bird experienced different sizes of bottlenecks, thereby allowing a comparison across species of the effects of bottleneck size on fitness. Briskie & Mackintosh (2004) exploited this system to examine reproductive success in pre- and post- bottlenecked populations, and found that hatching success was significantly lower in populations that had been founded by less than 150 individuals; hatching success was only equal to pre-bottleneck populations in species founded by more than 600 individuals. Whether other

fitness traits, such as the functioning of the immune system, might be similarly compromised are unknown.

STUDY AIMS

In this study, I investigate the relationship between bottleneck size and immune function in introduced New Zealand birds. My objective is to investigate whether populations of species that experienced more severe bottlenecks during their introduction exhibit reduced immunity as a result. Six species of bird, all introduced to New Zealand in the late 1880's, and each founded by a different number of individuals (meaning they experienced a range of population bottlenecks) are included in the study. To assess immune response, individuals were challenged with the immunostimulant phytohaemagglutinin (PHA), which is a well established test of immune function in birds (Goto et al., 1978; McCorkle et al., 1980; Smits et al., 1999). My prediction is that if population bottlenecks lead to a reduction in immunocompetence, then species that were founded by less individuals will exhibit lower responsiveness to challenge with PHA than species that have experienced larger bottlenecks. This study should help inform conservation practitioners, both when dealing with populations that have already experienced a bottleneck, and when making decisions regarding the numbers of individuals used to found new populations.

METHODS

STUDY POPULATIONS

I studied immunocompetence in the following species: greenfinch, house sparrow, dunnock, chaffinch, goldfinch and redpoll (scientific names in Table 1). All six species are small (10-30 g) passerines that were introduced to New Zealand in the late 1880's by Acclimatisation Societies. The number of individuals released by the societies was carefully recorded for each species (Thompson, 1922), and varied from 66 for the greenfinch to 599 for the redpoll. For the purpose of this study, the total number released and recorded as successfully established is used to indicate the bottleneck each species experienced (see Table 1 for details). All species are now common, and form contiguous populations across mainland New Zealand (Briskie and Mackintosh, 2004; Robertson et al., 2007). Birds were sampled from agricultural and viticultural field sites in the regions around Kaikoura and Blenheim, South Island, New Zealand (42°23' S, 173°37' E; 41°28' S, 173° 57' E, respectively) in the austral autumns (March-May) of 2006 and 2007.

GENERAL METHODOLOGY

The PHA assay requires two measurements of patagial thickness to be taken at a standardised interval (12 h in this study): once before the injection and once after the injection. Due to the difficulty in re-capturing wild, free-flying birds the test necessitates holding the bird for the duration of the standardised interval. Birds were caught in mist-nets and potter traps (baited with wild bird seed) after 12 noon, to minimise stress by allowing time for birds to feed in the morning. All birds were fitted with a metal ring, aged, sexed, and had basic biometrics taken (wing chord, mass, moult score), and then

held until the evening of the day of capture, when the immune assay was performed. If individuals were caught within 2 hours of sunset, they were kept in a cloth bird-bag in a quiet room, but if caught earlier in the afternoon, birds were caged and supplied *ad libidum* with water and wild bird seed. Avian immunity appears to exhibit seasonal fluctuations in response to challenge by PHA (Møller et al., 2003; Owen and Moore, 2006), therefore all work was carried out in the autumn, when adult birds could be held in captivity over night without disrupting breeding activities.

IMMUNE ASSAY

PHA is a lectin found in plants, and present in particularly high concentrations in the red kidney bean (*Phaseolus vulgaris*), where it is thought to act as a defence against herbivory (Martin et al., 2006b). It is mitogenic to many vertebrate cell types, particularly T lymphocytes, and hence acts as an immunostimulant. The subcutaneous injection of PHA activates T cells and stimulates the local infiltration and proliferation of a number of cell types involved in immunity (Martin et al., 2004). The localised swelling caused by injection of PHA is seen as a measure of immune function – a larger swelling indicates a stronger response. Thus by measuring the size of a swelling in response to PHA injection, an aspect of immunity can be assessed.

The PHA assay methodology herein follows the simplified protocol, which eliminates the use of a control wing, as recommended by Smits et al (1999). In their technical report, Smits et al (1999) used data from 7 different studies (608 individuals) of 5 different species, to compare determination of the 'PHA response' in two different ways. Firstly, calculating the response as the increase in thickness of the PHA injected wing, minus the change in thickness of a control wing (injected with phosphate buffered

saline, PBS), and secondly eliminating the control wing and calculating the change in thickness of the PHA-injected wing only (called PHA-PBS and PHA only, respectively). They found the two methods resulted in very closely correlated measures in all 7 studies, and concluded that the PHA response was so much greater than the (non) response to PBS, that the control wing was unnecessary, and instead the pre-injection thickness of the PHA injected wing should be used as the control. Smits et al (1999) note several advantages to eliminating the control wing, including a reduction in handling time (and hence potentially increased sample sizes), decreased handling-related stress on the birds, and a decrease in the coefficient of variation due to measurement inaccuracies. They also propose that the use of the term 'control' for the PBS injected wing only applies to the experimental assessment of whether PHA is the active ingredient within the injected solution (and not PBS), and does not function as a more general experimental control (Smits et al., 2001). Since the publication of this report the simplified protocol has been adopted by a large number of immunoecologists (in a recent Web of Science search the technical report was cited by almost 200 studies).

In contradiction to these arguments (for eliminating the PBS control wing), in studies such as this, where several species are being compared, there would appear to be a benefit to measuring the other wing to control for species specific changes such as dehydration. However, the other wing may not represent an independent control, as a recent study in poultry found that PHA induced a systemic response to the inflammation (Adler et al., 2001). This may mean that both wings may be affected by the injection of PHA. Furthermore, as *functio laesa* (loss of function) is one of the 5 cardinal signs of inflammation (Punchard et al., 2004), certain individuals of a species or certain species

may favour the other, 'control' wing, thereby negating the use of this as a true control. On balance, it would seem that the inclusion of a 'control' wing introduces as many variables as it controls for, and hence, in this study the simplified protocol was followed.

Preceding injection with PHA, the underside of the left wing web (patagium) of each bird was swabbed with alcohol, to clear the area of feathers and to sterilise the injection site, and a mark was made with permanent ink. Thickness of the patagium was measured using a digital micrometer precise to 0.001 mm (Mitutoyo, 395-371, Tokyo, Japan), that was steadied in a clamp attached to a table-top. The bird was held immobile, with the left wing outstretched and the bare patch of the patagium exposed and positioned over the lower contact point of the micrometer. An assistant gradually closed the micrometer until contact caused the skin of the patagium to twist slightly, the micrometer reading was made at this point of contact (See Figure 9 in Chapter 4). All measurements were taken by the same individual (S.E. Allen).

At the beginning of each field season 50 mg of PHA (L8754, Sigma) was combined with 50 ml of liquid Phosphate Buffered Saline (PBS) (P4244, Sigma) in a sterile container, aliquoted into 1.5 ml vials and immediately frozen. Each vial was subsequently defrosted as required (for 20 min at room temperature). Immediately following measurement of the patagium, the PHA solution was administered subcutaneously using a 27g needle, at the marked injection site, in a dose appropriate for the species, based on body size (J.E. Smits pers. comm.; see Table 1). Following administration the bird was placed in a cotton bird bag, and held over night in a quiet, safe room. The patagium was re-measured the following morning, 12 hours after the PHA was administered. Traditionally the PHA response is measured 24 hours after

injection. However, a study of the temporal dynamics of this immune response (measured from 6 to 72 hours) found no significant change in mean patagium thickness following the initial increase at 6 hours (Navarro et al., 2003). Thus, a 12 hour period was chosen for this study to minimise disturbance to the birds.

The patagium on a small passerine is extremely thin (in the order of 0.1 mm) and it is acknowledged that obtaining this measurement is the most error-prone aspect of the PHA assay (Smits et al., 1999). To increase the accuracy of my measurements, I took either 3 or 5 repeat measurements of each patagium and a mean of three measurements was calculated. For those individuals in where 5 readings were taken, the two outlying measurements were subsequently excluded so that all estimates of patagium thickness were based on 3 measurements. Patagium thickness was highly repeatable (Lessells and Boag, 1987) across each set of measurements on each individual (see Table 1).

Immune response (IR) was quantified as the relative increase in thickness of the patagium:

$$IR = (PT_{post} - PT_{pre})/PT_{pre}$$

where PT_{post} and PT_{pre} are the mean patagium measurements post- and pre-injection respectively. A positive IR score indicates the patagium has swollen in response to the PHA injection, with higher IR scores corresponding to a greater relative increase, interpreted as a stronger immune response. Birds that exhibited a negative IR were excluded as this can mean that either the administration of the injection was unsuccessful (S. Allen unpubl. data) and/or the birds became dehydrated whilst held overnight (JE Smits pers. comm.).

IMMUNOSENESCENCE

In adult birds, immune function may decrease with age (Haussmann et al., 2005; Lavoie et al., 2007; Lozano and Lank, 2003; Palacios et al., 2007). Individuals included in this study were aged as either 'Hatch Year' (HY), which are birds less than 1 year old, 'After Hatch Year' (AHY), which are birds over 1 year old (adult), or as 'Unknown Age' (UNK), based on plumage characteristics (Svensson, 1992). As the exact ages of adults were unknown, and bottleneck effects could lead to differential longevity, a data set was also created of HY only birds. This allowed interspecific comparison of immune response, without the potentially confounding effects of immunosenescence in the adult age group.

STATISTICAL ANALYSIS.

Full data set

I used general linear mixed-effects models to examine the relationship between population bottleneck and immune response, with IR as the response variable. Mixed-effects models allow the analysis of observations structured in groups, when within-group errors are correlated and have unequal variances, via the specification of fixed and random effects (Crawley, 2007).

Although my primary interest was to examine if severity of bottleneck had an influence on immune response, a number of other variables required inclusion in the model. Sex, age, and moult stage (Martin, 2005; Martin et al., 2006a; Moreno et al., 2001) have previously been found to influence immunological responses in birds. Birds that were caged prior to injection, as opposed to being held in a bird bag, could also have

differed in levels of activity and stress, which has been shown to affect PHA response (Ewenson et al., 2003). PHA dose was administered at levels appropriate to each species (see above), however, the two species that experienced the most severe bottlenecks (greenfinch and house sparrow) were in higher dose classes, so I included dose per gram (calculated as dose (µl)/ pre-injection mass (g)) to control for this in the analysis. Body size did not need to be included in the model, as the response variable is calculated as a relative, not absolute, size increase. The maximal model thus included bottleneck size, sex, age, moult (fitted as two levels: Yes or No), caging regime (fitted as two levels: Yes or No) and dose/gram. IR and bottleneck were log-transformed to correct for heteroscedasticity and deviation from normality (Crawley, 2007). The random effects within a mixed model specify the underlying structure of the data. Species and year were included as random effects (nesting species within year), thereby accounting for any consistent inter-year or inter-species differences.

A maximal model was fitted (with main effects, no interactions), and explanatory variables were removed one at a time, using a stepwise deletion method, based on the Akaike information criteria (AIC) (Crawley, 2007). The backwards elimination of variables continued until a minimum adequate model was obtained.

HY Only Data set

The model fitting process was repeated as above with the restricted data set of HY only birds, using a general linear mixed effects model with year and species as random effects. IR was the response variable, and bottleneck, sex, caging, and dose per gram were main effects in the maximal model. The house sparrow was excluded from this data set, as it is the only species in this study to experience a post-juvenal moult (Svensson,

1992) and thus were the only HYs in moult (accordingly moult was not included as a variable in the maximal model). Model simplification was conducted as above.

Phylogenetic control

Comparing immune responses across several species can potentially create problems of phylogenetic non-independence (Bennett and Owens, 2002; Harvey and Pagel, 1991)in that closely related species may be more similar to one another than by chance alone, due to sharing a common ancestor. To control for this, independent contrasts were used on the mean IR values (mean IR per year, averaged) using Comparative Analysis of Independent Contrasts (CAIC) (Purvis and Rambaut, 1995) . A phylogeny was constructed from Sibley and Ahlquist (1990) and a regression conducted of mean IR contrasts on bottleneck size contrasts. The correlation was forced through the origin, as recommended (Harvey and Pagel, 1991)

Comparison with source populations in Europe

Whilst conducting a corresponding test of immune response in non-bottlenecked European birds of the same species was beyond the scope of this study, it was possible to compare my results to published data. Møller et al. (2006) conducted a study investigating immune response to PHA in a number of bird species in Northern Jutland, Denmark. Four of the species tested in Denmark (greenfinch, house sparrow, chaffinch and dunnock) are those used in my current study, enabling some comparisons to be made. The methods and protocols differ between the two studies in a number of ways (e.g., dose, measurement protocol, season), excluding the possibility of a direct intraspecific comparison. However, an alternative is to merely consider whether the mean immune

response to PHA differs between species in Denmark, as it does in New Zealand (see Results). As there are only four species, it would be ambitious to draw any strong conclusions. However, examining if there are differences between the species (irrespective of magnitude or direction) is still instructive in determining whether there is a pattern worth further investigation. If there are differences in IR between the species in Europe, then this would indicate that species effects could be confounding bottleneck effects in my New Zealand data. On the other hand, if there are no differences between the European species, then this would strengthen any observed effect of bottleneck on IR in the New Zealand data set.

The data provided in Møller et al. (2006) consists of the mean immune response (x), standard error (y) and sample size (n) per species, where x is calculated as the mean change in wing web thickness of the inoculated wing, minus the change in thickness of the other, control wing. Thus a vector of n random numbers from a normal distribution, with mean x and standard error y was generated for each species, and combined to form a simulated data set, on which an ANOVA was run, with immune response as the response variable and species as the explanatory factor. This process was reiterated 1000 times, and the percentage of significant ($p<0.05$) outcomes calculated (significance would indicate there was a difference in immune response between species). For consistency, a corresponding data set was simulated for the New Zealand species (rather than using the actual New Zealand data) and an ANOVA run. The mean immune response (x) for the New Zealand data set was calculated as mean change in wing web thickness of the inoculated wing (this differs from the above calculation of IR, as it is not a relative

increase of wing web thickness) . Again, this was reiterated 1000 times and the percentage of significant ($p<0.05$) outcomes calculated.

All statistics were carried out using R v2.6.2 (R Development Core Team, 2008), except for the CAIC analysis.

RESULTS

Complete data set

Immune Response (IR) varied in relation to bottleneck size, but in the opposite direction predicted ($P<0.05$); the response was lower in species that had experienced larger (less severe) bottlenecks (Figure 1). The minimal adequate model included bottleneck size, cage regime, dose/gram, and moult as main effects (Table 2). Birds that were caged prior to the immune assay exhibited a dampened immune response compared to individuals kept in bags ($P<0.05$). A positive relationship was found between dose/gram and IR, and birds that were moulting had a decreased IR. Whilst none of these trends were significant, inclusion in the model improved overall fit (based on AIC scores). IR did not vary with sex or age, and inclusion of these variables did not improve model fit.

Hatch year (HY) only data set

Bottleneck size was the only explanatory variable retained in the minimal model for the HY-only data set (estimate ± SE = -0.36 ± 0.15, $P < 0.05$), and again was negatively correlated with IR. None of the other variables - caging, dose per gram and sex - included in the maximal model improved model fit, and were accordingly removed from the final model.

Phylogenetic control

The negative relationship between immune response and bottleneck size was significant after controlling for phylogeny. An ANOVA for the regression line (forced through the origin) was significant ($F = 63.5$, $df = 1, 4$, $P = 0.0013$), and the slope negative (-0.002), indicating an inverse relationship between the two variables.

Comparison to European data set.

ANOVAs were run on the two simulated data sets (Denmark and New Zealand, using the figures in Table 3) to see if there were significant differences between species in either country (irrespective of the trend or direction of those differences). This process was repeated 1000 times for both data sets. In the simulated data set for New Zealand, species differences were significant in 58.5% of 1000 iterations, whilst in the Danish data set, species differences were only significant in 9.9% of the 1000 iterations. This means that species differences were rarely (9.9%) detected in the Danish data set, and suggests that the differences found in my current study are not due to species affects alone.

DISCUSSION

The purpose of this study was to elucidate how immune function is affected by population bottlenecks of differing sizes, with the prediction being that response to PHA challenge would be lower in more severely bottlenecked populations. Contrary to predictions, the reverse relationship was found, with the response elicited by PHA higher in species that had experienced a more severe (smaller) bottleneck. For example, the greenfinch in New Zealand is descended from a population founded by just 66 individuals, and it exhibited a larger immune response than the redpoll which was founded by nearly ten times the number of birds (599 individuals). This negative relationship remained significant after controlling for other biologically relevant variables, and when the potentially confounding effects of immunosenescence and phylogeny were controlled for. The existence of a relationship, albeit negative, between bottleneck size and IR in this study implies that immune function is affected by the size of a population bottleneck. Whilst the inference that more bottlenecked populations are more immunocompetent cannot be discounted, in light of past findings this seems an unlikely interpretation of the results, and instead may reflect the complex mechanisms under-lying immune function.

Previous work on immunocompetence and bottlenecks has looked at levels of inbreeding within a population (Hale and Briskie, 2007; Reid et al., 2003; Reid et al., 2007), or compared bottlenecked with source populations within the same species e.g., (Hale and Briskie, 2007). In my study however, I used an inter-specific approach where different species are represented by differing sizes of bottlenecks, and thus it is necessary

to disentangle the difference between species from the effect due to bottlenecks. The use of statistical techniques have been employed to achieve this (i.e., mixed-effects models, and phylogenetic contrast), however the ideal scenario would have been to directly compare immune responses of the species under study in New Zealand to their non-bottlenecked conspecifics in Europe. Whilst this was beyond the scope of this study, comparisons can be made to previously collected data on European birds. Møller et al. (2006) studied the PHA response of a number of passerine species in Denmark, including four of our study species. Using the summary data provided in that study, and the corresponding summary data for New Zealand, two data sets (Europe and NZ) were simulated. In a thousand simulations, the European populations were only found to differ in their immune response 9.9% of the time, whilst in New Zealand, in the same number of simulations, species differences in immune response were detected in 58.5% of the iterations. This lends some support to the observed differences in immune response in New Zealand populations being due to bottleneck effects, rather than species effects *per se*. Clearly, this would be greatly strengthened by a direct comparison of the source population (i.e. birds from the UK) using the same methodology and protocols as the current study.

IMMUNE RESPONSE AS AN INDICATOR OF CURRENT INVESTMENT

The immune system is locked in a constant battle with the pathogens and parasites with which an individual comes into contact. The two sides of this combat have reciprocal co-evolutionary effects and the specific immunological challenges a population faces will, to some extent, mould immune response. A population that is prone to higher rates of parasitism or disease might be expected to invest more in immune function, than

less parasitised populations (e.g. Lindström et al., 2004; Tschirren and Richner, 2006) Thus the immunological response to PHA may be an indication of current investment in immunity, rather than capability (or 'immunocompetence') *per se*. That being the case, it may be that more severely bottlenecked species experience higher parasite and pathogen pressure, and are therefore preferentially investing in immune functions. To investigate this further would require the quantification of current parasite and pathogen burdens these populations are experiencing (See Chapters 1 & 2). However, even this may not be fully informative if increased immune investment is an adaptive shift to counter increased immunological challenge; this could be in response to past challenges (e.g., at some point since the introduction event) as opposed to present day levels of parasitism and disease. Nonetheless, examining immune responses in the context of parasite and disease load in bottlenecked populations would be highly informative.

If immune responses are energetically costly then increased investment in immune function may be to the detriment of other life history traits (Sheldon and Verhulst, 1996), and indeed this has been demonstrated in a number of studies (Ardia, 2005; Moreno et al., 1999; Sanz et al., 2004). Species that have experienced severe bottlenecks might therefore preferentially invest in immune response, and one would expect to see trade-offs with other traits. In the case of reproductive success this might be true, as hatching success was found to be markedly lower in introduced birds in New Zealand that had experienced bottlenecks of less than 150 birds (Briskie and Mackintosh, 2004). It would be illuminating to investigate the dynamic between other measures of life history traits and immunological investment in populations that have experienced bottlenecks.

DIFFERENTIAL INVESTMENT IN IMMUNE COMPONENTS

The avian immune system, as in any vertebrate, is comprised of two integrated arms – the innate (non-specifc) and the acquired (pathogen-specific), which are triggered by infection or tissue trauma (Wakelin and Apanius, 1997). Innate responses exhibit no memory and are produced at the same intensity at re-exposure to the trigger. Acquired immune responses, of which there are two main types (antibody and T-cell mediated), do exhibit memory and thus mount a more efficient response on subsequent exposure (Kennedy and Nager, 2006; Wakelin and Apanius, 1997). Ecologists using the PHA assay have traditionally interpreted the induced swelling as a measure of acquired, T-cell mediated, immune function, based on past work on poultry (Goto et al., 1978; McCorkle et al., 1980). However, a recent study (Martin et al., 2006b) investigating the underlying cellular response to PHA in wild birds (*Passer domesticus*), suggests that the swelling is a more complex and dynamic process, involving both innate and acquired arms of the immune system. Kennedy and Nager (2006) suggest that a significant proportion of the swelling induced by PHA is produced by the non-specific, innate response, and that the contribution by the cell-mediated, acquired arm of the immune system is minor, at least on first exposure to the mitogen. This is of some significance, as there is evidence that differing components of the immune system are differentially activated, and in fact trade-offs may exist between different components (Hõrak et al., 2006; Kennedy and Nager, 2006; Norris and Evans, 2000). It may be that populations exhibiting an increased PHA response (such as the more severely bottlenecked species in my study) are compensating for a reduction in other aspects of immunity.

There is much evidence that there is a genetic component to immunity (Wakelin and Apanius, 1997) and furthermore, different aspects of the immune system appear to be controlled by different groups of genes (Acevedo-Whitehouse and Cunningham, 2006). For example, the genes of the major histocompatibility complex (MHC) have long been implicated in immune responses; genes of the MHC class III subgroups appear to regulate aspects of innate immunity, whilst acquired immunity is regulated by MHC class I & II genes (Acevedo-Whitehouse and Cunningham, 2006). The reduction in genetic diversity associated with population bottlenecks may therefore have differing effects on immunity. Components of the immune system may differ in their sensitivity to genetic impoverishment, and thus components more robust to genetic changes may up-regulate to compensate for reduced function in other arms.

Maintaining and activating an immune system is energetically costly (Lochmiller and Deerenberg, 2000; Lochmiller et al., 1993), and different components of the immune system may have differing energetic requirements (Lochmiller and Deerenberg, 2000). If populations that have experienced a bottleneck are resource constrained, then certain arms of the immune system may be favoured, either due to them being less energetically costly or more essential. The immune response elicited by PHA may be energetically 'cheap', when an individual can't 'afford' to fully activate all immunological components or it may be a preferential response in an energetically constrained environment. Indeed, Lochmiller and Deerenberg (2000) argue that the initial, non-specific acute response to infection is the most important in terms of fitness and life history of an individual, and the PHA response may reflect this aspect of immunity (as suggested by Kennedy and Nager (2006)).

In this study, population bottlenecks may affect the energy budgets available to populations. Hence, if the more severely bottlenecked populations are on a lower budget they may invest more in either 'cheaper' immunity or in essential components, which could translate as a higher PHA response relative to less bottlenecked populations (that are less dependent on the aspects of immunity that PHA measures). Clearly, to elucidate differential investment in immunological components in bottlenecked populations necessitates the simultaneous assessment of multiple immune parameters. The range of immunological assays is increasing, and it is now possible to evaluate differing immune components using a single blood sample (Matson et al., 2006), enabling just such an assessment to be made.

CONSERVATION IMPLICATIONS & CONCLUSION

The good news, from a conservation perspective, is that avian populations experiencing moderately small bottlenecks in the range I studied here are still capable of mounting an immune response. The most severe bottleneck examined in this study was a population founded by just 66 birds, which is comparable to many current translocation schemes (Wolf et al., 1996), and they displayed the largest immune response. In a global survey of translocation schemes (Griffith et al., 1989; Wolf et al., 1996) it was found that in 1993, the median number of individuals released was 50.5 (an increase from 31.5 in 1987). However, of the 336 surveys returned, it was found the 32% of the programs released 30 or fewer animals. A previous study by Hale and Briskie (2007) found a severely bottlenecked population (5 individuals) of New Zealand robins appeared to be considerably immunocompromised and so, at present, caution should be urged for conducting translocations with such low numbers.

The fact that there was a relationship between immune response and severity of bottleneck (albeit a negative one) suggests that population bottlenecks do impact on immunity, but that the interaction is a complex one. The larger immune response in the more bottlenecked populations may be an indication of increased investment in immunity, due to increased parasite and pathogen pressure. An alternative, but not mutually exclusive explanation is that components of the immune system may be differentially affected by population bottlenecks. The immune system is a complex and dynamic system, and this study clearly points the way to further work that is required to assess the impact of population bottlenecks. Simultaneously investigating several immunological components, whilst assessing a population's current parasite and pathogen challenge should prove extremely illuminating, as will comparing the introduced population directly to their source populations. The need for further research in this area is urgent, as emerging infectious disease, habitat loss and environmental stress increase the pressures on threatened and endangered species.

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TABLES

Table 1 Species investigated: bottleneck size, sample size, dose and repeatability statistics for patagium measurement (the intraclass correlation coefficient).

Species	Common name	Bottleneck[a]	n	Dose PHA-PBS 1mg ml^{-1}	Repeatability Pre-injection	Repeatability Post-injection
Carduelis chloris	Greenfinch	66	36	40	r=0.92, p<0.0001	r=0.97, p<0.0001
Passer domesticus	House Sparrow	111	38	40	r=0.99, p<0.0001	r=0.99, p<0.0001
Prunella modularis	Dunnock	284	6	25	r=0.92, p<0.0001	r=0.87, p<0.0001
Fringella coelebs	Chaffinch	377	22	25	r=0.88, p<0.0001	r=0.94, p<0.0001
Carduelis carduelis	Goldfinch	517	27	25	r=0.91, p<0.0001	r=0.94, p<0.0001
Carduelis flammea	Redpoll	599	7	25	r=0.87, p<0.0001	r=0.96, p<0.0001

[a] bottleneck calculated as total number of birds introduced into NZ per species, excluding introductions that were recorded as unsuccessful (Lever, 1987)

Table 2. The minimum adequate model (MAM) for the relationship between PHA response and bottleneck size, from a general linear mixed-effects model, for the full data set. The MAM was derived by backward deletion based on AIC scores, from a full model that also included Sex and Age. Year and Species were included as random effects. The estimates are linear parameter estimates (SE is the standard error), P-values in bold are significant. Dose per gram and moult, whilst not significant improved the fit of the model, and thus were retained.

Predictor		Estimate	SE	P-value
(Intercept)		1.14	0.76	0.14
Log (Bottleneck)		-0.41	0.12	**0.0083**
Caged–	No	0	-	
	Yes	-0.61	0.19	**0.0018**
Dose per gram		0.76	0.44	0.0867
Moult	No	0	-	
-	Yes	-0.31	0.21	0.1460

Table 3 Parameters used to simulate the two data sets for comparison between Europe and New Zealand. Sample size (n), mean immune response and standard error (se), refer to methods for calculation of mean immune response.

Species		Denmark[b]		New Zealand[c]	
	n[a]	Mean immune response (mm)	se	Mean immune response (mm)	se
Carduelis chloris Greenfinch	12	0.21	0.02	0.19	0.04
Passer domesticus House sparrow	13	0.23	0.04	0.33	0.03
Prunella modularis Dunnock	9	0.21	0.03	0.06	0.10
Fringella coelebs Chaffinch	8	0.25	0.06	0.10	0.02

[a] Sample sizes used for both data sets taken from Møller et al. (2006)
[b] Data from Møller et al. (2006)
[c] Data from current study

FIGURES

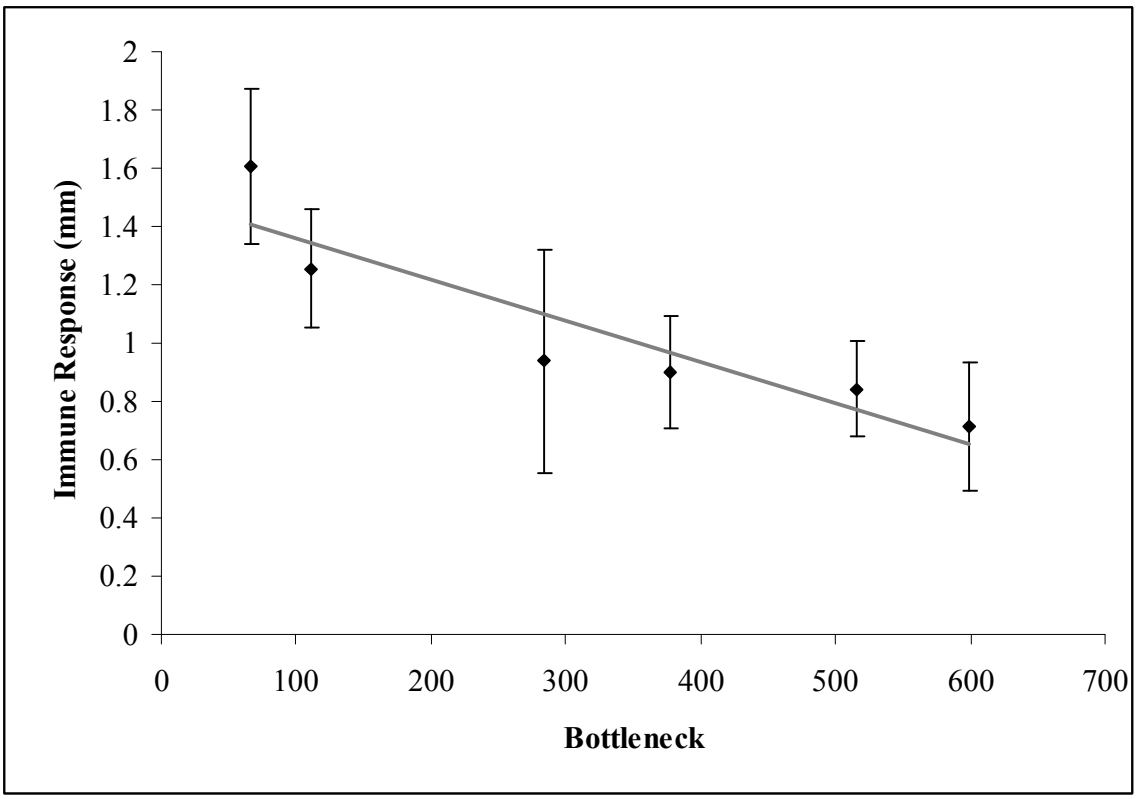

Figure 1. Mean Immune Response against Bottleneck Size. IR (mean ± SE) is averaged across both years. In the statistical analysis both variables were log transformed.

CHAPTER 4

A COMPARISON OF NESTLING IMMUNOCOMPETENCE BETWEEN TWO INTRODUCED BIRDS THAT EXPERIENCED DIFFERENT POPULATION BOTTLENECK SIZES

ABSTRACT

Population bottlenecks are hypothesised to cause a reduction in genetic diversity and increased inbreeding, and thus have negative fitness implications, including a reduction in immunocompetence. Bottlenecks are frequently experienced by species under conservation management, and thus determining fitness consequences is of crucial relevance. This study compared the immunological responses of nestlings (to the immunostimulant phytohaemagglutinin, PHA), in two related bird species (the myna, *Acridotheres tristis*, and the starling, *Sturnus vulgaris*) that were introduced into New Zealand in differing numbers (myna 70 birds, starling 653 birds), and hence experienced bottlenecks of differing severity. The prediction that myna nestlings would exhibit a lower immune response than starlings (as they originated from the more bottlenecked population) was not supported. No significant difference in immune response was found, and this was true after controlling for levels of ectoparasitic infestation. In addition, I found no evidence that growth rates were differentially affected in the two species following immune challenge. This suggests that relatively severe bottlenecks do not lead to a decrease in the component of immunity measured by the PHA assay. However, sample sizes of the current study were relatively small and interspecific competition may have confounded the results. This study highlights the need to consider interspecific interactions when conducting cross species comparisons.

INTRODUCTION

Population bottlenecks – the decline and subsequent increase of a population's size – are a common occurrence in conservation, yet the fitness implications of these events are far from understood. Whenever an endangered population is brought 'back from the brink', or when individuals of a threatened species are translocated to found a new population, a bottleneck occurs. The reduction of a population to a small number is hypothesized to cause a reduction in genetic diversity, both in terms of decreased allelic diversity, and decreased heterozygosity as a result of inbreeding (England et al., 2003; Keller and Waller, 2002). These predicted genetic effects have now been demonstrated in a number of wild populations (Ardern et al., 1997; Bodkin et al., 1999; Bouzat et al., 1998; Bradshaw et al., 2007; Hajji et al., 2007; Hoelzel, 1999; Hudson et al., 2000; Keller et al., 2001; Nyström et al., 2006; Packer et al., 1991; Zhang et al., 2004). Furthermore, a decrease in genetic diversity has been correlated with a reduction in fitness (Reed and Frankham, 2003), and increased extinction risk (Frankham, 2005). For example, in a recent study comparing heterozygosity of threatened and non-threatened related taxa, Speilman et al. (2004) confirmed that the heterozygosity of threatened species was on average 35% lower than in the non-threatened counterparts.

Whilst it is clear that population bottlenecks, via inbreeding and genetic impoverishment, may have severe consequences to a population's fitness, and ultimately to its survival, the question of relevance for conservation managers is how are these fitness costs manifested, and at what severity or size of bottleneck? Knowing how fitness costs may vary with bottleneck size could thus help with the design of management strategies for endangered species.

One fitness trait that may be negatively affected by population bottlenecks is the capability of an individual to mount an immune response (i.e., its immunocompetence). The immunological system provides complex and dynamic protection against a wide array of parasites and pathogens, and is at the core of an animal's ability to survive and reproduce. Reduced genetic diversity and/or inbreeding has previously been linked to reduced immunocompetence and increased susceptibility to pathogens (Coltman et al., 1999; Hawley et al., 2005; Pearman and Garner, 2005; Reid et al., 2003; Reid et al., 2007; Sanjayan et al., 1996). However, the effects of population bottlenecks on immunity have rarely been explicitly investigated. An exception to this is work done by Hale and Briskie (2007), who found that a bottlenecked population of the endemic New Zealand robin (*Petroica australis*), displayed significantly weaker responses to an immune challenge (in the autumn) than their source population. However, the bottleneck the robins experienced was extremely severe (the population was founded by 5 individuals), and whether a similar response occurs in populations that have been subject to less severe bottleneck sizes, or of another species, is unknown.

This study aims to compare the immunological responses of nestlings, in two wild bird species, introduced into New Zealand in the late 1800's (Lever, 1987; Thompson, 1922), that have experienced differing sizes of bottlenecks during their establishment. The myna (*Acridotheres tristis*), a species that passed through a severe bottleneck (< 70 birds), is compared with the starling (*Sturnus vulgaris*), a species that passed through a moderate bottleneck (650 birds) (Lever, 1987). These two species provide an ideal model system as both are cavity nesters (allowing a large sample of nests to be followed simultaneously), both have similar ecologies and life history strategies (Feare and Craig,

1999), and both are in the same family (thus minimising any phylogenetic effects). The genetic differentiation of both mynas and starling populations in New Zealand from their source populations (in India and United Kingdom (UK), respectively) has previously been assessed (Baker and Moeed, 1987; Ross, 1983), and suggests that whilst starlings exhibit similar genetic variation as their UK counterparts (measured via proportion of polymorphic loci and expected heterozygosity), mynas exhibited a reduction when compared to Indian populations (Merilä et al., 1996). This suggests that of the two species, only the myna (that experienced a severe bottleneck of 70 individuals) suffered a reduction in genetic variation. However, these studies employed allozymes to estimate levels of genetic diversity between native and introduced populations and given the low resolving power of allozymes (particularly in birds, Crochet, 2000), further studies employing higher resolution molecular markers (e.g., microsatellites) are warranted.

To assess immune response, nestlings were challenged with the immunostimulant, phytohaemagglutinin (PHA), which is a well established test of immune function in birds (Goto et al., 1978; McCorkle et al., 1980; Smits et al., 1999). The immune stressors a nestling faces may impact on the immune response exhibited (e.g. Gwinner et al., 2000), accordingly ectoparasitic infestation was also assessed. Finally, as mounting an immune response may be costly for chicks (Lochmiller et al., 1993; Zuk and Stoehr, 2002), and may be traded-off against important functions such as growth (Fair et al., 1999), the mass change (during immune challenge) in chicks of the two species was measured.

My prediction was that nestlings of the less severely bottlenecked species (starlings), would mount stronger immune responses than the more severely bottlenecked species (myna), after controlling for the effects of ectoparasitic infection. Furthermore, I

predicted that starling chicks would be better able to cope with the increased energetic requirements of mounting an immune response than mynas, and hence their growth (as measured by mass change) would be less affected by the immune challenge.

METHODS

STUDY POPULATION

Starlings (*Sturnus vulgaris*) and the mynas (*Acridotheres tristis*) were both released in New Zealand (NZ) in the 19th century and are now common. Both belong to the family Sturnidae (Feare and Craig, 1999) and share similar life histories. A total of approximately 650 starlings were brought to NZ from the UK (Lever, 1987; Thompson, 1922) and they are now abundant across NZ (Robertson et al., 2007). The myna is native to India, but the mynas introduced to NZ were originally sourced from an established introduced population in Australia (Baker and Mooed 1987). Approximately 70 individuals were successfully released (Lever, 1987; Thompson, 1922) and are now common on the North Island (Robertson et al., 2007).

I studied a nest box population of the common myna and the starling, located on farmland at Limestone Downs, North Island, New Zealand (37°29' S, 174°46' E). Fifty nest boxes were erected in June 2005 and a further 130 in January 2006, making a total of 180 boxes. The nest boxes for both species were identical apart from the size of the entrance hole (65 mm for mynas and 55 mm for starlings), and had hinged lids to enable access to the chicks. My study took place from November 2006- March 2007, and constituted the first season the boxes were in use for the majority of nest boxes. Starlings commence breeding earlier than mynas in New Zealand (Heather and Robertson, 1996),

and our study population had completed (or were completing) their first brood at commencement of the study, whilst mynas were beginning their first broods. Hence, data was collected from second broods only for starlings, but both broods for mynas. Immunological and parasitic indices can differ between first and second broods (Christe et al., 2001; Sorci et al., 1997) and so comparisons between species were only conducted on second broods. However, climatic conditions may also influence these same indices (Christe et al., 2001), and as second brood starlings were raised at the same time as first brood mynas, the same analyses were conducted on second brood starlings vs. first brood mynas. See table 1 for sample sizes and other summary statistics.

Nest boxes were visited regularly during the pre-laying period to ascertain lay date, and hatch date. Once hatched, nests were visited every three days, from day 2 (day 1= hatch day), and chicks were measured as part of a separate study. I refrained from visiting the nests close to fledging, to prevent forced fledging of the young, and as mynas have a longer nestling period than starlings, myna nest visits ended on day 20 - 23 whilst visits to starling nests ended on day 17 - 20.

MEASUREMENTS OF MITE LOAD

Chicks

Nestlings were examined every 3 days (starting on day 2) for the presence of blood feedings mites (Order: Acarina), until they became too feathered to allow reliable assessment (until day 11 for starlings and day 14 for mynas). As chicks were extracted from the nest they were comprehensively examined and assigned a 'chick mite score' (where 1 < 5 mites, 2 < 10 mites, 3 < 20 mites, 4 < 30 mites, 5 < 50 mites, and 6 = 50+

mites). Nestlings were also examined for the presence of other ectoparasites; ticks (Order: Ixodida) and chewing lice (Order: Phthirpatera) were found, but in very low numbers (lice ~ 6 nests, ticks ~ 1 nest), and were subsequently not included in the study.

Nests

Total mite infestation per nest box was estimated every three days, from day 8 (both species) until day 20 (starlings) or day 23 (mynas). Nestlings were removed from the nest box (to be measured for a separate study), and a sheet of white A5 paper was placed within the nest box, and the lid closed. The paper was left in the box for 20 min (mean ± SD, 22 ± 8), and on removal from the box the number of mites on the paper were either counted by eye, or, if numbers were too high, pictures were taken and subsequently counted. The number of mites was then divided by brood size, to give the final 'nest mite score'.

IMMUNOLOGICAL ASSAY

Immune response was assessed by challenging nestlings with Phytohaemagluttinin (PHA) – an immunostimulant that when injected causes a localised inflammatory response, the swelling response being commensurate with a stronger immune response (Goto et al., 1978; McCorkle et al., 1980; Smits et al., 1999). The procedure involves the subcutaneous injection of PHA into the bird's wing web (patagium), and measuring the resultant swelling response 24 hours later.

As immune response may vary in accordance with developmental age, I wished to compare immune response in nestlings of the two species at a similar developmental stage. Mynas have a longer nestling period than starlings (mynas 25 - 30, starlings 21 -

23) (Counsilman, 1974; Feare and Craig, 1999; Heather and Robertson, 1996), and to account for this difference, I conducted the immune response tests on day 16 for starlings and day 19 for mynas. At these ages, the nestlings are at a similar developmental stage.

Prior to injection of PHA, the left and right wing webs of the nestlings were swabbed with ethanol to clear the area of feathers and to sterilise the injection site and a mark made with permanent ink (see Figure 1). Thickness of the left and right patagium was measured using a digital micrometer precise to 0.001 mm (Mitutoyo, 395-371, Tokyo, Japan). All measurements were taken by the same individual (S. Allen), and repeated three times (measurements were highly repeatable $r^2 = 0.79 – 0.98$, $P < 0.001$). Following measurement, the left wing web was injected with 100 µl of PHA dissolved in phosphate buffered saline (PBS) at a solution of 1mg ml^{-1}, whilst the right wing was not injected and thus acted as a control for differential growth rates between individuals. Nestlings were returned to the nest box, and 24 hours later both wings were re-measured.

Immune response (IR) was quantified as the mean difference of the increase in thickness of the left and right patagium, relative to the initial thickness of the left patagium:

$$IR = \text{left } (PT_{post} – PT_{pre}) - \text{right } (PT_{post} – PT_{pre})/PT_{pre}$$

Where PT_{pre} and PT_{post} is the mean patagium measurement pre-and post-injection, respectively. A positive IR score indicates the patagium has swollen in response to the PHA injection, with higher IR scores corresponding to a greater relative increase, and hence a stronger immune response

At every nest, only half the brood were challenged with PHA; this was to accommodate a concurrent study that required some nestlings remain free from immunological challenge. These nestlings underwent the same wing web measurement process, and thus acted as an additional control. Starlings and mynas had brood sizes of 2 – 5 chicks (mynas 3 - 5, starlings 2 - 4). When brood sizes were even, exactly half of the chicks were tested (1 out of 2, 2 out of 4), while for odd-numbered brood sizes, more than half were challenged (i.e., 2 out of 3, 3 out of 5). Because selecting chicks from the nest was non-random, with larger chicks being preferentially selected (S. Allen pers. obs.), the allocation of treatment (PHA or non-PHA) was based on nestling mass at 2 days prior (day 14 for starlings and day 17 for mynas). PHA treatment was allocated to the heaviest chick, then the third heaviest and so on. Chicks were weighed just prior to injection and again, when re-measured 24 hours later.

STATISTICAL ANALYSES

I used general linear mixed effects models (GLMM) to examine if there were species differences in mite loads. Mixed effects models allow the analysis of observations structured in groups, when within-group errors are correlated and have unequal variances, via the specification of fixed and random effects (Crawley, 2007). The use of mixed effects models in my study enabled the use of individual chick data, whilst specifying the non-independence of chicks from the same nest, thereby avoiding pseudoreplication.

As explained above, for completeness I conducted species comparisons on two data sets - second broods of both species, and on first brood mynas vs. second brood

starlings, however, unless differences between the two data sets were found, all results presented are for second broods only.

Mite Load

The number of mites infesting a nest box may change over the course of the nestling period; this temporal change was accordingly investigated in this study. As the two species differed somewhat in their nestling periods (21 days for starlings and 25 days for mynas), but were measured at the same time intervals (every 3 days), a new variable, 'period' was created, of measurement day relative to total nestling period (i.e., measurement day/ total nestling period). Chick mite scores were averaged per nest box, and thus are a representation of the average mite load per chick, at each period. Nest mite scores (log +1 transformed) and chick mite scores (log+1 transformed) were both analysed using a GLMM, with period (and period^2 the quadratic term), species, and first order interactions as fixed effects, and nest box specified as the random effect. Only second broods for both species were analysed. The model was simplified using a backwards stepwise deletion method (at significance $P < 0.05$).

Differences in mite infestation, (quantified by both chick mite scores and nest mite scores) between the two species was analysed using GLMMs. The difference in mite infestation (chick or nest score) between species was re-analysed using a GLMM with species as a fixed effect, and a specifying a random effect structure of nest within period, to account for the temporal and spatial pseudoreplication.

Immune Response

Initially, immune response (IR), measured as the relative increase in patagium thickness (see above) of nestlings that had been challenged with PHA was compared with the 'immune response' on the non-challenged nestlings (calculated in the same way). Immune challenge (categorised as either yes or no) was specified as the fixed effect, with nest box as a random effect, and IR as the response variable. IR was significantly higher ($P < 0.05$) in the immune challenged nestlings of both species, indicating that the PHA challenge elicited an immune response in both species under study.

Species differences in IR were investigated using a GLMM with chick IR (log+10 transformed) as the response variable, species (starling or myna) as the fixed effect, and nest box as the random effect. One starling chick was excluded from the final analyses, as it exhibited an abnormally large response to the immunological assay (however preliminary analyses with the chick included did not change the significance of the results). Hypersensitivity reactions are known to occur rarely in individuals challenged by PHA (Smits et al., 1999), and the chick subsequently fledged successfully.

The effect of mite infestation on immune response in the two species was then analysed. Due to the temporal influence on mite scores, mean values were calculated for both nest and chick infestation measurements and these new variables (mean nest score & mean chick score) were used in subsequent analyses. For chicks, the average of the last two measurement days (days 8 & 11 for starlings, days 11 & 14 for mynas) were used, and for nest box scores, averages of the two measurements before and after the PHA challenge were used (days 14 & 17 for starlings, and days 17 & 20 for mynas). GLMMs were used to test mite infection, with IR (log +10 transformed) as the response variable,

species and mean nest score (or mean chick score), and the interaction term were fitted as main effects, and nest as a random effect.

Mass Change

The effect of PHA challenge on mass gain in nestlings was also investigated. Mass gain was calculated as the percentage change in mass of individual chicks from the time of PHA administration, to when the chicks were re-measured 24 hours later. Initially single species analyses were conducted, comparing mass change in immune challenged chicks to non-challenged chicks (GLMM, mass change as response variable, immune challenge as explanatory and nest ID as random effect). An interspecific comparison was then conducted, where mass change was fitted in a GLMM as a response variable, and 'challenge' (yes or no), species, and interaction between the two fitted as fixed effects, with nest as the random factor.

RESULTS

Mite Loads

The mean mite load per chick increased significantly ($P < 0.001$) over the period measured (mynas – days 2 - 14, starlings days 2 - 11; Table 2, Figure 2). Starling chicks had significantly higher mite loads throughout the nestling period measured ($P = 0.001$).

The infestation level in nest boxes, corrected for brood size, changed significantly over the period measured (mynas days 8-23, starlings days 8-20), and was best described with a quadratic term (Table 3, Figure 3). Mite infestation was not significantly different between the two species ($P = 0.2$) for this period of the nestling period.

Immunological assay

No significant differences were found between the two species in response to PHA ($P = 0.77$) when a GLMM was fitted, with immune response (IRlog) as the response variable, and species as the fixed effect, and nest ID as the random (Figure 4, Table 1).

The effect of individual chick mite load (mean of days 8 & 11 for starlings, days 11 & 14 for mynas) on immune response was investigated, by fitting chick load, species and the interaction between the two in a GLMM (with nest ID as the random effect). None of the terms were significant, indicating that a nestling's mite load prior to immune challenge had no effect on immune response, for either species (Figure 5).

The effect of nest box infestation (mean of days 14 & 17 for starlings and days 17 & 20 for mynas), corrected for brood size, on immune response (log+10 transformed) was also investigated. A GLMM with IR as the response variable, nest mite load, species and the interaction between the two was fitted as fixed effects, with nest ID as the random effect. None of the terms were significant, indicating the level of mite infestation had no effect on immune response in either species (Figure 6).

Mass change

The effect of immune challenge on percentage mass gain was first examined in the two species separately. Immune challenge had no significant effect on mass change in either species (starling: $P = 0.3$, myna: $P = 0.9$; Figures 7 a & b).

A GLMM, with IR (log+10 transformed) as the response variable, species, mass change and the first order interaction as fixed effects and nest ID as the random effect was run to determine if mass change had any effect on the immune responses of the two species. None of the terms were significant, indicating that an individual's change in

mass had no affect on its immune response, and that this did not differ between species (Figure 8).

DISCUSSION.

In this study I compared the immune response in two bird species that had previously experienced population bottlenecks of considerably different severity and found that nestling immune response did not differ between the two species. On average myna nestlings (the more severely bottlenecked species) mounted a similar response as starling chicks. Starlings experienced higher levels of infestation by haematophagus mites (Order: Acarina) than mynas, but immune response was not correlated with mite burden, and the two species' responses remained the same after controlling for mite infestation. Furthermore, the immune challenge did not have any effect on nestling mass gain during the immune assay period (24 hours) for either species, and mass gain did not differ between species.

The immune response was measured as the amount of localized swelling induced by administration of PHA, an immunostimulant, that may activate both innate and acquired (T-cell mediated) aspects of the immune system (Kennedy and Nager, 2006; Martin et al., 2006). Overall, it would appear that nestlings from a population that had experienced a relatively severe bottleneck (70 individuals) were equally as able to mount an immune response of this nature as nestlings from a less bottlenecked population. A previous study found that ndividuals from a population of New Zealand robins that had passed through a severe bottleneck (of 5 individuals) were immunocompromised compared to their source population (Hale and Briskie, 2007). It may be that a bottleneck of 5 birds causes a decrease in immunocompetence, whilst less severe bottlenecks (e.g.,

in this study 70 birds) do not have the same negative consequences. Under this interpretation, my results would suggest there is little negative effect of bottleneck sizes on immunocompetence at least above founding populations of 70 or more individuals.

On the other hand, the disparity in results between our two studies could arise for a number of other reasons, any of which might mask underlying differences in immunocompetence between starlings and mynas. For example, the study of robins was conducted on adult birds in the autumn season. In the autumn, adult birds, after experiencing a breeding season and moult, may be under greater immunological, energetic, and environmental stress than nestlings being provisioned by their parents in a nest box, and hence differences in immunocompetence may be more emphasized. Furthermore, the robin study compared two conspecifics populations, whilst this study compared two different species. Mynas and starlings are closely related species (Feare and Craig, 1999) which should minimise phylogenetic differences but there may still be species-specific differences in immune function that mask any subtle immunological effects of population bottlenecks. The sample sizes of my study were also quite small (number of nestlings tested; mynas n = 13, starlings n = 8), and there was considerable individual variation in immune response within a species (see Figure 4) Larger samples sizes may be required to detect interspecific differences. Finally, the comparison of immune responses of myna and starling populations in New Zealand with their non-bottlenecked source populations (India and UK, respectively) would overcome the difficulties in interspecific comparison, although such a study might be confounded by habitat and climate differences.

Avian immunity is a complex and dynamic system, and the PHA test only measures one small part of an animal's immunocompetence. There is evidence that differing components of the immune system are differentially activated (Hõrak et al., 2006; Kennedy and Nager, 2006; Norris and Evans, 2000), hence whilst two populations can exhibit similar PHA responses they could differ in other aspects of their immunity. The simultaneous assessment of several components of the immune system (e.g., innate, and cell-mediated and humoural immunity) would be informative in this instance. The use of multiple tests would be especially important from a conservation perspective to ensure that small population bottlenecks, such as the 70 birds experienced by the myna, really do not have any serious consequences on immunocompetence across the full spectrum of possible immune responses.

An individual's immune system is locked in a constant battle with the pathogens and parasites it encounters, and individuals from a population prone to higher rates of parasitism or disease might be expected to invest more in immune function than less parasitised populations (e.g. Lindstrom et al., 2004; Tschirren and Richner, 2006). The immunological response of an individual to PHA may thus be an indication of that individual's current investment in immunity, and should be assessed in the context of the current immunological stressors an individual is experiencing. In other words, understanding the immune response of an individual requires knowing its past and current exposure to potential parasites and pathogens.

In this study I assessed the ectoparasite burden the nestlings experienced as an index of their exposure to potential stressors. The only ectoparasites found regularly on the nestlings and in the nest boxes were blood-feeding mites. Lice (Order: Phthiraptera)

and ticks (Order:Acarina) were rarely found on nestlings, and hence ectoparasite burden was quantified entirely by mite infestation. A previous study of nestling starlings in Europe found that chicks from nests with high mite loads had stronger immune responses (Gwinner et al., 2000), and these authors hypothesized that the chicks may be sensitized by the blood feeding activities of the mites. In my study, nestling starlings from second broods had significantly higher levels of mite infestation than mynas during the early stage of the nestling period, but this difference was non-significant for nest box infestation later in the nestling period. I found no relationship between mite load and immune response, neither when considering mite infestation at the time of PHA administration, nor when comparing the mite load individual chicks experienced earlier in the nestling period. Again, the small sample size of the current study (number of nests, myna n = 6, starling n = 5) limits my power to detect such an effect. However, no relationship was found between immune response and mite load when the analysis was repeated solely on mynas, but including both broods and boosting the sample size (22 nests, 44 chicks). Differences in the quantification of the level of infestation, or indeed the actual mite load experienced by the chicks in the two studies may well explain the discrepancy.

Ectoparasites are far from the only factor that may challenge a bird's immune system; whilst in the nest they may be exposed to a plethora of pathogens, including endoparasites, bacteria, fungi, viruses, and haematozoa (Slomczynski et al., 2006). However to conduct a full audit of nestling immunostressors is challenging in the extreme, and rarely achievable in wild populations. It seems likely that the starling and myna nestlings in this study were exposed to similar parasites and pathogens, as they

inhabited the same area, used the same nest boxes, and have similar diets (Higgins et al., 2006). Indeed, whilst mite infestation was on average somewhat higher in starlings compared to mynas early in the nestling period, the infestation patterns were almost identical (see Figures 2 & 3), and when infestation of nest boxes later in the nestling period was investigated the difference between the two species disappeared, suggesting similar parasitic infection profiles. Nonetheless, it would be worth conducting further surveys of other potential pathogens as far as feasible.

Mounting an immune response is energetically costly (Lochmiller and Deerenberg, 2000; Lochmiller et al., 1993) and a number of other factors may influence a nestling's ability to respond to an immunostimulant. During the course of the field work it was noted that a high level of interspecific competition existed between mynas and starlings, and that mynas were dominant (SE Allen and CA Debruyne, pers. obs.). This observation has also been made in a previous study of mynas in New Zealand (Counsilman, 1974). In Australia, Pell and Tideman (1997) quantified the 'winners' and 'losers' in a series of aggressive interspecific interactions between the myna and starling (and other species), during the period of nest site selection and reproduction, and they found mynas won all interactions with starlings (18 out of 18). In the case of my study, such interspecific competition may confound the potential fitness effects of population bottlenecks. If mynas out-compete starlings, then starling nestlings may experience greater environmental stress than myna chicks (e.g., they may be less well provisioned, have 'poorer' nest sites). Hence the starling's ability to mount an equal immune response to the myna, whilst experiencing a less favourable rearing environment may indicate superior fitness. It would be illuminating to investigate this further, and would require

the comparison of mynas and starlings, that cohabit (as in this study) with populations that are isolated from one another. This issue highlights the need to consider interspecific interactions, when conducting cross species comparisons.

It is hypothesised that during the nestling period, trade-offs exist between the growth and immunity (Saino et al., 2002). Indeed several studies have demonstrated reduced nestling growth in response to antigenic challenge (Fair et al., 1999; Soler et al., 2003; Whitaker and Fair, 2002). This study compared mass gain in immune challenged chicks and their non-challenged brood mates, in the two species, during the challenge period of 24 hours. I predicted that immune-challenged myna chicks (i.e., the more severely bottlenecked species) may be more energetically constrained than starlings, and thus gain less weight than immune-challenged starlings. However, no difference was detected in weight gain between chicks that had been immune challenged, and ones that had not in either species. This may indicate that the swelling response elicited by PHA was not energetically costly, or that mass gain was not measured over an adequate period, or that another function of growth (e.g., feather growth) was affected instead.

My study detected no difference in nestling immune response between the starling and myna, despite the two populations experiencing considerably different sized bottlenecks during their introduction to New Zealand. This would suggest that populations that experience a relatively severe bottleneck (70 individuals) are equally as immunocompetent as less bottlenecked populations. However, this finding should be interpreted with some caution, as the sample sizes of the current study were relatively small, and interspecific competition may have confounded the results. This study

highlights the need to consider interspecific interactions when conducting cross species comparisons.

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TABLES

Table 1. Summary statistics of the bottleneck size, sample size (of nests and chicks that were immune challenged) and immune response (IR) of nestling mynas and starlings. See text for calculation of IR (note that mean IR is overall mean per species, per brood).

Species	Brood	Bottleneck[a]	Nests (n)	Chicks (n)	Mean IR (± SE)
Myna	1	70	16	31	0.98 ± 0.18
	2	70	6	13	1.04 ± 0.18
Starling	2	653	5	9	0.90 ± 0.24

[a] Data from Lever (1987)

Table 2. Results of a GLMM investigating the effects of nestling period and species on the mite load per chick in bottlenecked populations of the myna and starling (Nest ID is fitted as a random effect).

Predictor	Estimate	SE	p-value
(Intercept)	-0.224	0.162	
Species - Myna	0	--	--
- Starling	0.797	0.185	0.0012
Period	2.351	0.318	<0.0001

Table 3. Results of a GLMM investigating the effects of nestling period (including a quadratic term - 'period^2') and species on the nest box mite load in bottlenecked populations of mynas and starlings (Nest ID is fitted as a random effect).

Predictor	Estimate	SE	p-value
(Intercept)	4.34	1.98	
Species - Myna	0	---	
- Starling	0.95	0.69	0.20
Period	20.01	6.62	0.0048
Period ^{2}	-13.92	5.31	0.0131

FIGURES

a)

b)

c)

Figure 1. Conducting the PHA assay on a myna nestling:

a) swabbing the patagium to prepare the area;

b) measuring the thickness of the patagium using a stand-mounted micrometer;

c) administering the sub-cutaneous injection of PHA

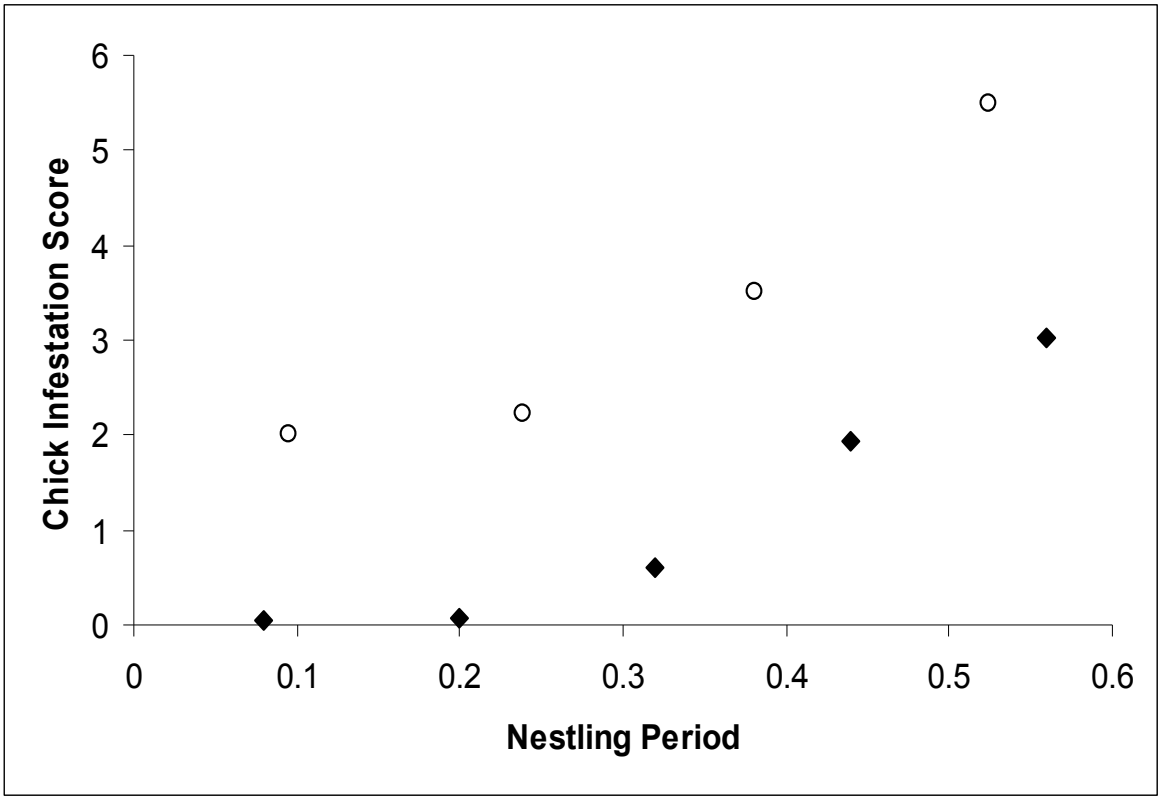


Figure 2. Mean chick mite load against nestling period (standardised as chick age/21 for starlings and chick age/25 for mynas). Starlings open circles (○) and mynas filled diamonds (♦). See text for definitions of chick infestations scores.

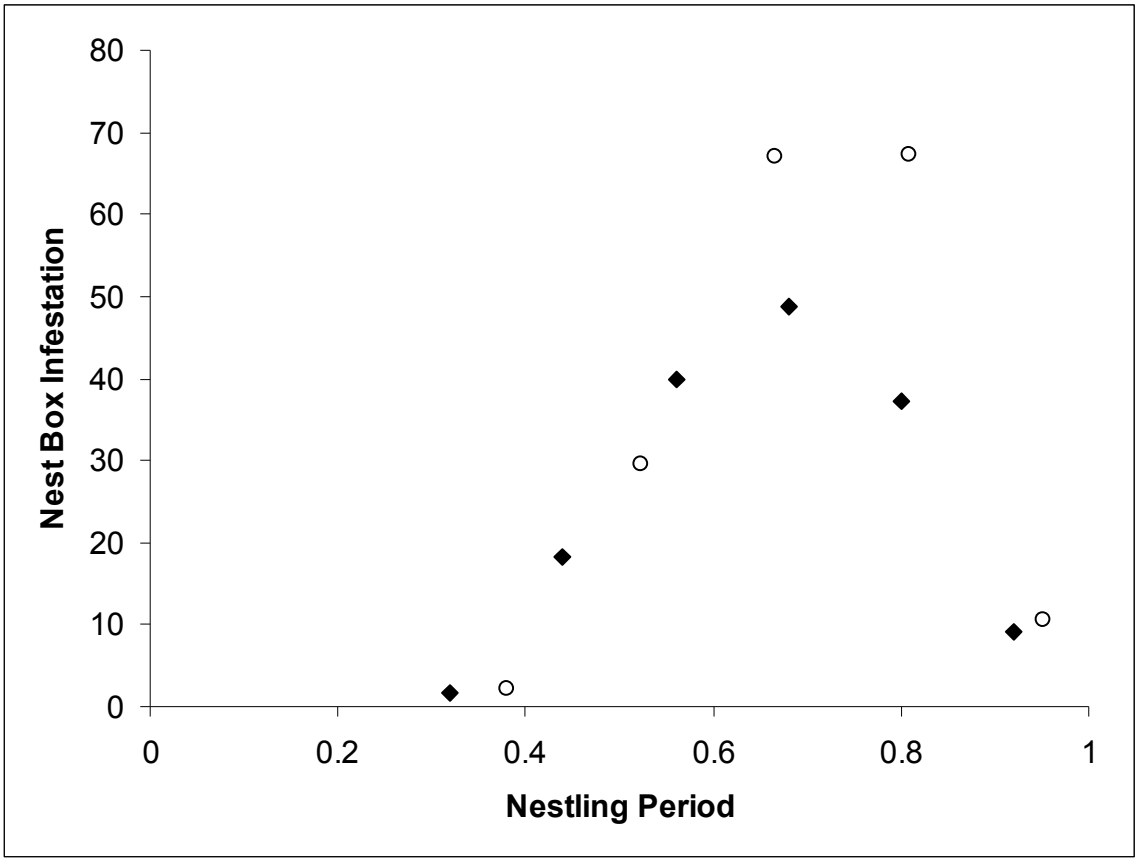


Figure 3. Mean nest box load of mites against nestling period (standardised as chick age/21 for starlings and chick age/25 for mynas). Starlings open circles (○) and mynas filled diamonds (♦).

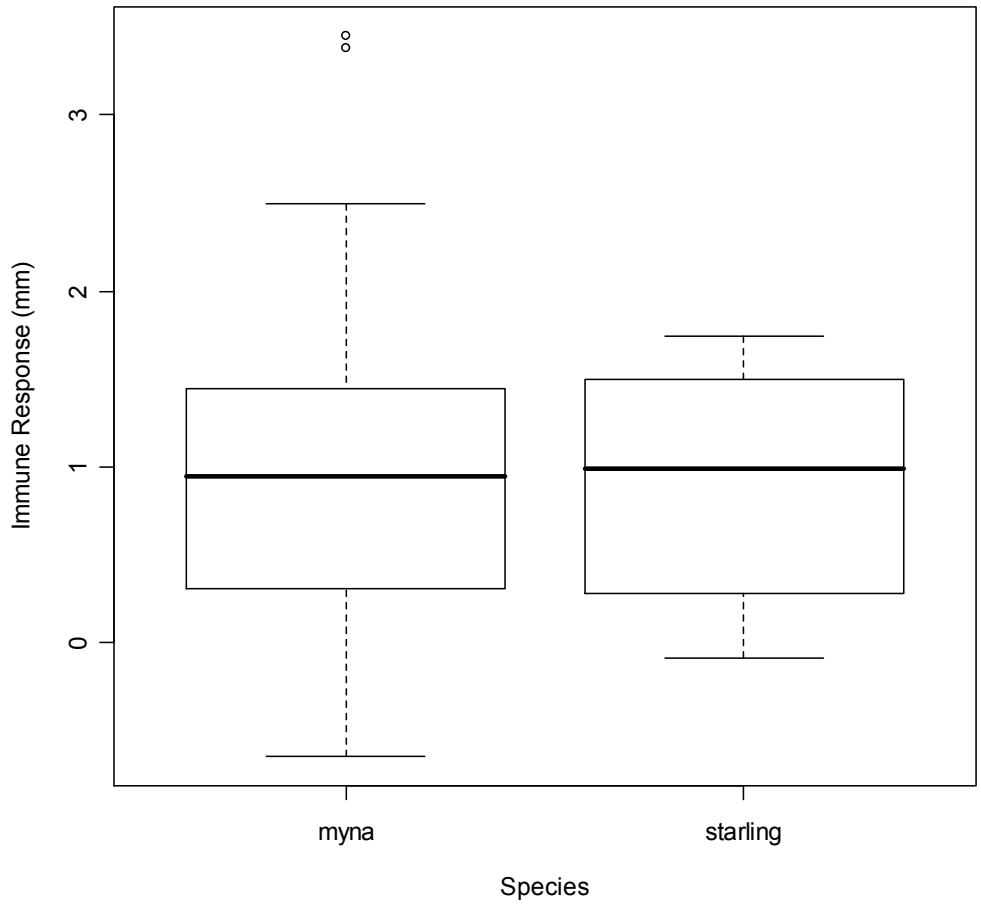


Figure 4. Box and whisker plot of the relative immune response (see text for details of calculation of immune response) in second brood nestling starlings and mynas. Horizontal line indicates median immune response (per species), bottom and top of box represents 25th and 75th percentiles respectively, dashed vertical lines show either the maximum, or 1.5 times the interquartile range (whichever is smaller), and data points beyond this value are marked as individual points

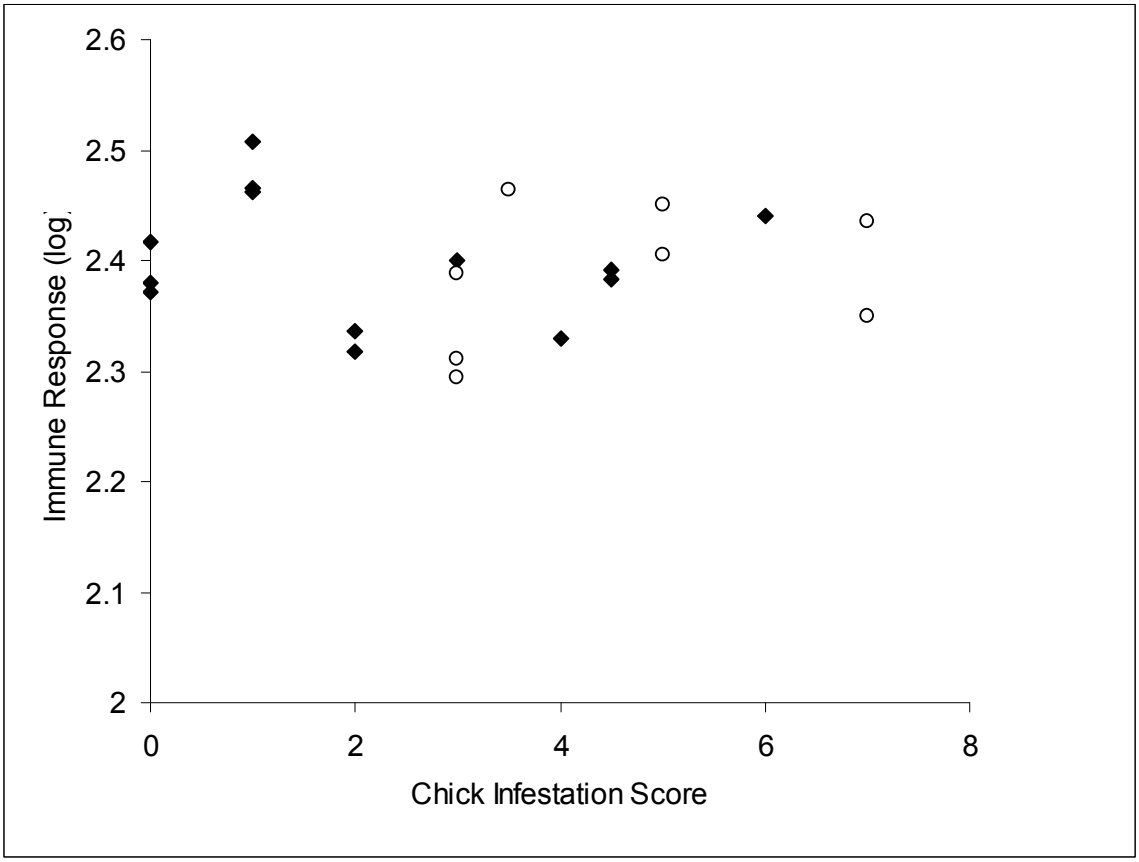


Figure 5. Chick infestation score (mean per chick of scores from days 8 & 11 in starlings and days 11 & 14 in mynas) against immune response. Starlings open circles (○) and mynas filled diamonds (♦).

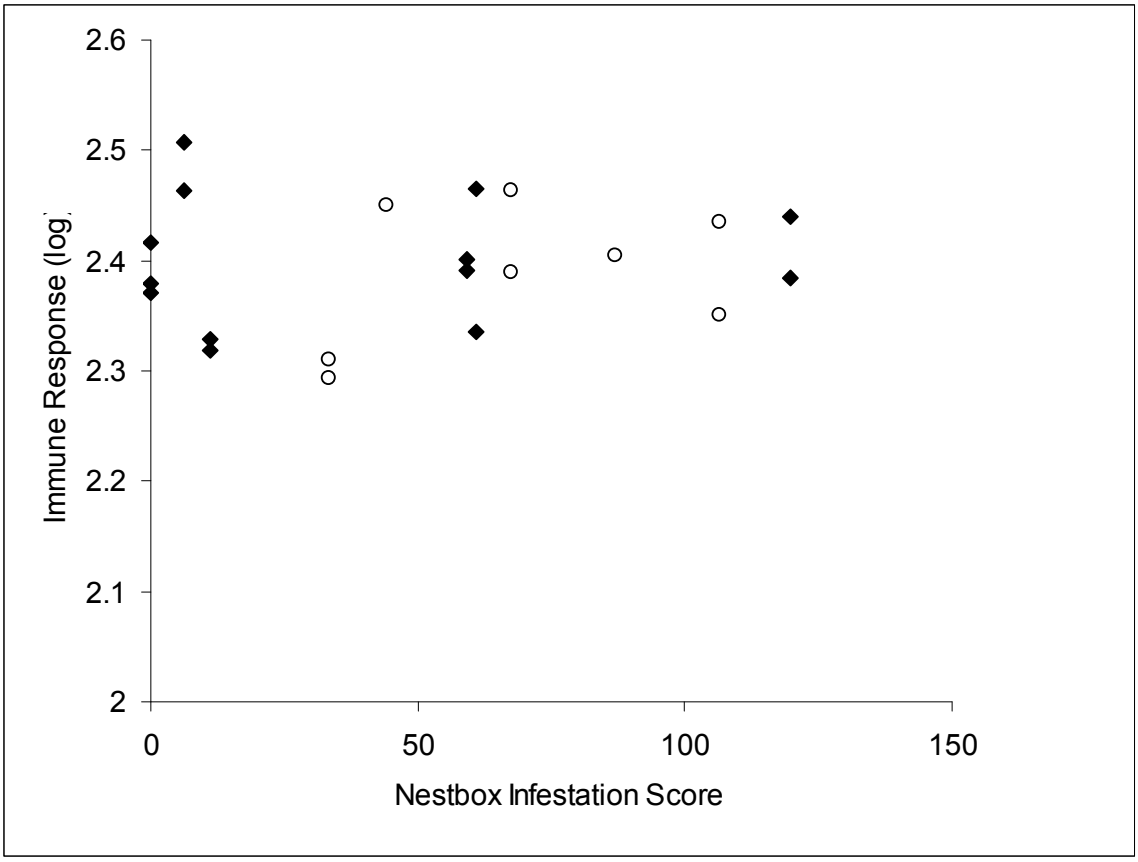
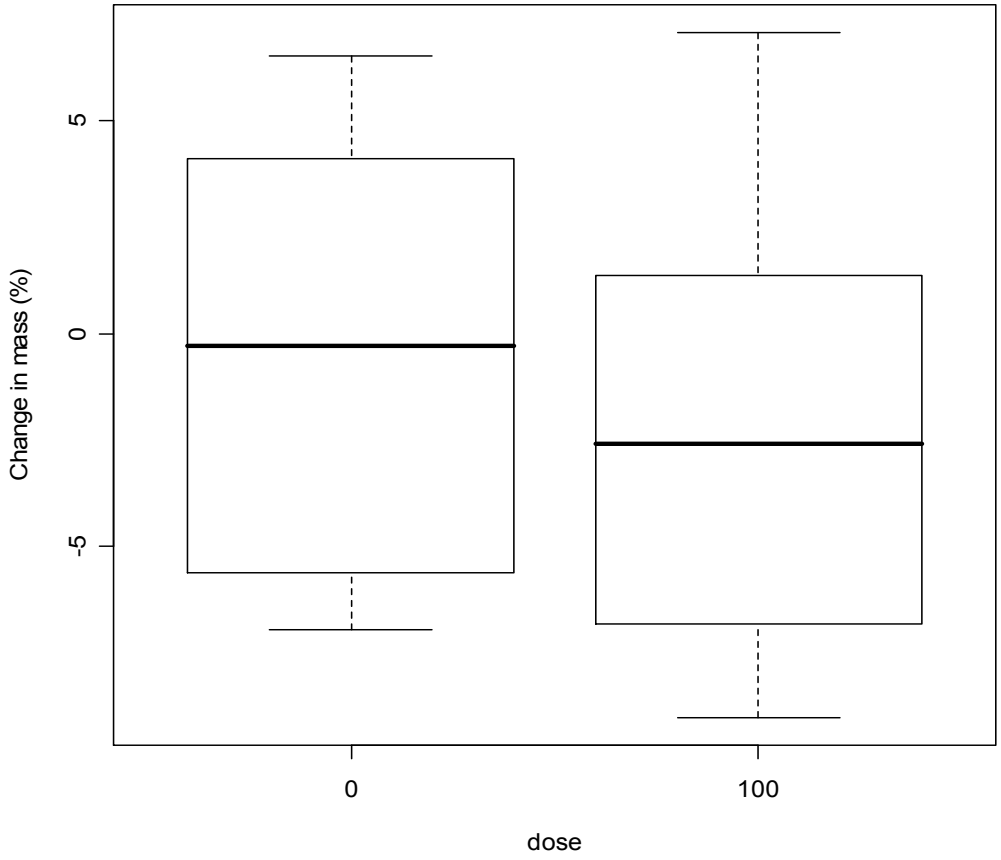


Figure 6. Nest box infestation score (mean nest score from days 14 & 17 in starlings and days 17 & 20 in mynas) against immune response. Starlings open circles (○) and mynas filled diamonds (♦).

a) Starling



b) Myna

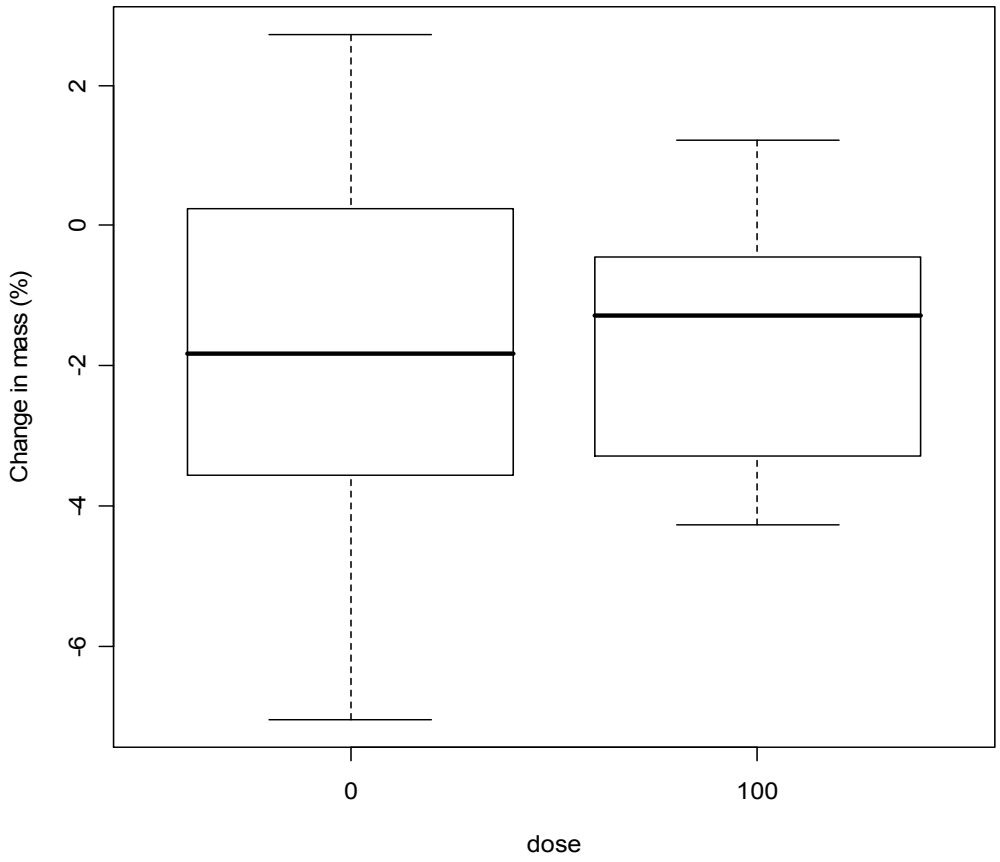


Figure 7. Change in mass (expressed as %) in starling (a) and myna (b) chicks over a 24-hour period following immune challenge. Dose 100 = chicks that were administered with PHA, Dose 0 = control chicks. Note that data are not nest box means, but include all chicks. See Figure 4 for explanation of Box and Whisker plots.

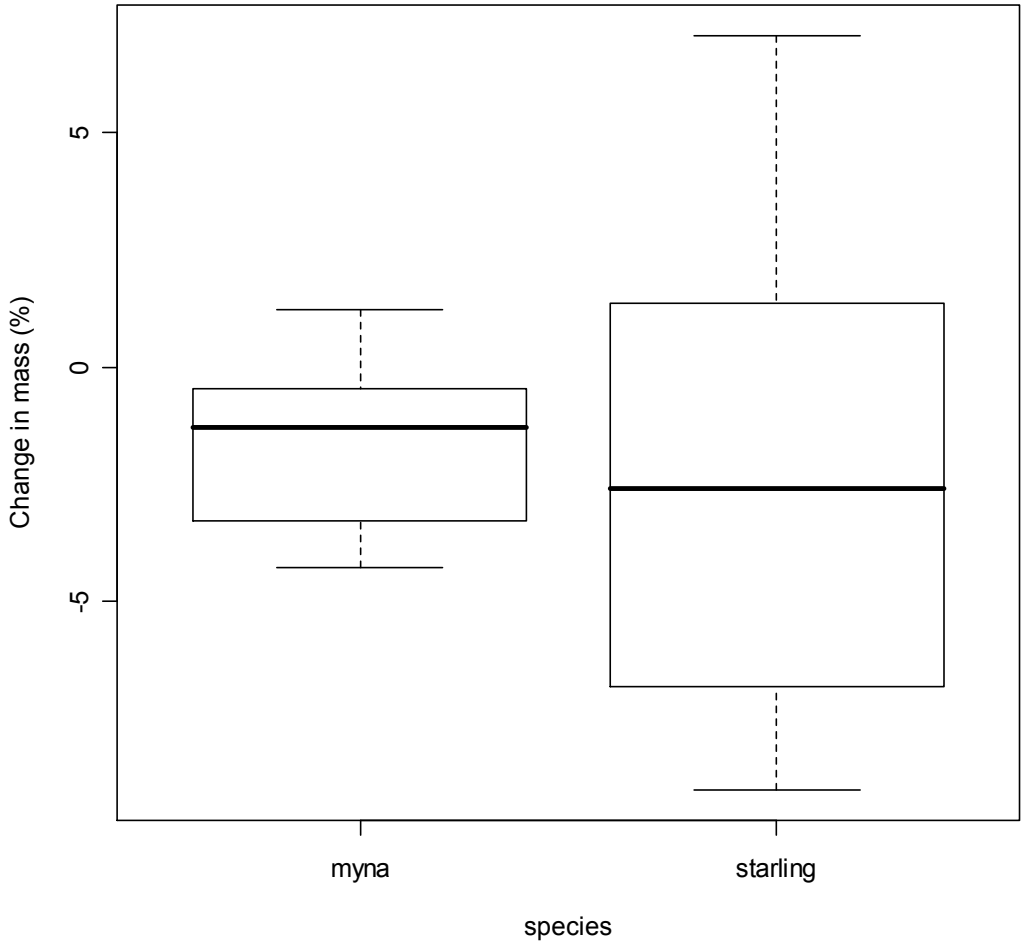


Figure 8. Change in mass (expressed as %) of immune challenged chicks over a 24-hour period following administration of PHA. NB: Data are not nest box means, but include all chicks. See Figure 4 for explanation of Box and Whisker plots

DISCUSSION

In this thesis I investigated levels of parasitic infection and immunocompetence in wild populations of introduced bird species in New Zealand that had experienced a range of population bottleneck sizes. My aims were two-fold; firstly to assess if population bottlenecks are linked to increased parasite loads and/or decreased immunocompetence, and secondly, to assess at what severity of bottleneck size these effects become evident. The ultimate goal of this work was to further our understanding of the problems faced by populations founded by low numbers of individuals or that experience a period of severe population decline (i.e., endangered and translocated species), and to thereby optimise the success of conservation projects dealing with such populations.

In terms of pathogenic and parasitic infection, I found evidence that both ectoparasitic load, and the prevalence of avian malaria were related to the severity of bottleneck a population experienced. The ectoparasite burden of chewing lice (Order: Phthirpatera, Suborders: Amblycera and Ischnocera) on introduced bird species in New Zealand was significantly affected by the severity of the bottleneck that each species had experienced, when compared to their source populations in the UK (Chapter 1). This relationship was found to be driven primarily by Amblycera, which may be the more virulent suborder of chewing lice, meaning they probably exert higher fitness costs than Ischnocera. Species in NZ that were introduced in low numbers, and therefore had experienced more severe bottlenecks, had significantly higher prevalence (i.e., number of birds carrying lice) of Amblycera when compared to their non-bottlenecked counterparts

in the UK, while loads were similar between the two countries at more moderate bottlenecks. Intensity of infestation (i.e., the number of parasites per infected individual), whilst not related to the severity of the bottleneck in amblyceran lice, was in the majority of cases higher in NZ than the UK. Furthermore, when differences in genera richness were investigated, I found that species that had experienced severe bottlenecks had higher genera richness in New Zealand, whilst the less bottlenecked species had greater diversity of genera in the UK. The prevalence of avian malaria (*Plasmodium* spp.) was also related to bottleneck size; within New Zealand I found that the number of individuals infected with *Plasmodium* parasites was higher in species that had experienced the more severe bottlenecks (Chapter 2).

Overall my results would suggest that the more severely bottlenecked species had higher levels of ectoparasitic and haematazoan infection. These parasites differ hugely in their life histories and mode of transmission (Clayton and Moore, 1997; Loye and Zuk, 1991), which lends support to the theory that bottleneck size is the common denominator. Birds experience a plethora of parasitic attack; indeed, it is estimated that birds (as a taxon) may support more than 58,000 species of parasites (Cromptom, 1997), and in this study I investigated a fraction of this potential parasite load. Whether population bottlenecks influence infection rates by other types of parasite (e.g. fungal, viral, bacterial), is at present unknown, but the evidence presented here, from two diverse parasite taxa, suggests a similar effect might be expected.

The relationship I found between population bottlenecks and immunocompetence was more complex. In chapter 2, I compared leucocyte profiles between NZ and UK populations of introduced species, and discovered that species in NZ that had experienced

more severe bottlenecks had significantly higher HL (Heterophil/Lymphocyte) ratios than their UK counterparts, and that this difference was correlated with severity of bottleneck (i.e., the most severely bottlenecked species exhibited the biggest difference). HL ratio is known to increase in response to a number of stressors, including infectious diseases and environmental disturbance (Ots and Hõrak, 1998). Whilst this can't be used as a direct assessment of immunocompetence (Norris and Evans, 2000), it suggests populations with a higher HL ratio, are under some form of increased stress (be that immunological, psychological or environmental). I also examined the haematological responses of species within New Zealand to malarial infection, comparing differences between infected and non-infected groups. Bottleneck size was correlated with changes in two haematological responses (total white blood cell, and differential lymphocyte count). As bottleneck size increased (became less severe) these responses increased in infected individuals, suggesting that less bottlenecked species were more able to mount an immune response to malarial infection (i.e., were more immunocompetent). The converse interpretation of these findings is that the more bottlenecked species were less impaired by malarial infection, and thus did not invest in an immunological response to the infection. This inference cannot be discounted but it seems the less likely of the two interpretations. Taken together, the raised HL ratio in species experiencing severe bottlenecks, and the lack of haematological response in malaria infected individuals of these same species, suggests that these populations may be immunocompromised as a result of the population bottleneck they experienced.

The experimental challenge of individuals with a novel immunostimulant can provide a more standardized approach to assessing immunocompetence (Norris and

Evans, 2000), than the observation of haematological parameters. In chapter 3, I examined the differences in immune response between six introduced bird species to the mitogen phytohaemagglutinin (PHA). Again I found a relationship existed between bottleneck and immunity, but in the opposite direction to that predicted; the more bottlenecked species exhibited the larger (i.e., stronger) responses. This finding highlights the complexity of making interpretations about the 'immunocompetence' of an individual, based on tests assessing one component of immunity in isolation (Salvante, 2006). Considering the findings of Chapter 1 (increased ectoparasite load), the larger immune response in the more bottlenecked populations may be an indication of increased investment in immunity, due to increased parasite and pathogen pressure. An alternative, but not mutually exclusive explanation is that components of the immune system may be differentially affected by population bottlenecks, for either genetic or energetic reasons.

In my final data chapter (Chapter 4), I examined the immune response to PHA in nestlings of two closely related species that differed in the bottleneck size they experienced, and placed this immune response in the context of ectoparasitic infestation. I found no differences in immune response between the two species (myna and starling), despite them experiencing considerably different bottlenecks (70 vs 653 birds, respectively). However, this result may have been confounded by interspecific competition, highlighting a potential problem of conducting cross species comparisons.

Taken together my findings have important implications for the two questions I set out to answer with this thesis and that bear directly on the conservation relevance of population bottlenecks. Firstly, do population bottlenecks have negative impacts on parasite load and immunocompetence? The evidence presented here strongly suggests

that populations that experience a significant reduction in size are more prone to infection by avian malaria and infestation by ectoparasites, at least in the introduced bird species of New Zealand. The relationship between immunity and bottleneck size was more ambiguous, which no doubt reflects the complex and dynamic nature of the vertebrate immune system. Nevertheless, I conclude that population bottlenecks do appear to impact on immunity, but that this impact may differ depending on the component of immunity under study, and the current pathogenic pressures on the population under question.

The second, and more practical question to conservation managers is – when is a bottleneck, really a bottleneck? In other words, at what severity of bottleneck do the negative consequences become evident, and hence, what is the minimum number of individuals required to found a new population to avoid these problems. It is to be expected that different aspects of a population's fitness (and indeed different populations/species) will be differentially affected by bottlenecks – there is unlikely to be a 'magic number' below which populations will falter and fail, above which they will be gloriously healthy and sustainable. However, in two differing aspects of this study that allowed direct comparison between the source (non-bottlenecked) population and the bottlenecked population (i.e. ectoparasite load and HL ratio), the three most bottlenecked species (greenfinches, house sparrows and dunnocks, bottlenecks of 66, 111 and 284 respectively) experienced raised parasitism and HL ratio. HL ratio in birds can be used as an indication of a general stress response (including immunological stress) (Maxwell, 1993; Ots et al., 1998), and it may be that increased ectoparasite load is driving the raised stress levels, or conversely that birds under environmental or immunological stress are

more prone to ectoparasitic infection. Regardless of the mechanisms underlying this relationship, the conclusion that can be drawn is that a population founded by low numbers may be prone to higher ectoparasite loads, and higher general stress levels, than a healthy, less bottlenecked population. Conclusions are harder to make from my findings on immunocompetence and avian malaria, as no direct comparison between the source populations in the UK was possible. However, the populations experiencing the more severe bottlenecks had a higher prevalence of malaria and lower immune response. Conversely, these same species had the highest response to the PHA assay, although this may be reflection of the increased parasite/pathogen load.

Briskie and MacKintosh (2004) investigated this same question of minimum bottleneck size in terms of levels of hatching failure in introduced and native species in New Zealand, and concluded that hatching failure was significantly increased in species that had passed through bottlenecks of less than 150 individuals, and did not reach pre-bottleneck levels until a population was founded by 600 individuals. Thus my findings of bottlenecks falls within the range suggested by this earlier study.

In a global survey of translocation schemes (Griffith et al., 1989; Wolf et al., 1996) it was found that in 1993, the median number of individuals released was 50.5 (an increase from 31.5 individuals in a similar survey done in 1987). In this study, only greenfinches, with a bottleneck of 66 birds, fell in the range used in translocation schemes (Lever, 1987). This species consistently exhibited significant differences when compared to their con-specifics in the UK, in both parasite load and haematological parameters, and had a higher prevalence of avian malaria and lowered leucocyte response to infection than less bottlenecked species. If the greenfinch is representative of other

species that have passed through similar sized bottlenecks used in translocation schemes, then it is possible similar problems might be widespread. That being said, greenfinches are extremely successful in New Zealand today despite their small bottleneck (MacLeod et al., 2008; Robertson et al., 2007). It may be that whilst I found statistically 'significant' differences in various measures of parasite load and immunocompetence, these differences don't translate to being biologically significant; they may not cause a decrease in fitness. Alternatively, greenfinches would be even more successful if the were not limited by their past bottleneck, or New Zealand may present such a benign environment (MacLeod et al., 2008) that they are successful despite their fitness handicap. If anything, the fitness effects of population bottlenecks are likely to be more pronounced in endangered species, as they are likely to experience greater environmental stress than the introduced species under study here. A rule of thumb then, for conservation practitioners considering how many individuals to translocate, might be the 'bigger the better', and over a couple of hundred would be best. Obviously, in the case of many vulnerable species, this will not be possible, however, an understanding of the added problems populations founded by small sizes face will surely aid in the management of these species.

As in most studies, the number of questions created by the findings far exceeds the numbers answered. The relationship between immunity and population bottleneck was particularly complex (as would be expected in such a diverse system), and future studies simultaneously investigating several components of immunity (e.g. Matson et al., 2006) in introduced birds would be extremely illuminating. Whilst this study established a link between both ectoparasitic load and avian malaria and the size of the population

bottleneck, no inference could be made of the actual fitness costs to the individual or population experiencing these increased pathogenic pressures. Studies examining the fitness consequences of these parasites in introduced birds should be achievable and informative.

Whilst the evidence is mounting that introduced bird species in New Zealand do experience fitness costs in relation to their past bottleneck ((Briskie and Mackintosh, 2004; Debruyne, 2008), whether the population bottlenecks experienced by these species is reflected in their genetic diversity is yet to be answered. Past genetic studies are ambiguous (Baker, 1992; Baker and Moeed, 1987; Baker et al., 1990; Merilä et al., 1996; Parkin and Cole, 1985; Ross, 1983), and all employed allozymes which may have a low resolving power (Crochet, 2000). Further studies employing higher resolution molecular markers (e.g., microsatellites) are warranted.

Finally, there are a multitude of questions still to be answered in regards to the long-term effects of population bottlenecks. Introduced bird species could also prove to be an excellent study system to address these questions (Briskie, 2006), as many populations of introduced birds have persisted for upwards of several hundred years, which may translate into a similar number of generations. Understanding how such populations have survived, despite some showing negative fitness consequence as a result of a bottleneck, may help guide the development of management strategies to ensure the long-term survival of the 1200 species of endangered native birds around the world that are each now facing a population bottleneck crisis through continued human activities.

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