Conservation biology and genomics of a flagship endangered species: the Mauritian pink pigeon *Nesoenas mayeri*

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Abstract

The reduction of natural populations is a major conservation problem and the main cause of increased extinction risk. I investigated conservation issues that limit the growth of the pink pigeon population, including infection with *Trichomonas gallinae*, biased sex ratios and the reduction in both reproductive fitness and life history traits. In particular, I examined the association between these issues in the pink pigeon and genome-wide genetic variation using 45,841 single nucleotide polymorphisms (SNPs) generated using restriction site associated DNA sequencing (RAD-seq).

The average observed and expected heterozygosity (Ho and He respectively) were low, at 0.27 and 0.28 respectively. Rapid genetic loss has increased inbreeding depression in the population. The effective population size was found to be particularly small in one subpopulation, Ile aux Aigrettes. Examining genome-wide heterozygosity for both immune and non-immune genes between males and females showed that males have a higher level of gene variation than females, which may explain the male-biased sex ratio in fledglings that this study found to be significant. A significant negative association was found between genome-wide heterozygosity and infection with *Trichomonas gallinae*. The longevity and body weight of adult birds and fledgling success showed a significant positive relationship with the level of genome-wide heterozygosity. Reproductive success, in terms of the number of nests, eggs laid and hatched young that died before fledging, did not show any significant relationship with the level of genome-wide heterozygosity. However, using genome-wide association studies (GWAS), this study identified a genomic region close to the progesterone receptor gene (PRG) that potential affects egg-laying in the pink pigeon.

The findings of this thesis suggest an association between the problems limiting the growth of the pink pigeon population and a reduction in genome-wide variation, suggesting that the pink pigeon may be entering a vortex that may drive the species to extinction and thus emphasising the urgent need for conservation management to avoid its extinction.

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Chapter 1

General introduction

1.1 Conservation of natural populations

Extinction of wild species is increasingly recognised as a serious, global conservation biology concern. Anthropogenic factors, such as exotic species introduction (Blackburn *et al.,* 2004) and habitat loss (Manne *et al.,* 1999), have significantly contributed to the reduction in numbers of natural species. However, there is evidence that extinction rates in small populations including island bird species are significant higher compared to large populations on the mainland (Loehle and Eschenbach, 2012). This may be due to stochastic factors, including environmental, demographic and genetic stochasticity, which can interact to hasten extinction processes in small populations (Frankham *et al.,* 2010). Genetic factors play an important role in the maintenance of natural populations. Conservation genetics is the field that uses genetic techniqes to estimate genetic components of endangered and small natural populations and identify any necessary management intervention strategie (Frankham, 2003; Frankham *et al.,* 2010). This field has emerged from the development of both evolutionary genetics and quantitative genetic theory (Frankham *et al.,* 2010). These have contributed to this field by providing theoretical frameworks to explain the mechanisms that lead to evolutionary changes and influence the pattern of genetic variance in populations, and also by providing tools to describe genetic variation within and between populations (Loeschcke *et al.,* 1994). The role of genetics in populations was an early observation of Darwin (1868), who indicated that inbreeding may negatively affect small isolated populations, and of Voipio (1950), who was concerned about the influence of genetic drift on small populations as a result of hunting and habitat destruction (In Allendorf *et al.,* 2012). However, conservation genetics was introduced formally as modern research field by Frankel (1974), and then by Frankel and Soule' (1981), who were interested to apply genetic theory to conservation biology (In Frankham *et al.,* 2010; Allendorf *et al.,* 2012). Subsequently, in the 1980s and 1990s, a surge of research in the field of molecular genetics revolutionised the field of conservation genetics (e. g. Vrijenhoek *et al.,* 1985; Wildt *et al.,* 1987; O'Brien, 1994; Paetkau and Strobeck, 1994).

1.2 Extinction vortices and small populations

Stochastic factors, including environmental, demographic and genetic stochasticity can interact to accelerate extinction processes in small populations (Frankham *et al.,* 2010). Such positive feedback loops have been called “extinction vortices”, and four distinct vortices have been described by Gilpin and Soule' (1986). These four extinction process were explained by Tomback *et al.* (2001). The first extinction vortex is a demographic variation that occurs as a result of random environmental events which can decrease population size (*N*) and impact population growth rate. Reduction in population size (*N*) and increasing demographic stochasticity lead to fragmentation between populations within a single species, which is the second extinction vortex. Fragmentation causes isolation, increasing the risk of local extinction. A reduction in effective population size (N_e) causes the third vortex, inbreeding depression and loss of genetic variation. The lower the effective population size (N_e), the greater the amount of inbreeding. Over generations, inbreeding will lead to loss of genetic variation due to decreased heterozygosity and increased homozygosity (recessive lethal alleles). In turn, further decreases in effective population size (N_e) lead to genetic drift (loss of genetic variation requiring adaptation to environmental conditions), the fourth vortex. This means that species can be threatened by the increase of detrimental genetic variation and the reduction of adaptive variation (Hedrick, 2001). Adaptive variations are heritable changes in amino-acid substitution in response to natural selection (Ouborg *et al.,* 2010). Detrimental genetic variation can often be added to the gene pool of a population by mutation, gene flow or genetic drift, causing inbreeding depression in strong cases or genetic load in weak cases (Kohn *et al.,* 2006).

The effect of a reduction in population size has been demonstrated by comparing heterozygosity levels in threatened taxa with those in non-threatened taxa, and this has shown lower heterozygosity rates in the former in 77% of comparisons (Spielman *et al.,* 2004b). These results were more recently confirmed by James et al. (2015), who found a significant reduction in genetic diversity in island species compared to mainland species. This is because threatened species are more prone to increase levels of both homozygosity and deleterious alleles, leading to a decline in population fitness (Oosterhout *et al.,* 2000; Avdi and Banos, 2008). This can result in decreased resistance to environmental stressors (Reed *et al.,* 2002), and elevated susceptibility to infection

and diseases (Trinkel *et al.,* 2011). For example, as a result of being isolated and having small population sizes, endangered bird species endemic to islands, including the Mauritius kestrel (*Falco punctatus*) (Ewing *et al.,* 2008), the black stilt (*Himantopus novaezelandiae*) (Hagen *et al.,* 2011) and the Hawaiian goose (*Branta sandvicensis*) (Paxinos *et al.,* 2002; Veillet *et al.,* 2008), have suffered from inbreeding and consequently low genetic diversity. In addition, genetic drift and inbreeding can increase genetic divergence between isolated populations of the same species which may lead to outbreeding depression (e. g. when crossing of individuals between populations is conducted in recovery programmes) (Ouborg *et al.,* 2010). For example, hybridization between female pink salmon endemic to Auke Creek, Southeast Alaska, and males endemic to Pillar Creek, Kodiak Island, led to outbreeding depression affecting population fitness (Gilk *et al.,* 2004).

1.3 Genetic approaches in conservation biology

The field of conservation genetics has achieved remarkable advances in the past few decades. Along with evolutionary genetics theory, this has been driven by the development of genetic approaches to examine a population through variation in individual genotypes using molecular markers.

Genetic markers refer to inherited measurable characteristics, which may be linked to other characteristics that are difficult to measure, and detect genetic attributes of individuals (de Vicente and Fulton, 2003). The first genetic markers to have been used by biologists were morphological traits, after the rediscovery of Mendelian genetics at the beginning of the last century (Charlesworth, 2010). Biochemical markers were established in the field of population genetics early in the second half of the last century when protein-based technology (allozymes) was used to detect protein variation in *Drosophila* and humans (Harris, 1966; Hubby and Lewontin, 1966). This molecular tool has been used on a large scale for studying genetic variability in many species, and for different purposes such as studying allozyme variation and mating systems (Apollonio and Hartl, 1993), the effect of selective hunting on allele frequencies in red deer (*Cervus elaphus*) (Hartl *et al.,* 1991), and population differentiation in anadromous fish (Fleischer *et al.,* 1983). In spite of their low cost and suitability for several applications in conservation genetics, allozymes are tissue-based molecular markers which means

that the expression of genes may be influenced by differences in developmental stages and the types of tissues that are used (de Vicente and Fulton, 2003). In addition, there are limitations to the use of this type of marker to conduct genetic association studies and mapping because the number of informative marker loci is very small (Schlötterer, 2004).

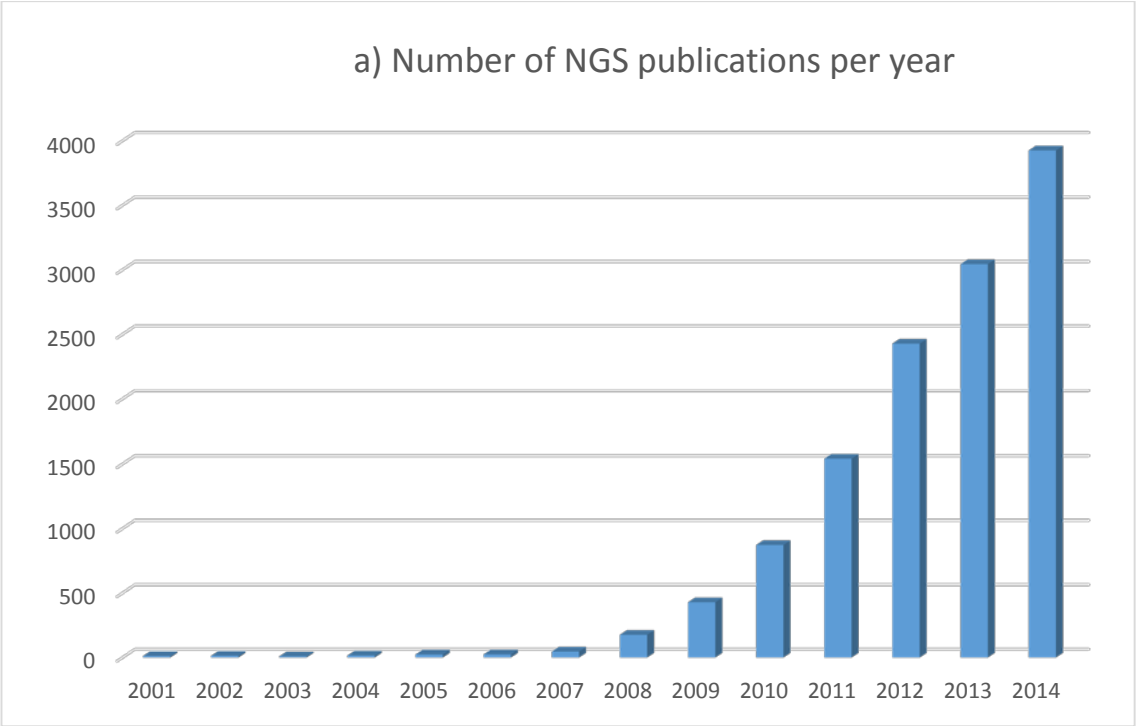
The field of conservation genetics has been revolutionised by the advent of DNA-based markers, which provide important genetic information for natural populations using several different molecular markers. These markers can be classified into three categories depending on type of technique required to generate them (Allan and Max, 2010): (i) Non PCR-based markers are generated by restriction enzyme techniques including restriction fragment length polymorphism (RFLP) (Liu, 2007b). These are co-dominant markers that rely on detecting variation at genomic sites that can be cut using restiction enzymes (de Vicente and Fulton, 2003). PCR-based markers include minisatellites (Jeffreys *et al.,* 1985), amplified fragment length polymorphisms (AFLPs) (Liu, 2007a), random amplified polymorphic DNA (RAPD), microsatellites (short tandem repeat sequences of 1-6 nucleotides) in length (Gupta *et al.,* 1996) and single nucleotide polymorphisms (SNPs) (Gupta *et al.,* 2001). (iii) Direct DNA sequencing of targeted genomic regions (Allan and Max, 2010) using a sequencing platform developed by Sanger (1977), and Maxam and Gilbert (1977). The introduction of bioinformatics and automation of DNA sequencing has significantly increased the number of nucleotide sequences in databases (reviewed in Hutchison, 2007). Mitochondrial DNA (mtDNA) has been widely sequenced to study evolutionary history (e. g. Garnery *et al.,* 1992; Bernatchez, 2001), phylogeny and taxonomy (e. g. Leisler *et al.,* 1997; Caccone *et al.,* 1999).

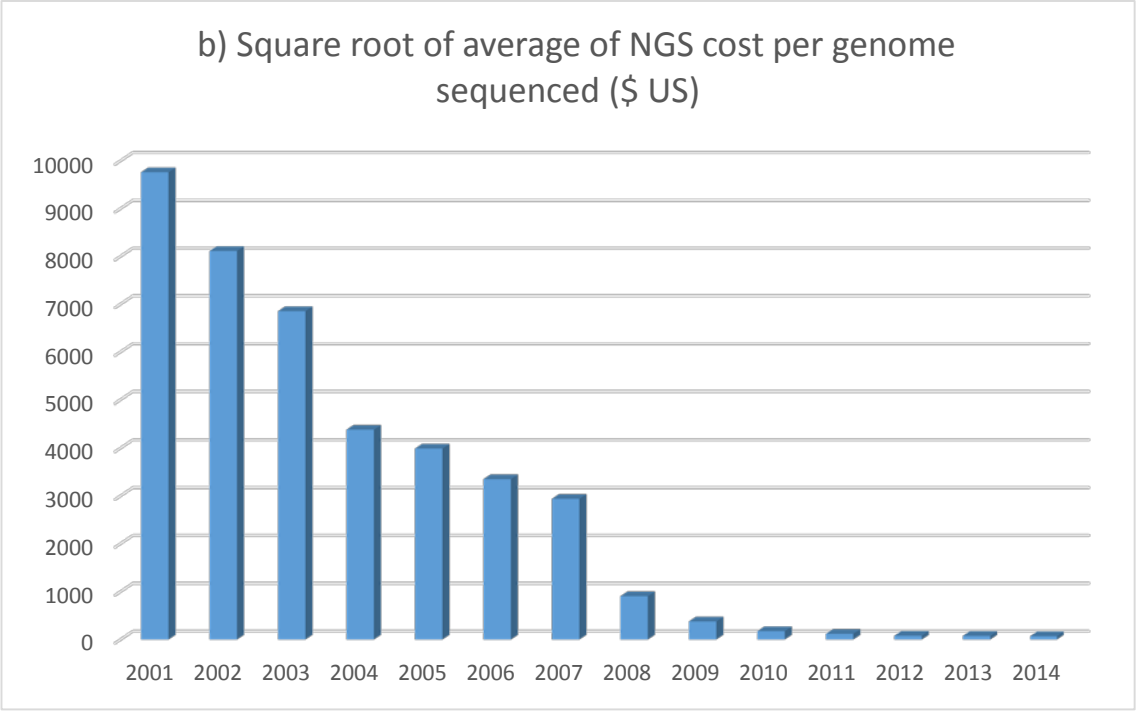
The aforementioned genetic methods have been used extensively in the field of conservation biology to study the dynamics of natural populations and in recovery programs, measuring factors such as genetic diversity in bottlenecked and endangered species (González-Porter *et al.,* 2011; Kim *et al.,* 2011; Yang and Jiang, 2011; Zschokke *et al.,* 2011), the evaluation of inbreeding and inbreeding depression (Hagen *et al*., 2011; Spiering *et al*., 2011; Willoughby *et al*., 2015), effective population size (Sato and Harada, 2008), gene flow (Méndez *et al*., 2011) and phylogenetic relationships (e. g. Gibson and

Baker, 2012). However, these approaches are limited by having few markers across the genome, and are costly in time and outlay for development of genetic markers for each new species including marker identification population and deployment (Davey and Blaxter, 2010). In addition, there has been little agreement regarding the use of neutral markers such as microsatellites for small populations to detect selection of adaptive variation in natural populations (Van Oosterhout *et al*., 2004; Ouborg *et al*., 2010). In order to accurately assess adaptive variation, taking into consideration the potential effects of selection and environment conditions, genome-wide estimations of variation need to be performed (Hedrick, 2001; Kohn *et al*., 2006; Ouborg *et al.*, 2010). Recently, it has become possible to overcome these issues with developments in genomic approaches which have reduced costs and facilitated access to genomic resources even for non-model species, which has led to a shift from conservation genetics to conservation genomics (Primmer, 2009; Avise, 2010; Ouborg *et al*., 2010).

1.4 Genomic approaches and conservation genomics

Since the middle of the last decde, a surge in research and reviews has paid particular attention to the role of genomic technologies in the field of conservation biology (e. g. Kohn *et al*., 2006; Romanov *et al*., 2006; Stinchcombe and Hoekstra, 2008; Primmer, 2009; Romanov *et al*., 2009; Allendorf *et al*., 2010; Avise, 2010; Künstner *et al.*, 2010; Ouborg *et al*., 2010; Vanderssteen *et al*., 2010; Pollinger *et al*., 2011; Bowden *et al*., 2012;) (Figure 1.1a). In addition to the advances in the field of bioinformatics, a reduction in the costs of new genomic techniques, such as reduced-representation sequencing and whole-genome sequencing, has contributed to transforming the study of natural populations from the genetic level to genomic level (Allendorf *et al.*, 2010; Ouborg *et al*., 2010) (Figure 1.1 a-b).





(Figure 1.1 a-b) The growth of publications relating to Next Generation Sequencing and population genomics per year from publication data from PubMed, retrieved using keywords: "high-throughput sequencing" OR "deep sequencing" OR "next generation sequencing". b) The average costs of NGS per genome from 2001 to 2014 (Wetterstrand, 2014).

''Population genomics'' is a relatively a new term for the field that uses genome-wide genetic variation to study natural populations (Charlesworth, 2010). It did not appear in literature until 1990s, when thousands of genetic markers in a specific genomic region could be analysed together in a single individual (Black IV *et al*., 2001; Ellegren, 2014). Before the advent of next-generation sequencing, most studies of natural variation at the genomic level had been performed on populations of species for which there existed a reference genome, including humans (*Homo sapiens*) and some model organism species such as *Escherichia coli*, yeast (*Saccharomyces cerevisiae*), the worm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*), the zebrafish (*Danio rerio*) and the mouse (*Mus musculus*) (Primmer, 2009; Pool *et al*., 2010). This is probably because traditional genomic technologies available up to that time, such as Sanger-based genome sequencing, required cloning *E. coli* and led to results which were costly both in time and expenditure (Hutchison, 2007). More recently, genomic data of these models, and additional ecological models and commercial species, have been used to develop genomic markers such as microsatellites, or SNPs, to study the evolutionary genetics in other wild related species (e. g. Miller *et al*., 2012). For example, Backström *et al.* (2008) used assembled genomes of chicken (*Gallus gallus*) and zebra finch (*Taeniopygia guttata*) to develop 242 gene markers for several bird species including Tengmalm's owl (*Aegolius funereus*), the peregrine falcon (*Falco peregrinus*), the collared flycatcher (*Ficedula albicollis*), the blue tit (*Parus caeruleus*) and the great reed warbler (*Acrocephalus arundinaceus*).

For natural population studies, it is worthwhile sampling thousands of polymorphic markers across the entire genome (Luikart *et al*., 2003). Developments in sequence platforms such as 454 (Roche), Solexa (Illumina), and SOLiD (Applied Biosystems) have enabled the generation of gigabases of DNA sequences in a short time and at a lower cost (Chain *et al*., 2009; Davey and Blaxter, 2010). This has resulted in the possibility of assembling entire small size genomes for a few individuals (Hernandez *et al*., 2008). However, it would be unnecessary and extremely expensive to assemble large genomes of hundreds of individuals (Narum *et al*., 2013).

A considerable current advance which has been brought to the field of conservation genomics is next-generation sequencing. This approach uses restriction enzyme

digestion in order to discover, sequence and genotype massive numbers of markers over the genomes of hundreds of individuals across populations, even when genomic references are not available (Davey *et al*., 2011). These methods can be divided into three groups, which are reduced-representation sequencing, including reduced-representation libraries (RRLs) and complexity reduction of polymorphic sequences (CRoPS), restriction-site-associated DNA sequencing (RAD-seq), and low coverage genotyping, including genotyping by sequencing (GBS) and multiplexed shotgun genotyping (MSG) (reviewed in Davey *et al*., 2011).

Using bioinformatic tools, the generated sequence reads from these methods can then be assembled into longer contiguous genomic sequences by using the *de novo* assembly approach which compares all the sequence reads before building longer contiguous sequences from the overlap (Catchen *et al*., 2011). For example, using the RRLs method, Bers *et al.* (2010) detected 20,000 SNPs in the great tit (*Parus major*) from over 16 million short sequence reads that were assembled using the *de novo* assembly approach. Alternatively, the reference-based assembly approach can be applied to map all the sequence reads against a reference genome of the same or a related species (Ng and Kirkness, 2010). For example, large contigs from 9 to 42 Gb of Illumina short-read data of four divergent *Arabidopsis thaliana* strains were assembled against a reference genome sequence of the same species that was released a few years earlier (Schneeberger *et al.*, 2011).

In addition to the cost and the DNA quality and quantity, choosing one method from these techniques depends on the conservation questions being addressed and the extent of genomic data which is required to answer these questions (reviewed in Allendorf *et al*., 2010). The reduced-representation library (RRL) method is more suitable for sequencing pools of DNA samples from multiple individuals to detect genomic markers at moderate depth of coverage within a population, rather than in each single individual (Davey *et al*., 2011). For example, Leaché *et al.* (2013) used the reduced-representation library (RRL) method to sequence a pool of 22 species of North American *Sceloporus* lizards to test species divergence with gene flow.

In another study this method was used to genotype pools of individual great tits (*Parus major*) of two populations, located in the United Kingdom and the Netherlands, to study differentiation between populations (Van *et al*., 2012).

Low coverage genotyping methods, including GBS and MSG methods, are used to sequence large numbers of genomic markers at low coverage for each individual (Davey *et al*., 2011). However, because they are based on low sequencing depth, it is difficult to obtain accurate genotypes for outbred populations as a result of increased heterogeneity levels (Chen *et al*., 2013). However, they are appropriate for some applications including genetic mapping and QTL studies, in which genotypes of parents are known (Davey *et al*., 2011). For example, the GBS method has been used for constructing genetic maps and detecting QTL related to temperature tolerance and body size in families of diploid Chinook salmon (*Oncorhynchus tshawytscha*) (Everett and Seeb, 2014).

The RAD-seq method can sequence DNA samples from multiple individuals to detect genomic markers at high coverage and depth in each individual across a population (Davey *et al*., 2011). However, unlike other NGS Methods that use just one or two restriction enzymes (RE) for sequencing large regions surrounding each restriction site, RAD-seq can use more than two REs to sequence high genome-wide marker density and reduce the bias of fragment length coverage, which makes it a useful tool for ecological population genomics (Davey and Blaxter, 2010; Davey *et al*., 2011; Narum *et al*., 2013).

1.5 Applications of RAD-seq in population genomics

The RAD-seq technique can genotype and discover thousands of SNP markers in tens or hundreds of individuals across a population in order to address important research questions for the conservation of natural populations. Below are reviews of some case studies which applied the RAD-seq technique to detect genome-wide genetic variation related to population genetic parameters and fitness in natural populations.

1.5.1 Evolutionary relationships among and within species and populations

It is expected that gene flow barriers between populations which live in a variety of environmental conditions will increase the degree of genetic differentiation among

these populations via genetic drift or local adaptation (Pérez-Espona *et al*., 2008). However, it has been found that there is a likelihood of divergence in the face of gene flow (Nosil, 2008). For instance, Tennessee cave salamanders (Plethodontidae: Gyrinophilus) emerged from spring salamanders (G. porphyriticus) through divergence in the face of gene flow (Niemiller *et al*., 2008). Conservation genomics approaches such as the RAD-seq technique are available now to provide accurate interpretation of divergence in the face of gene flow through a high number of markers for neutral and adaptation sites. For example, one phylogeographic study which benefited from RAD-seq data detection methods was conducted by Emerson *et al*. (2010), who examined genetic divergence in the pitcher plant mosquito, *Wyeomyia smithii,* a species that distributed geographically to southern and northern groups. These two groups were distinguished genetically using mitochondrial cytochrome oxidase subunit I (COI). However, the patterns of genetic differentiation between 20 populations within these two groups cannot be detected by the COI sequences. Alternatively, RAD-seq was applied to generate 3,741 SNPs from 126 individuals belonging to 21 populations. SNPs were found to vary between populations within the groups (Emerson *et al*., 2010). Another example of using RAD-seq to discover and genotype single nucleotide polymorphisms (SNPs) to study genomic divergence in the existence of gene flow was conducted by Guo *et al*. (2015), in the three-spined stickleback (*Gasterosteus aculeatus*) in the Baltic Sea. They utilized these SNP markers to assess the patterns of genetic diversity and differentiation among ten populations that live in different degrees of salinity and temperature in the Baltic Sea. Discovering such genome-wide patterns of differentiation among these populations indicated that local adaptation gives rise to heterogenic genomic divergence despite gene flow.

1.5.2 Measuring inbreeding depression and identifying adaptive loci

Recent developments in genomic approaches, including the RAD-seq technique, have extended the ability to obtain heterozygosity fitness correlations (HFCs) and to identify loci that influence related fitness traits in natural populations.

HFCs can be performed accurately using genome wide heterozygosity obtained from large numbers of genome wide genetic markers (Miller *et al*., 2014). A study by Hoffman *et al.* (2014) showed a significant negative correlation between genome wide

heterozygosity, generated using the RAD-seq technique, and the inbreeding coefficient in oldfield mice, (*Peromyscus polionotus*) compared to heterozygosity obtained using a smaller number of microsatellite markers, in the presence of pedigree data. However, when the authors applied this comparison to a wild population of harbour seals (*Phoca vitulina*), based on 27 microsatellites and 14,585 SNPs, they found a significant negative HFC for parasite infection on SNPs compared to a weak correlation on microsatellites, in the absence of pedigree data (Hoffman *et al*., 2014).

Identification of genomic regions that explain trait variation or signals of natural selection can be achieved by comparing sets of contigs with previous sequences in existing databases, such as the National Center for Biotechnology Information (NCBI) (e. g. in Hand *et al.,* 2015), or using several methods including genome-wide association studies (GWAS) and quantitative trait loci (QTL) (reveiwed in Angeloni *et al*., 2012). QTL mapping requires crossing individuals that differ genetically for the measured trait to generate F1 individuals that are crossed between themselves, or to one of the parents, in order to create backcross progeny to generate an F2 (Stapley *et al*., 2010). The RAD-seq technique has been used to discover and genotype SNPs to map polymorphic loci associated with adaptive variation in the three-spined stickleback (*Gasterosteus aculeatus*) (Hohenlohe *et al.*, 2010), *Lolium perenne* (Pfender *et al*., 2011) and eggplant (*Solanum melongena L.*) (Barchi *et al*., 2012). GWAS is an approach used to scan SNPs across the whole genome of many individuals in order to find loci associated with a particular phenotypic trait or disease (Ellegren, 2014). It has been conducted with the RAD-seq technique to identify genes for fur phenotypes in white and Bengal tigers (*Panthera tigris tigris*) (Xu *et al.*, 2013) and to identify genes associated with the tendency to migrate both within and between two wild populations of migratory steelhead and resident rainbow trout (*Oncorhynchus mykiss*) (Hecht *et al*., 2013).

1.6 Diseases in natural populations

Wild species are exposed to many parasitic diseases that include an extensive range of both macroparasites, such as helminths and arthropods, and microparasites, such as bacteria, viruses, protozoa and fungi (Tompkins *et al*., 2011). Parasitic diseases emerge in wildlife populations by transmission of infectious vectors from reservoir domestic animals that occasionally overlap with wild populations, and by anthropogenic

translocation and introduction of wild species to new regions, which renders the endemic species vulnerable to novel infectious vectors (Daszak *et al*., 2000; Dobson and Foufopoulos, 2001). However, the prevalence and persistence of infectious diseases within susceptible populations is subject to the contact rate between host individuals (Fichtenberg *et al*., 2009), which is either dependent on the host population density or independent of host population density (Johnson *et al*., 2011). In the case of density-dependent transmission, the infectious disease continues to spread until population size is below the density threshold, the point at which the pathogen cannot infect more individuals (McCallum *et al*., 2001). However, when transmission is independent of host population density (called frequency-dependent transmission), the infectious disease continues to spread even when population size is below the density threshold (McCallum *et al*., 2001).

Parasitic diseases can negatively affect general public health, livestock fitness and biodiversity (Cox-Witton *et al.*, 2014), and can also have an impact on the population dynamics of natural populations by decreasing the survival rate of adults and juveniles in those populations (Newey *et al.,* 2007; Lachish *et al.,* 2009; Descamps *et al.,* 2011; Höner *et al.,* 2012;). However, studies of parasitic diseases in free-living wild species are relatively few (e. g. Schloegel *et al.,* 2006; Bunbury *et al.,* 2008). This could be due to the practical difficulties of catching and screening individuals in free-living wild populations (Bunbury, 2006), or perhaps because their importance has overlooked until they have become a threat to human health, or to domestic and agricultural animals (Daszak *et al.,* 2000). Most of the studies only investigated diseases in wild mammalian populations (e. g. Laurenson *et al.,* 1998; Laurenson *et al.,* 2003; Gelling *et al.,* 2011; Zhang *et al.,* 2011) and birds (e. g. VillanÚA *et al.,* 2006; Bunbury *et al.,* 2008; Savage *et al.,* 2009; Atkinson and Samuel, 2010; Robinson *et al.,* 2010; Bunbury, 2011; Garamszegi, 2011; Park, 2011). The majority of efforts in the field of wildlife disease have aimed to study the role of infectious diseases in conservation biology, including evolutionary strategies that drive co-evolution between host and parasite (Ricklefs, 2010; Lauron *et al.,* 2015), co-infection with other parasites (Varela-Stokes *et al.*, 2006), and susceptibility and resistance to pathogens (Benskin *et al*., 2009; Pedersen and Babayan, 2011).

1.7 Genetics and disease resistance

Diseases are important factors for increasing the extinction risk of small, endangered populations (Frankham, 2003; Didham *et al*., 2005; Van Oosterhout *et al*., 2007). Theoretically, disease can cause species extinction if infection occurs in a small population with frequency-dependent transmission (De Castro and Bolker, 2005; Smith *et al.,* 2006; Pedersen *et al.,* 2007; Prentice *et al.,* 2014), and this is due to their high susceptibility to infection which is often related to the existence of inbreeding and low genetic variation (Spielman *et al.,* 2004a; Ellison *et al.,* 2011).

The role of genetics in disease processes has been well studied in the laboratory under controlled conditions and with limited diseases (Pedersen and Babayan, 2011). More recently, however, attention has been paid to the application of genetic methods to wild populations that are threatened by pathogens, such as the co-evolution between host and parasite (Penczykowski *et al*., 2011) and the relationship between major histocompatibility complex (MHC) genetic variation and disease resistance (Westerdahl, 2007; Alcaide *et al*., 2010; Radwan *et al*., 2010; Kennedy *et al*., 2011; Ujvari and Belov, 2011).

As an evolutionary force, natural selection drives species in natural populations to adapt with environmental changes. It occurs when individuals ''struggle against'' several natural factors such as infectious diseases which play an important role in natural selection, more than other factors including predation / hunting (Haldane, 1992). However, when the population size is small, genetic drift may affect the variation of a gene/genes even if it is under a selection process (Hedrick, 2004). To assess variation at adaptive loci which are often under selection, neutral loci such as microsatillite markers cannot provide important information on adaptive markers (Schwensow *et al*., 2007). Unlike neutral markers, adaptive markers in the major histocompatibility complex (MHC) have been considered to be highly adaptive loci (Schwensow *et al*., 2007). MHC is a group of genes which code for cell-surface glycoproteins; they play a major role in the immune systems of vertebrates (Miller *et al*., 2011). In birds, there are two classes of MHC: class I, predominantly associated with antigens (peptides) from intracellular pathogens (e.g. viruses and some protozoa), and class II, predominantly associated with presentation of antigens (peptides) from extracellular pathogens (e.g. bacteria) (Alcaide *et al*., 2010; Westerdahl, 2007). MHC genes are highly polymorphic and this

polymorphism is most likely maintained by pathogen-driven selection and by the mating choices of females that prefer to mate with males which have genes compatible with their own (Bernatchez and Landry, 2003; Ujvari and Belov, 2011). Development of resistance to pathogens can occur when the individuals within a population are subjected to heavy selection pressures through exposure to large numbers of different infectious diseases across different time periods (Alcaide *et al*., 2007). As a result, individuals with heterozygosity or rare alleles (MHC genetic diversity) have higher survival rates than homozygotic individuals because the heterozygotic individuals generally have a greater ability to tolerate high pathogen levels (Jeffery and Bangham, 2000).

Since the development of degenerate primers by Edwards *et al*. (1995), exon 2 of MHC class II genes, an important region for immune response that encodes the peptide-binding region (PBR), has been studied a great deal in some bird species, such as the little spotted kiwi (*Apteryx owenii*) (Miller *et al*., 2011), Petroicidae (*Petroica traversi*) (Miller and Lambert, 2004), the Galápagos penguin (*Spheniscus mendiculus*) (Bollmer *et al.,* 2007), the crested ibis (*Nipponia nippon*) (Zhang *et al*., 2006), the lesser kestrel (*Falco naumanni*) (Alcaide *et al*., 2008; Alcaide *et al*., 2010), the house sparrow (*Passer domesticus*) (Bonneaud *et al*., 2004), the little greenbul (*Andropadus virens*) (Aguilar *et al*., 2006), the house finch (*Carpodacus mexicanus*) (Hess *et al*., 2000), the Savannah sparrow (Passerculus sandwichensis) (Freeman-Gallant *et al*., 2002) and Darwin's finches (*Geospiza sp*.)(Sato *et al*., 2011).

The association between the prevalence of parasitic diseases and the genetic diversity in MHC within wild bird populations has been studied in the context of exposure to a single pathogen, particularly avian malaria (Westerdahl, 2005; Bonneaud *et al*., 2006; Loiseau *et al*., 2008; Loiseau *et al*., 2011). Overall, the MHC alleles investigated in these studies revealed negative association between variation in MHC genes and prevalence of malaria. Further, a study of this association in the context of exposure to multiple pathogens was reported by Alcaide *et al.,* (2010); they explored the variability of the MHC alleles of the Eurasian kestrel (*Falco tinnunculus*) and the lesser kestrel (*Falco naumann*). This study demonstrated that the variability of the MHC alleles of the Eurasian Kestrels that were exposed to greater numbers of pathogens was higher than

the variability of the MHC alleles of the lesser kestrels that were exposed to fewer pathogens.

1.8 Mascarenes, Mauritius and the pink pigeon

1.8.1 Mascarenes

The Mascarene Islands of Mauritius (1,865 km^2 at 20.25^{o}S 57.5^{o}E), Réunion (1,865 km^2 at 21^{o}S 55.5^{o}E) and Rodrigues (2,512 km^2 at 19.75^{o}S 63.5^{o}E) are tropical volcanic islands in the Indian Ocean east of Madagascar (Jones, 1987) (Figure 1.2). These islands have been home to a number of endemic species of plant and animal, most of which are now either totally extinct or seriously threatened as result of losing their native habitat since the islands were discovered in the early 16th century (Safford and Jones, 1998). It is estimated that the current total number of surviving angiosperm species in the Mascarene islands comprises of 150 species in Rodrigues, approximately 550 species in Réunion and 691 species in Mauritius, with some of these species being shared by two or all three of the islands (the Mauritius Herbarium database, 2015).

The current surviving native Tetrapod fauna of Mauritius comprises of 24 of 49 bird species, three of six mammalian species and seven of twelve lizard species. Three snake families and five chelonian species are also now extinct (Cheke and Hume, 2008) (Table 1.1). However, conservation management projects were established on both Mauritius and Rodrigues in 1972 in order to protect and carry out captive breeding and reintroduction programmes for the endangered and rare endemic species (Jones and Hartley, 1995). Some plants and rare bird species on Mauritius, including the Mauritius kestrel (*Falco punctatus*), Mauritius fody (*Foudia rubra*) and Mauritius pink pigeon, were prioritised to become an early focus of studies (Jones and Hartley, 1995).

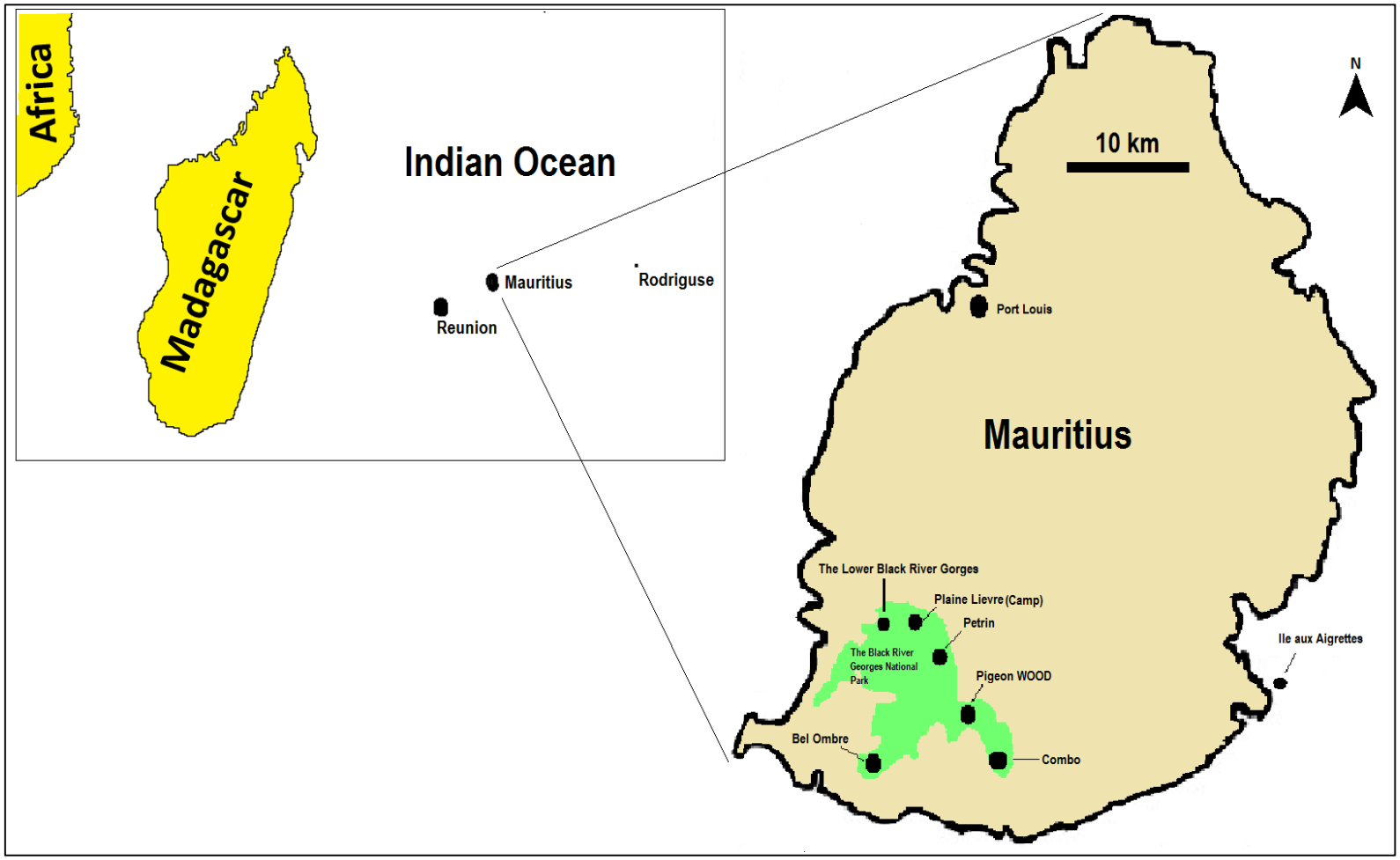


Figure 1.2. Map showing the location of the Mascarene Islands of Mauritius, Réunion and Rodrigues; also shown are the seven pink pigeon subpopulations in Black River Gorges National Park and on the Ile aux Aigrettes of Mauritius (Adapted from: Swinnerton *et al.,* 2005)

Table 1.1 The native tetrapod of Mauritius (Adapted from: Cheke and Hume, 2008).

		Species	Status
Mammals	Fruitbats	Black-spined Flying- fox *Pteropus niger*	Surviving/rare and vulnerable
		Golden Bat *Pteropus rodricensis*	Extinct
		Rougette *Pteropus subniger*	Extinct
	Microbats	Mascarene Free-tailed Bat *Mormopterus acetabulosus*	Surviving/common
		Grey Tomb Bat *Taphozous Mauritianus*	Surviving/common
		Dugong *Dugong dugon*	Extinct
Birds	Petrels and shearwaters	Wedge-tailed Shearwater *Puffinus pacificus*	Surviving/common
		Tropical Shearwater *Puffinus baillioni*	Extinct
		'Round Island' (Trindade) Petrel *Pterodroma arminjoniana*	Surviving/rare
		Kermadec Petrel *Pterodroma neglecta*	Surviving/rare
		Barau's Petrel *Pterodroma baraui*	Never present, but related form existed
		Bourne's Petrel *Pterodroma* sp. (undescribed)	Never present, but related form existed
		Bulwer's Petrel *Bulweria bulwerii*	Surviving/rare
	Cormorants and allies	White-tailed Tropicbird *Phaethon lepturus*	Surviving/common
		Red-tailed Tropicbird *Phaethon rubricauda*	Surviving/common
		Abbott's Booby *Papasula abbotti*	Extinct
		Masked Booby *Sula dactylatra*	Surviving/ vulnerable
		Reed Cormorant *Phalacrocorax africanus*	Extinct
		Great Frigatebird *Fregata minor*	Extinct
		Lesser Frigatebird *Fregata ariel*	Extinct
	Herons and allies	Reunion Night Heron *Nycticorax duboisi*	Never present, but related form existed
		Mauritius Night Heron *Nycticorax mauritianus*	Extinct
		Rodrigues Night Heron *Nycticorax megacephalus*	Never present, but related form existed
		Dimorphic Egret *Egretta dimorpha*	Extinct
		Striated Heron *Butorides striata*	Surviving/common
		Greater Flamingo *Phoenicopterus roseus*	Extinct
	Ducks and geese	Reunion Sheldgoose Alopochen kervazoi	Never present, but related form existed
		Mauritius Sheldgoose *Alpochen Mauritianus*	Extinct
		Mascarene Teal *Anas theodori*	Extinct

		Species	Status
Birds	Raptors	Réunion Harrier *Circus maillardi*	Extinct
		Dubois's Kestrel *Falco duboisi*	Never present, but related form existed
		Mauritius Kestrel *Falco punctatus*	Surviving/fairly common
	Gulls and terns	Sooty Tern *Sterna fuscata*	Surviving/common
		Brown Noddy *Anous stolidus*	Surviving/common
		Lesser Noddy *Anous tenuirostris*	Surviving/common
	Rails and allies	Dubois's Wood-rail *Dryolimnas ugusti*	Never present, but related form existed
		Sauier's Wood-rail *Dryolimnas* sp. (not described)	Extinct
		Red Hen *Aphanapteryx bonasia*	Extinct
		Leguat's Rail *Erythromachus leguati*	Never present, but related form existed
		Mascarene Coot *Fulica newtoni*	Extinct
		Common Moorhen *Gallinula chloropus*	Surviving/common
	Pigeons	Dodo *Raphus cucullatus*	Extinct
		Rodrigues Solitaire *Pezophaps solitarius*	Never present, but related form existed
		Slaty Pigeon? *Alectroenas* sp. (unspecified)	Never present, but related form existed
		Pigeon Hollandais *Alectroenas nitidissima*	Extinct
		Reunion Pink Pigeon *Nesoenas duboisi*	Never present, but related form existed
		Mauritius Pink Pigeon *Nesoenas mayeri*	Surviving/endangered
		Rodrigues Dove (?*Nesoenas*) *rodericana*	Never present, but related form existed
		Malagasy Turtle Dove *Nesoenas picturata*	Surviving/common
	Parrots	Raven Parrot *Lophopsittacus mauritianus*	Extinct
		Leguat's Parrot *Necropsittacus rodricanus*	Never present, but related form existed
		Echo Parakeet Psittacula eques	Surviving/endangered
		Rodregues *Parakeet psittacula exsul*	Never present, but related form existed
		Thirioux's Grey Parrot *Psittacula exsul*	Extinct
		Dubois's Parrot *Psittacula* (?) *borbonicus*	Never present, but related form existed
	Owls	Reunion Lizard-owl *Mascarenotus grucheti*	Never present, but related form existed
		Commerson's Lizard-owl *Mascarenotus sauzieri*	Extinct
		Tafforet's Lizard-owl *Mascarenotus murivorus*	Never present, but related form existed

Table 1.1 continued

		Species	Status
Birds		Mascarene Swiftlet *Aerodramus francicus*	Surviving/ vulnerable
	Passerines	Mascarene Swallow Phedina borbonica	Surviving/fairly common
		Reunion Cuckoo-shrike Coracina newtoni	Never present, but related form existed
		Mauritius Cuckoo-shrike Coracina typica	Surviving/ vulnerable
		Reunion Bulbul (Reunion Merle) Hypsipetes borbonicus	Never present, but related form existed
		Mauritius Bulbul (Mauritius Merle) *Hypsipetes olivaceus*	Surviving/vulnerable
		Rodrigues Bulbul (Rodrigues Merle) *Hypsipetes* sp. (undescribed)	Never present, but related form existed
		Mascarene Paradise Flycatcher *Terpsiphone bourbonnensis*	Surviving/endangered
		Mascarene Grey White-eye *Zosterops borbonicus*	Surviving/common
		Reunion Olive White-eye *Zosterops olivaceus*	Never present, but related form existed
		Mauritius Olive White-eye *Zosterops chloronothos*	Surviving/endangered
		Reunion Fody Foudia delloni	Never present, but related form existed
		Mauritius Fody Foudia rubra	Surviving/endangered
		Rodrigues Fody *Foudia flavicans*	Never present, but related form existed
Reptiles	Chelonians	Reunion Tortoise *Cylindraspis indica*	Never present, but related form existed
		Mauritius High-backed Tortoise *Cylindraspis triserrata*	Extinct
		Carosse Tortoise *Cylindraspis vosmaeri*	Never present, but related form existed
		Mauritius Domed Tortoise *Cylindraspis inepta*	Extinct
		Rodregues Domed Tortoise *Cylindraspis peltastes*	Never present, but related form existed
		Green Turtle *Chelonia mydas*	Extinct
		Hawksbill Turtle *Eretmochelys imbricate*	Extinct
		Loggerhead Turtle *Caretta caretta*	Extinct

		Species	Status
Reptiles	Lizards	Telfair's Skink *Leiolopisma telfairii*	Extinct
		Arnold's Skink *Leiolopisma* sp. (undescribed)	Never present, but related form existed
		Didosaurus *Leiolopisma mauritiana*	Extinct
		Bojer's Skink *Gongylomorphus bojerii*	Extinct
		Reunion Slit-eared Skink *Gongylomorphus borbonicus*	Never present, but related form existed
		Macabe Skink *Gongylomorphus fontenayi*	Surviving/rare
		Bouton's Skink *Gongylomorphus boutonii*	Surviving/fairly common
		Mauritius Night-gecko *Nactus serpensinsula*	Extinct
		Reunion Night-gecko *Nactus* sp. (undescribed)	Never present, but related form existed
		Small Rodrigues Night-gecko *Nactus* sp. (undescribed)	Never present, but related form existed
		Lesser Night-gecko *Nactus coindemirensis*	Surviving/rare
		Reunion Forest Day-gecko *Phelsuma borbonica*	Never present, but related form existed
		Blue-tailed Day-gecko *Phelsuma cepediana*	Surviving/common
		Upland Forest Day-gecko *Phelsuma rosagularis*	Surviving/fairly common
		Manapany Day-gecko *Phelsuma inexpectata*	Never present, but related form existed
		Vinson's Day-gecko *Phelsuma ornata*	Surviving/common
		Guimbeau's Day-gecko *Phelsuma guimbeaui*	Surviving/fairly common
		Gunther's Day-gecko *Phelsuma guentheri*	Extinct
		Newton's Day-gecko *Phelsuma edwardnewtoni*	Never present, but related form existed
	Snakes	Keel-scaled Boa Casarea dussumieri	Extinct
		Burrowing Boa Bolyeria multicarinata	Extinct
		Carie's Blind-snake *Typhlops cariei*	Extinct

1.8.2 The pink pigeon

The pink pigeon (*Nesoenas mayeri*) (Prévost, 1843) of Mauritius is one of the most endangered pigeons in the world, and is a recovered bottleneck species (Jones *et al*., 1992). It has been estimated that the number of birds in the wild in 1975 was between 10 and 20, and there were only about 10 birds in the wild in 1990 (Swinnerton *et al*., 2004). During the 40 years following the establishment of recovery programmes on Mauritius and in Jersey Zoo in 1976 and 1977 respectively, the pink pigeon population of Mauritius has grown from an estimated number of 10-20 birds in 1975 (Jones, 1987) to approximately 400 birds today (Mauritius Wildlife Foundation, 2012). At the time of the population bottleneck the remaining birds occurred in a single subpopulation in a location called Pigeon Wood from which eggs and adults were taken to establish a captive breeding facility in Black River (Jones and Owadally, 1988). Six pink pigeon subpopulations on Mauritius have since been established from the founder stock (Mauritius Wildlife Foundation, 2012). The Pigeon Wood, Bel Ombre, Plaine Lievre (also called Camp) and Combo subpopulations are located in the Black River Georges National Park, the only suitable area of native forest remaining on the island (Jones, 1987). Another was established on Ile aux Aigrettes, Ile aux Aigrettes, a small islet of 27 hectares, off the eastern coast of Mauritius from which introduced mammalian predators had been eradicated (Jones, 1987; Jones *et al*., 1992). An additional two subpopulations in the Lower Black River Gorges and at Pétrin were set up in 2007 and 2012, respectively (Mauritius Wildlife Foundation, 2012) (Figure 1.2), and are not included in the present study.

1. 8. 3 Conservation problems of the pink pigeon population

As stated above, a highly successful recovery program has resulted in the recovery of this species from just a few wild birds in one small area to a relatively stable number of around 400 individuals in recent years. The goal of conservation programme for pink pigeons is to achieve a minimum number of 600 free living birds (Mauritius Wildlife Foundation, 2012). However, there have been several factors limiting the increase of the pink pigeon population including habitat loss and fragmentation, predation by introduced mammalian predators such as crab-eating macaques (*Macaca fascicularis*), feral

cats (*Felis catus*) and black rats (*Rattus rattus*) ((Jones, 1987), lack of natural food resources, diseases, and probably inbreeding depression. Below we mention some of the main research and management techniques used to counteract these factors.

1.8.3.1 Restoration of native forest habitat, control of predators and supplementary food

On Mauritius, 95% of forests have been lost since the increase in human population, the introduction of non-native species and the expansion of the cultivation of sugar in the 18th century, which led to the dwindling of forests to a small area in the south-west, representing 3.6% (6500 ha) of the island area, as a final area for surviving native species on the island (Jones, 1987). Due to its role in providing food and protection from predators for many native species on the island, restoration of original vegetation has become a top concern for conservation projects on Mauritius. This is evident from the attempts to manage native vegetation on Mauritius in the mid-1980s with aims to develop Reginald Vaughan and Octave Wiehe's work that began in the 1930s to minimise the influence of alien species (Jones, 2008). Between 1982 and 1998, Conservation Management Areas (CMAs) were established to remove alien exotic weeds and to manage and replant native trees (Jones, 2008). As a result, about 54.37 hectares, including 12 types of vegetation, have been weeded and fenced since 1930 (Jones, 2008). Habitat restoration continues to be a conservation priority on Mauritius.

Safford and Jones (1998) suggested three long-term strategies for maintaining and increasing populations of pink pigeons and other endangered birds on Mauritius. Firstly, by improving food availability over limited areas by the rehabilitation of native vegetation in carefully selected areas and the removal of invasive plants and exotic animals. Secondly, by using the Marooning technique on the Mauritian offshore islet Ile aux Aigrettes, where introduced mammalian predator species and invasive plants can be removed at a relatively low cost. Thirdly, by including exotic plant species when enhancing mainland habitat areas when these species are of more immediate value to native animal species than are native plants, and when they are capable of growing and surviving on Mauritius with minimal management (Safford and Jones, 1998). Safford (1997) found nesting success for the fody (*Foudia rubra*) in introduced Japanese red cedar (*Cryptomeria japonica*) was higher than in native trees, suggesting that these

cedar trees provide a refuge from nest predation, specifically crab-eating macaques (*Macaca fascicularis*). This result encouraged Carter and Bright (2002) to examine this strategy in terms of the pink pigeon, which showed that nest predation by macaques is significantly lower in non-invasive Japanese red cedar than in endemic trees. Dependence on a supplementary food strategy for the pink pigeon was investigated by Edmunds *et al*. (2008) who concluded that supplementary food is a low-cost strategy, and can increase the survival chances and productivity of pink pigeon populations. On the other hand, the study indicated that this strategy should be accompanied by long-term management strategies and monitoring programmes to adjust the strategy to match the changing conditions of expanding populations (Edmunds *et al*., 2008).

1.8.3.2 Bias in sex ratio

In theory, parents are expected to produce nearly equal proportions of daughters and sons, as long as parental fitness is maximized by parity in sex ratio (Fisher, 1930). However, observed adult sex ratios in most bird species tend to be skewed toward males (Greene and Fraser, 1998; Taylor and Parkin, 2008; KosztolÁNyi et al., 2011). Biased sex ratio could be due to the higher reproductive success of one sex over the other owing to factors such as cost of egg production in females (Øigarden and Lifjeld, 2013) or external environmental conditions such as hatching time and climatic factors during the breeding season (Graham *et al*., 2011), parasitic stress (Dama, 2012) and availability of food (Kilner, 1998; Merkling *et al.,* 2012; Rutz, 2012), which may influence parents to invest adaptively in their offspring (Trivers and Willard, 1973). However, even if parents produce equal proportions of sexes, endangered species can show significant sex ratio distortions due to stochastic variation in sex bias survival rates, possibly associated with inbreeding depression, which could exacerbate the risk of species extinction (Dale, 2001; Grayson *et al.,* 2014). Hence, sex ratios can also deviate from parity when differential mortality occurs either in the offspring stage (after hatching) (Clutton-Brock *et al.,* 1985; Griffiths, 1992; Torres and Drummond, 1997) or in adult and juvenile stages (after fledgling) (Székely *et al.,* 2014; Saunders and Cuthbert, 2015).

Sex ratio in the pink pigeon population was examined by Bunbury (2006), based upon cumulative data of 931 birds over a period of 17 years and she found a significantly skewed sex ratio in the overall adult population between 1987 and 2004. Another study,

conducted by Concannon (2014) on one subpopulation (the Ile aux Aigrettes), reported a male bias in the adult sex ratio of that subpopulation since 1997. However, neither of the above studies tested the deviation of the adult sex ratio from parity statistically. In addition, it is not clear which factors cause a potential sex bias ratio in the pink pigeon population.

1.8.3.3 Parasitic diseases

Trichomonosis and Leucocytozoonosis are the main parasitic diseases to have been observed in the pink pigeon caused by the protozoan parasites *Trichmonas gallinae* and *Leucocytozoon marchouxi*, respectively (Bunbury, 2006).

More than 52 species of *Leucocytozoon* were found in approximately 8% of birds (Bennett *et al*., 1992). These are host-specific to particular bird species or families, such as *L. simondi* in anseriforms, *L. smithi* in turkeys, *L. toddi* in Falconiformes, *L. ziemanni* in owls and *L. marchouxi* in Columbiforms (Özmen *et al*., 2005). A range of columbiforms have been found to be infected with *L. marchouxi*, such as the zebra dove, spotted dove and pink pigeon (Swinnerton *et al*., 2005b). Swinnerton *et al*. (2005b) found that prevalence of infection *with L. marchouxi* was 30% in 378 individuals which were screened in captivity and in the wild on Mauritius between 1994 and 2002. In another study, Bunbury *et al.* (2007a) found that prevalence of infection was 18.3% of 328 free-living pink pigeons screened in 2003. In a study by Swinnerton *et al*. (2005b), no significant difference in survival was found between infected and uninfected pigeons examined. Conversely, Bunbury *et al*. (2007a) reported a reduction in survival of birds infected with *L. marchouxi* compared to uninfected birds.

Trichomoniasis is a widespread disease affecting several bird species throughout the world, such as pscittacines, columbiforms and galliformes, as well as raptors and passerines. It generally affects the upper respiratory and digestive systems (Park, 2011). It has also been found that this disease is present in columbid species such as Pscittacines (McKeon *et al.,* 1997), Columbiforms (Bunbury, 2011; Stimmelmayr *et al.,* 2012), and Galliformes (Mantini *et al.,* 2009), as well as raptors (Sansano-Maestre *et al.,* 2009) and Passerines (Lehikoinen *et al.,* 2013). Different strains of *T. gallinae* have been found within the same host, some of which being very closely related to the human *Trichomonas vaginalis* (Gerhold *et al.,* 2008; Grabensteiner *et al.,* 2010). da Silva et al.

(2007) used a molecular approach to investigate genetic variability in the ITS1/5.8S/ITS2 regions within *T. gallinae*, isolated from pink pigeons and from Madagascar turtle-doves (*Streptopelia picturata*) in Mauritius. The sequencing and phylogenetic analyses did not show genetic distance between isolates. However, the RAPD analysis showed clear genetic variation between theses isolates (da Silva *et al*., 2007).

Transmission of *T. gallinae* occurs directly when the infected adult birds feed their squabs, or indirectly by sharing food and water sources (Bunbury *et al*., 2008). Lesions in the digestive and upper respiratory tracts can cause starvation or asphyxiation, which usually leads to the death of the infected birds (Bunbury *et al*., 2008).

The first case of trichomoniasis in the free-living pink pigeon was recorded at an early stage in the recovery programme on Mauritius by Jones *et al.* (1988). The study of trichomonosis was first carried out in one subpopulation, on Ile aux Aigrettes, by Swinnerton *et al*. (2005a) who found that 49% of squabs in that subpopulation were clinically infected, of which of 27% survived without treatment. In another uniquely complete study, Bunbury *et al.* (2008) investigated 90% (426 adult birds) of the entire pink pigeon population between 2002 and 2004, and showed that 84.3% of all screened pink pigeons were infected with *T. gallinae* (Bunbury *et al*., 2008). Likewise, the average prevalence of *T. gallinae* infection in other dove species in Mauritius (turtle dove, spotted dove and zebra dove) in the same period was 44.3% (Bunbury *et al.,* 2007b).

As explained previously in section 1.7, investigations of the association between susceptibility to disease and host genotype have been performed by investigating adaptation variation in MHC genes. However, these methods face many problems (reviewed in Babik, 2010). This includes the complexity of PCR primer design for non-model organisms that may have no sequence information available. Moreover, genotyping is a challenge because of the presence of pseudogenes (a copy of a gene that has no genetic function) and the difficulty in identifying alleles of target loci for individuals that are often present on multiple copies. More recently, these issues have been resolved by the advent of next-generation sequencing techniques which can improve genotyping of immune genes including MHC by increasing the length of reads to cover whole genes and differentiate true alleles from artefacts (Babik, 2010; Huchard *et al.,* 2012; Sommer *et al.,* 2013). Genome-wide association studies (GWAS) can also be

used instead of traditional genotyping methods to utilize genome-wide genetic markers such as single nucleotide polymorphisms (SNPs) to determine associations for a susceptibility or resistance to diseases (Thompson-Crispi *et al*., 2014).

1.8.3.4 Reproductive success

There are potential issues of utilizing only a few members of breeding individuals in captive populations for conservation and management programmes of threatened populations (Oosterhout et al., 2000). As a species that has recovered from a bottlenecked population, the pink pigeon is likely to suffer from inbreeding, which can lead to inbreeding depression (reduction of fitness). Consequently, it is important to estimate the level of inbreeding and inbreeding depression within and between subpopulations.

A study of reproductive success on Ile aux Aigrettes in 2004 showed a high mortality in hatchlings and fledglings. Fertility was 49.4 % of 443 eggs laid in that year. Hatchability was low, 73.1% of fertile eggs were hatched, 66% of nestlings died from trichomoniasis, and just 12.5% fledged (Bunbury 2006). The relationship between squab fledging success and prevalence of *T. gallinae* between 2002 and 2004 was negative (Bunbury 2006). Concannon (2014) reported a decline in fledging in IAA over time between 1993 and 2009, which was accompanied by a reduction in female age. Also, habitat management-related disturbance, specifically invasive weed management, has been found to have a negative influence of on adult survival (Concannon, 2014).

Reduction in survival and productivity in the pink pigeon is likely to be related to inbreeding. The inbreeding coefficient for captive birds was calculated in the early stages of a recovery programme for captive birds using pedigree data by Jones *et al.* (1989), who found low levels of productivity of eggs, hatchability, and fledging in pairs, which showed a high inbreeding coefficient. Inbreeding analysis was later repeated using the same methods (Swinnerton *et al.,* 2004)) in 1980s and 1990s to estimate the effect of inbreeding on demographic parameters in both captive and wild pink pigeon populations and showed significant effects of inbreeding on egg fertility and survival rates of squab, juvenile and adult birds. In addition, mDNA sequence variation in the d-loop region was investigated in a pink pigeon founder, and low diversity was found in mitochondrial DNA (Swinnerton *et al*., 2004).

In order to determine accurate inbreeding coefficients for pink pigeons and to examine the relationship between reduction in fitness and host genotype, it is necessary to use hundreds or thousands of polymorphic markers across the whole genome using restriction-site associated DNA sequencing (RAD-seq) techniques.

Previous studies of the pink pigeon population suggested that factors which affect this species are the reduction in hatchability due to infertility, high squab mortality due to trichomonosis and biased sex ratio. However, genetic factors that can increase susceptibility to diseases and reduce reproductive success, particularly increased genetic similarity between individuals, have not been investigated. Sex ratio is reported as biased in pink pigeon populations. However, until recently it was not known whether that deviation is significant statistically and what factors influence parity.

1.9 Aims and structure of this thesis

The primary aim of this thesis was to enhance the genetic management strategies for the pink pigeon population, specifically the plans for increasing population size, establishing new subpopulations and/or releasing new individuals. In particular, this thesis examines a number of research questions, which are addressed in four analytical chapters:

Chapter 2 Investigates sex ratio in the pink pigeon population and subpopulations to find out whether there is significant bias in sex ratio, whether the deviation exists at secondary level (squab level) rather than being at adult level, and whether that is related to sex-biased mortality of the squabs.

Chapter 3 Focuses on infection with *Trichomonas gallinae* using data collected from 418 individuals between 2002 and 2004 by Bunbury (2006), to examine whether there is spatiotemporal variation in the proportion of infection, and whether this differs between males and females. The individuals significantly less infected than by chance, based on the site and sex, are identified with the aim of exploring whether there is (immuno)genetic variation present in the population that could be subject to selection (chapters 4 and 5). The second objective is to determine whether the proportion of *T. gallinae* infection differs between sexes, which may affect sex ratio in the pink pigeon population. In addition, infection with *T. gallinae* was examined in 251 birds which died

before February 2010, with the aim of identifying whether there is significant variation in the longevity between birds that have been found to be positive to *T. gallinae* infection and whether this differs between sites.

Chapter 4 Calculates a number of population genetics parameters including observed and expected heterozygosity, effective population size (N_e), genetic drift and *F*-statistics.

Chapter 5 Estimates the relationship between genome-wide genetic variation and breeding success and infection susceptibility. In particular, it examines the correlation between SNP variation and fitness-related traits including longevity, body condition (weight), number of eggs, number of hatched eggs and number of young fledged. In addition, it examines the association between SNP variation in immune and non-immune genes and infection status. It also identifies genes/genomic regions to explain variation in number laid-eggs.

Chapter 6 Summarises the results from the previous analytical chapters and presents proposals for their implications on pink pigeon conservation management

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Chapter 2

Sex ratio in the pink pigeon (*Nesoenas mayeri*)

Summary

Sex allocation theory predicts that sex ratio can deviate from parity, for example as a result of sex-specific mortality causing biases in the secondary and/or adult sex ratio. Endangered species can show significant sex ratio distortions due to stochastic variation in sex bias survival rates possibly associated to inbreeding depression. This could exacerbate the risk of species extinction, and this process is known as one of the four extinction vortices. The pink pigeon (*Nesoenas mayeri*) population endemic to Mauritius is an endangered species that has recovered from an estimated number of 10-20 birds in 1975 to a near-constant census population of circa 400 birds today. A previous demographic study of the pink pigeon population reported a male-biased sex ratio across the period from 1987 to 2004**.** Here, I examined the significance of sex ratio deviation from parity in the pink pigeon population across the period from 1987 to 2008 in order to identify the cause for a potential sex bias ratio. The study confirms that the overall adult sex ratio across the total population has been male-biased since 1995. No evidence was found that either the adult mortality rate or longevity was sex-biased when analysed across all subpopulations. However, the analysis of the sex ratio of 322 squabs in one subpopulation, the Ile aux Aigrettes, between 2004 and 2008 showed that, although the sex ratio of the squabs was unbiased, the overall fledgling sex ratio was significantly male-biased in 1995, 1997, 2001, 2006 and 2007. The current finding, of a male-biased fledging ratio, will further exacerbate the extinction risk.

2.1 Introduction

2.1.1 Sex ratio in natural population

Mayr (1939) pointed out that bias in the sex ratio is common in wild bird populations, a finding that has been confirmed by several later studies (reviwed in Donald, 2007). A significant bias in the sex ratio of squabs was detected in 17% of the 114 species analysed, including both dimorphic and monomorphic species, as well as migratory and non-migratory birds (Donald, 2007). Even more remarkably, the adult sex ratio deviated from parity in 65% of 173 species investigated (Donald, 2007) with males outnumbering females in all cases.

Sex ratios in birds are classified into three groups (Székely *et al.,* 2014): the primary sex ratio, which is defined as the ratio of males to females in embryos; the secondary sex ratio, which is the male to female ratio when the birds are chicks and juveniles; and the adult sex ratio, which represents the ratio of males to females among breeding adults (Székely *et al.,* 2014).

In many organisms, including birds, it might be expected for parents to produce an approximately equal proportion of daughters and sons, as long as they are equally costly for parents to produce (Fisher, 1930). Offspring sex ratios can be biased due to adaptive evolutionary processes and the (indirect) results of natural selection, or they can be more stochastic in nature as a result of inherent differences in viability and survival rates (Trivers and Willard, 1973). Deviations from an equal sex ratio could be due to larger reproductive success of sons or daughters in relation to specific external and internal environmental conditions, which may influence parents to invest adaptively in their offspring (Trivers and Willard, 1973). For example, factors like the availability and composition of food (Rutz, 2012; Merkling *et al.,* 2012; Kilner, 1998), clutch size (Øigarden and Lifjeld, 2013), hatching time and quality of breeding season (Graham *et al.,* 2011), parasitic stress (Dama, 2012) and metabolic rates (Teather and Weatherhead, 1988) may influence parents to overproduce one sex. This is true when the expected fitness benefit gains differ between sons and daughters (Bradbury and Blakey, 1998; Nager *et al.,* 2000; Whittingham and Dunn, 2000) or when the expected cost of rearing is associated with size sexual dimorphism (Kalmbach *et al.,* 2001; Benito and GonzÁLez-SolÍS, 2007). Natural selection can directly or indirectly alter the sex ratio, causing, for example, low-quality mothers to produce more daughters than sons (Trivers and

Willard, 1973). Stochastic or non-adaptive deviations from an equal sex ratio are particularly pertinent for conservation genetics, given that such deviations can increase the amount of genetic drift by reducing the effective population size (Grayson *et al.,* 2014). Inbreeding is thought to distort sex ratios by reducing the proportion of the homogametic sex, although a study by Frankham and Wilcken (2006) concluded that directional distortions in sex-ratio are not a consistent signal of inbreeding depression.
In birds, the homogametic sex is the male, which has ZZ chromosomes. Females are the heterogametic sex, with ZW chromosomes. This means that females have the potential to control the adjustment of the primary sex ratio (Oddie, 1998). It has been suggested that adjustment of sex ratio can occur during the stage of the development of the ovum prior to ovulation through one of the following mechanisms: (1) non-random segregation during meiosis, when either the Z or W chromosome is retained as the preferred sex chromosome, or (2) random segregation during meiosis, when a fully developed follicle carrying the undesirable sex chromosome is resorbed (i.e., compensated with the next follicle) before ovulation (Komdeur *et al.,* 2002; Alonso-Alvarez and Velando, 2003; Pike and Petrie, 2003; Pike, 2005; Rutkowska and Badyaev, 2008).

Offspring sex ratios can also depart from unity when differential offspring mortality occurs (Clutton-Brock *et al.,* 1985; Griffiths, 1992; Torres and Drummond, 1997). Different factors can lead to sex-specific offspring mortality, including a differential growth rate between sons and daughters during early life stages (Martins, 2004; Lemons *et al.,* 2012), egg size (Stenzel *et al.,* 2011), clutch size (Lima, 1987), sex-specific infanticide (Heinsohn *et al.,* 2011) and sibling competition (Bortolotti, 1986).

Adult sex ratio in most bird species is male biased (Greene and Fraser, 1998; Taylor and Parkin, 2008; KosztolÁNyi *et al.,* 2011). However, the primary underlying cause of bias in the sex ratio is usually sex-specific adult mortality (Székely *et al.,* 2014). In mammals, this bias in single-sex mortality can be explained by the differences in survival rates that have been found to be lower in the homogametic sex (which is male in birds) because it more prone to potential recessive deleterious mutations compared to heterogametic sex (Maklakov and Lummaa, 2013). In birds, the differences in survival rates have been found in relation to sex biases in parental care costs (Owens and Bennett, 1994) or male-male competition for mating (Promislow *et al.,* 1992; Liker and Székely, 2005).

Moreover, sex-specific density (Michler *et al.,* 2011) and differences in sensitivity to environmental pollutants between males and females could cause sex-specific adult mortality (Martínez-Abraín *et al.,* 2006). In addition, diclofenac poisoning (Arshad *et al.,* 2009) and exposure to radiation (Møller *et al.,* 2012) have been suggested to cause sex-specific mortality.

Small populations tend to decline more rapidly nearer to the time of their extinction (Fagan and Holmes, 2006). One of the reasons this can happen is because demographic and genetic factors that can contribute to low reproductive rates which can lead to over-production of one sex due to chance (Grayson *et al.,* 2014). Such positive feedback loops have been called extinction vortics (Gilpin and Soule', 1986). This kind of extinction vortex is defined as a disturbance that lowers the effective population size (Ne) which can make a population more vulnerable to additional disturbances. In turn, those subsequent disturbances will lead to further decreases in Ne and so forth until extinction.

2.1.2 Sex ratio in pink pigeons

During the 40 years following the establishment of a recovery programme on Mauritius and in Jersey Zoo in the late 1970s, the pink pigeon population of Mauritius has grown from an estimated number of 10-20 birds in 1975 (Jones, 1987) to approximately 400 birds today (Mauritius Wildlife Foundation, 2012).

Previous studies have reported a biased sex ratio in the adult pink pigeon population (Bunbury, 2006; Concannon, 2014). Bunbury (2006) found a significantly skewed sex ratio in the overall adult population between 1987 and 2004. This finding was based upon cumulative data of 931 birds over a period of 17 years. In this study, we further investigate this finding analysing annual sex ratio in the adult population. The aforementioned study also calculated an adult sex ratio for each sub-population. It reported a sex ratio skewed towards females in three sub-populations (Pigeon Wood, Ile aux Aigrettes and Plain Lievre) when the sub-population size was small; however, these ratios shifted to have male biases when the sub-population size increased (Bunbury, 2006). Another study, which was conducted by Concannon (2014) on one sub-population (the Ile aux Aigrettes), reported a male bias in the adult sex ratio of the studied sub-population since 1997. However, neither of the above studies tested the deviation of the adult sex ratio from parity statistically. In addition, it is not clear what

factors cause a potential sex bias ratio in the pink pigeon population. A study of reproductive success in one Mauritian sub-population, the Ile aux Aigrettes, showed that the majority of hatchlings (87.5%) died before reaching the fledgling stage (Bunbury, 2006). This raises the question of whether the persistent male bias in the adult pink pigeon population occurred as a result of a sex ratio skewed toward males at the primary or secondary level or because of the variations in longevity or mortality rates among adult males and females. I predict that the sex ratio at the adult level would continue to deviate from parity if: (a) the sex-biased mortality in adults is significant, (b) longevity differs significantly between adult females and males, (c) the sex ratios at the hatchling and fledgling levels are biased. Although pink pigeon adults show visible sexual dimorphism in their size and behaviour, this dimorphism is absent among juveniles (Bunbury, 2006). To investigate whether the hatchling sex ratio reflects the adult sex ratio, I used genetic approaches to determine the sex of 322 squabs, including those that died hours after hatching, in the Ile aux Aigrettes sub-population between 2004 and 2008. Squabs were only accessible in nests in this lowland forest sub-population in contrast to the four other upland forest sub-populations at Plaine Lievre, Bel Ombre, Pigeon Wood and Combo. I predict that hatching and fledgling sex ratios in the Ile aux Aigrettes sub-population are consistent with the adult sex ratio in the same sub-population.

2.2 Materials and methods

2.2.1 Study sites

At the time of the population bottleneck the remnant birds occurred in a single sub-population called Pigeon Wood from which eggs and adults were taken to establish a captive breeding facility in Black River (Jones and Owadally, 1988). Six pink pigeon sub-populations on Mauritius have since been established from the founder stock (Mauritius Wildlife Foundation, 2012)

The Bel Ombre, Plaine Lievre (also called Camp) and Combo sub-populations are located in the Black River Georges National Park. Another was established on Ile aux Aigrettes, a small islet off the eastern coast of Mauritius from which introduced mammalian predators had been eradicated (Jones, 1987; Jones *et al.,* 1992) (Figure 2.1). An additional two sub-populations were set up in 2007 and 2012, respectively, in the Lower

Black River Gorges and in Pétrin (Mauritius Wildlife Foundation, 2012), and are not included in the present study.

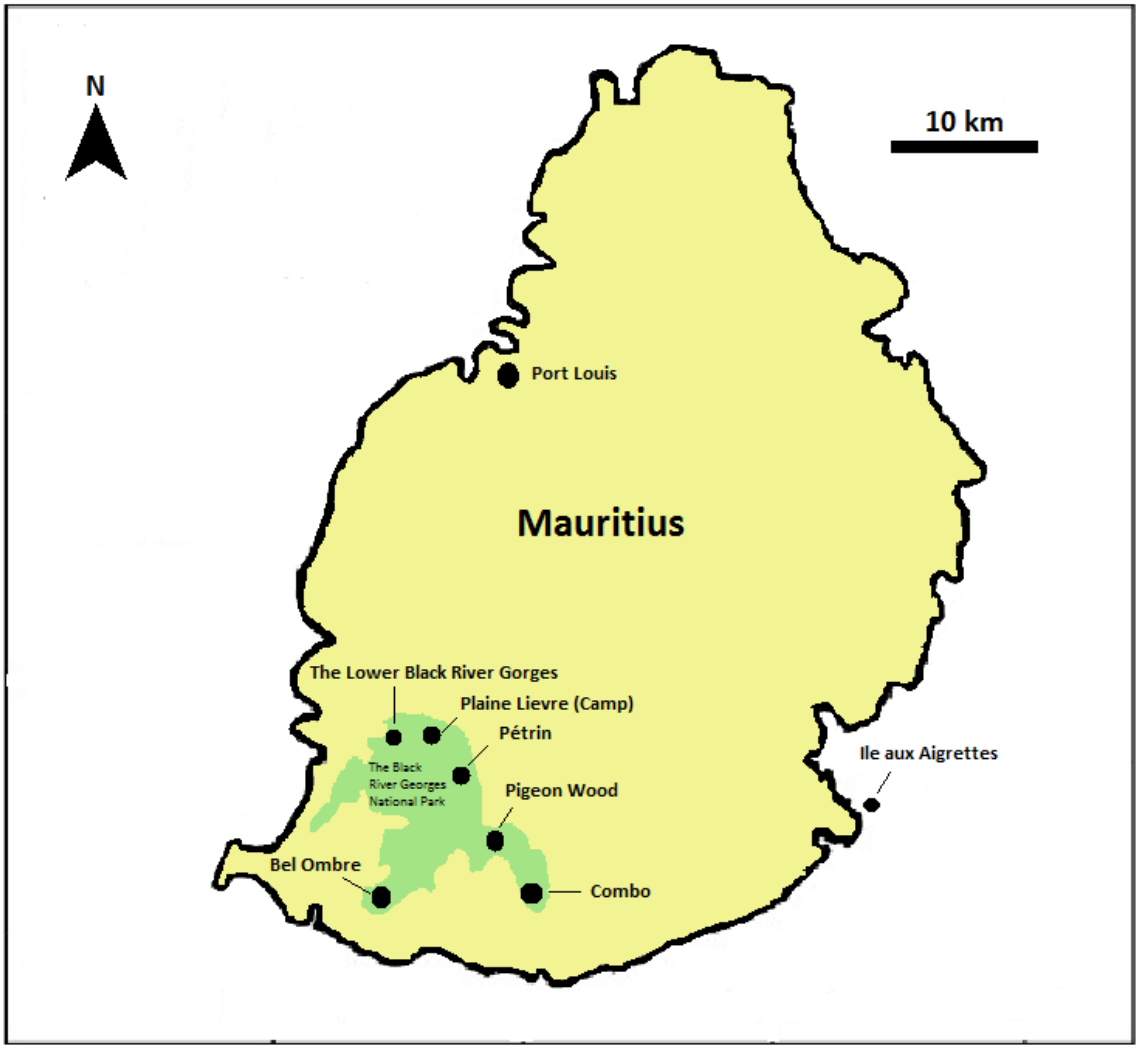


Figure 2.1 Map showing the location of seven subpopulations on Black River Gorges National Park and the Ile aux Aigrettes on Mauritius. (Adapted from: Swinnerton *et al.,* 2005).

2.2.2 Sampling and DNA extraction

To determine the sex of adults, I relied on Mauritius Wildlife Foundation pink pigeon sub-populations record data collection sheets for information on the release of captive bred birds, observational monitoring and monthly counts from 1987 to 2008. Each squab was given a unique identification number after hatching before ringing as juveniles at age of 10 to 12 days by the Mauritian Wildlife Foundation and the National Parks and Conservation Service of Mauritius (Bunbury, 2006). Although juveniles can reach sexual maturity at around six months of age, they do not show marked sexual dimorphism in

size or colour until they mate and display reproductive behaviours, including male vocalizations (Bunbury, 2006). To identify sex in squabs, a total of 278 clutches from 32 pairs were sampled in the Ile aux Aigrettes sub-population between October 2004 and September 2008 and blood samples were collected from 322 squabs. 26 known sex adult samples were used, of which 13 were males and 13 were females, as a control. The capture procedure and blood collection methods have been previously described elsewhere (Bunbury *et al.,* 2007; Bunbury, 2006). In this method, the outer claw was clipped and at least one blood drop was collected as sample from each bird before treating the bird with antibiotic powder and returning it to the nest. DNA was extracted from the blood samples using DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's instructions.

2.2.3 PCR amplification, electrophoresed and alleles calling

PCRs were performed to amplify Chromo-Helicase-DNA-binding (CHD) genes. These amplifications relied on one primer pair from earlier protocols: P2 (F'-TCTGCATCGCTAAATCCTTT-'3) and P8 (5'-CTCCCAAGGATGAGRAAYTG-'3) (Griffiths, 1992). The forward primer was labelled with fluorescent dye (FAM). The PCR was performed in 30 µl volumes, containing 6 µl of 5x PCR buffer (each containing an optimized concentration of dNTPs and 7.5 mM $MgSO_4$), 1.6 µM of each primer, about 50 ng/µl of template DNA and 2.50 U of HotStar HiFidelity DNA Polymerase (2.5 units/µl)(Qiagen). The PCR applications were performed in a thermal cycler (Applied Biosystems) with initial denaturation at 95°C for 5 mins, then 40 cycles at 94°C for 45 sec, 48°C for 45 sec and 72°C for 45 sec. A final repeat was done at 72°C for 5 mins. The PCR products were electrophoresed on a genetic analyser (ABI 3730X1) to identify sex alleles, and then the Gene Mapper Software (ABI *v* 5) was used to size and genotype the sex alleles. The sexing of 26 control birds resulted in one band for males (370 bp) and two different-sized bands for females (345 and 370 bp). Hence, these bands were used to identify the sex of 322 squabs. The results of sexing were also confirmed using sexing by sequencing (Appendix 1).

2.2.4 Data analysis

The sex ratio was determined annually by calculating the number of males and females alive in year t. The researcher examined the probability that number of females was

equal to or less than the observed number using the binomial distribution function (BINOMDIST) with the probability (p) = 0.5. The probability values (p) were transformed using ($-\log_{10}$) to visualise year and sub-population variation in the sex ratio. Annual mortality rate was calculated as the proportion of birds alive in year t that died before t+1 (Girondot and Pieau, 1993). The association between sex and mortality rate was examined using Fisher's exact test. Because the data on longevity were not normally distributed (The Ryan Joiner test, similar to Shapiro-Wilk test: R=0.946, p<0.10), the data were transformed using the Johnson transformation to achieve normality (R = 0.998, p > 0.100). Longevity was calculated based on the date of hatching and the date of death. The variances in longevity were homogeneously distributed across the sub-populations and sexes (Levene's Test statistic = 1.82, p = 0.061); hence, a General Linear Model (GLM) was performed to test whether there were significant differences in longevity between sites and sexes across sub-populations. In this GLM, the longevity data were run as a response variable, and site and sex were run as random variables. Since the interaction Site x Sex was non-significant ($F_{4, 783} = 0.64$, p = 0.635), the model was run without the interaction Site x Sex. All tests were conducted in Minitab 17.

2.3 Results

2.3.1 Adult sex ratio

The adult sex ratio was calculated using a BINOMDIST function of all sub-populations and for each single sub-population with a probability (p) of female = 0.5.

Overall, the adult sex ratio is biased towards males between 1987 and 2008 (p = 0.001) (mean± SE =157.18± 1.66 and 121.5±1.89, for the number of males and females, respectively). Annual changes in adult sex ratio showed a significant bias toward males since 1995 ($p \leq 0.05$ for all comparisons) (Figures 2.2). The largest differences between the number of males and the number of females were in 2007 with 255 males (mean± SE =51± 9.39) compared to 183 females (mean± SE=36.6±6.26), and in 2001 with 289 males (mean± SE = 57.8±4.30) compared to 216 females (mean± SE = 43.2±4.5.15) (Figure 2.3).

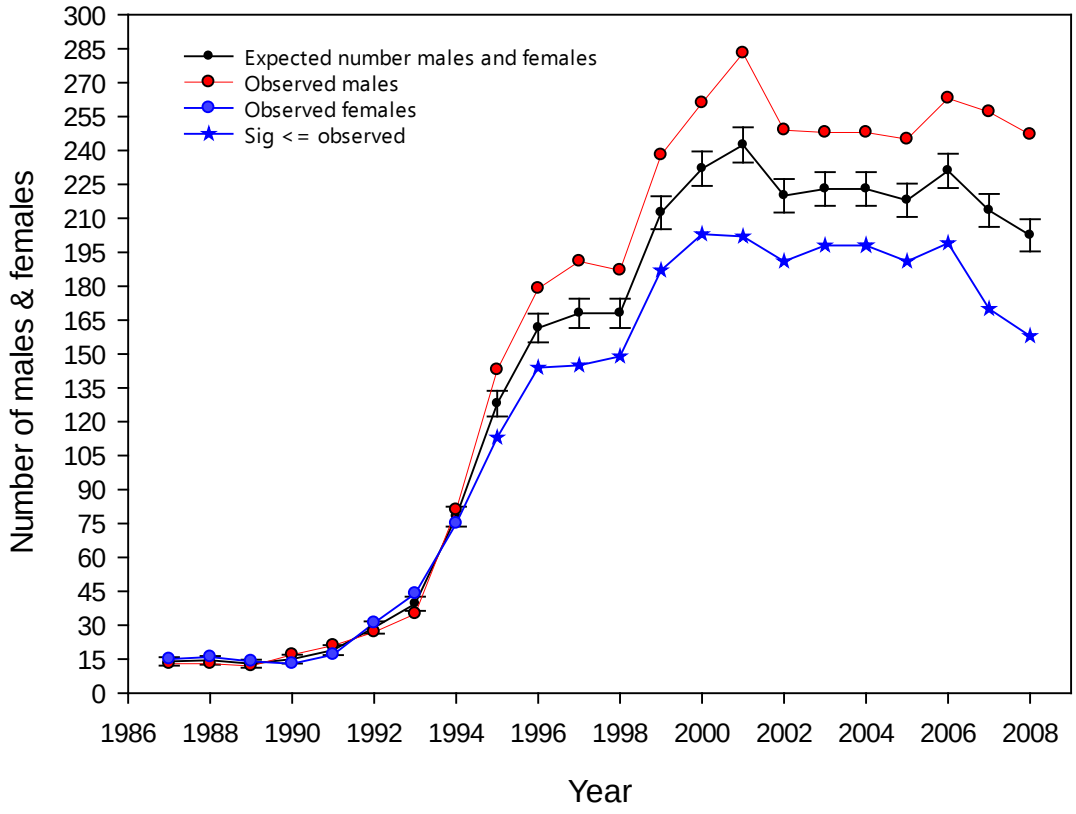


Figure 2.2 Changes in adult sex ratio across all sub-populations. Expected number ((number of males + number of females)* 0.5) ± (SE)) of female and male adults compared to observed numbers in all sub-populations between 1987 and 2008 .The standard errors were calculated using a bootstrap.

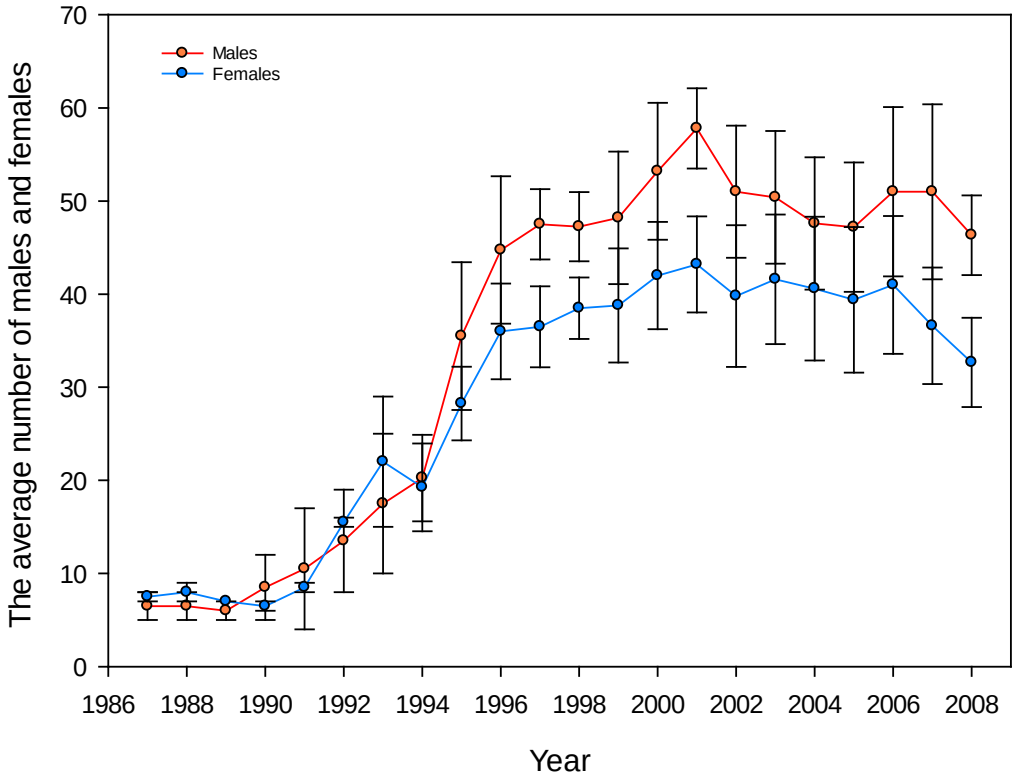


Figure 2.3 Changes in adult sex ratio in all subpopulations. Mean (±SE) of number of males and females in all subpopulations between 1987 and 2008.

The probability that the number of females was equal to or less than the observed number was examined in each single sub-population over 22 years between 1987 and 2008 (Figures 2.4 and 2.5 a-e). Two sub-populations (Pigeon Wood and Plaine Lievre) have been significantly biased towards males in different years. Specifically, Pigeon Wood shifted to become significantly male-biased in 1999 and has maintained this bias ever since (except in 2003 and 2004), and Plaine Lievre was significantly male-biased in four years (1995, 1996, 2007 and 2008). Bel Ombre and Ile aux Aigrettes sub-populations were male-biased in 2004 and 1999 respectively. I found no evidence that any sub-population was significantly female-biased at any point during the study period.

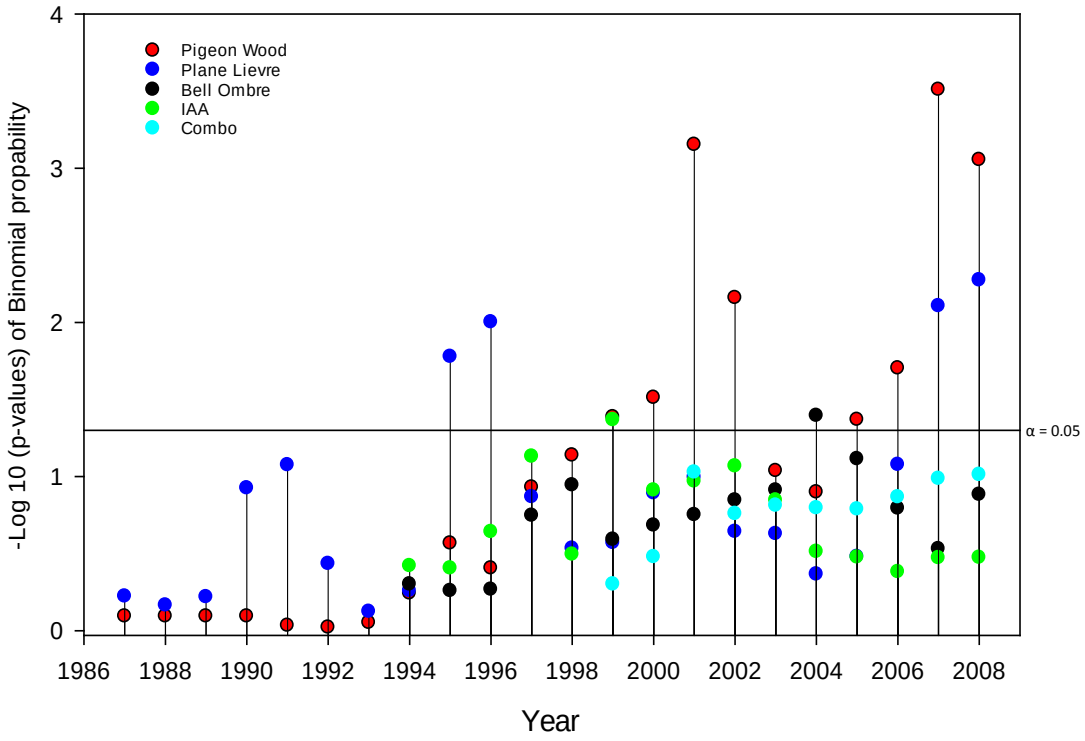
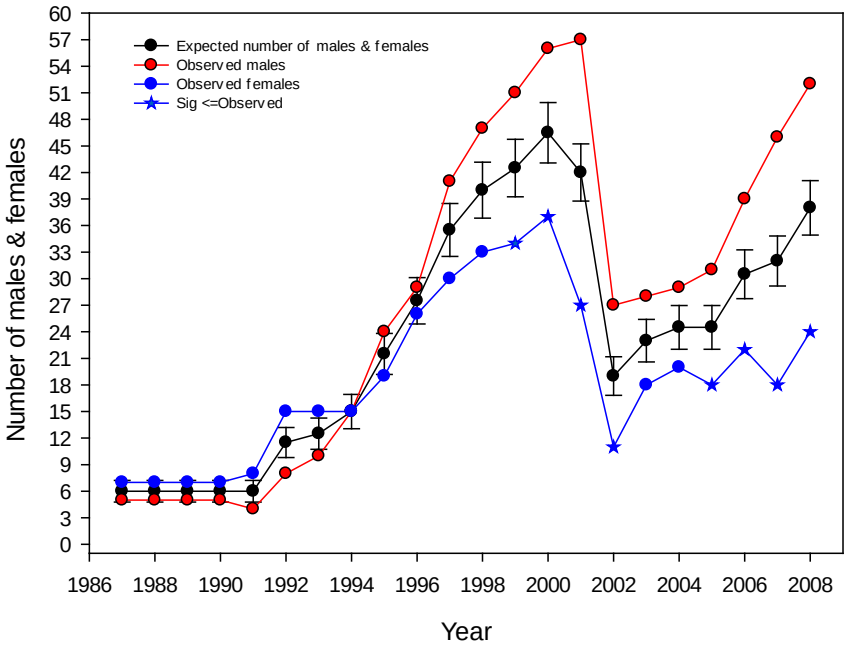


Figure 2.4 Changes in adult sex ratios in each sub-population. The probability (-log10 transformed) that observed female numbers were equal to or less than expected numbers, calculated using a BINOMDIST function in each sub-population with probability (p) of female = 0.5. Three sub-populations showed significant biases toward males in different years.

a



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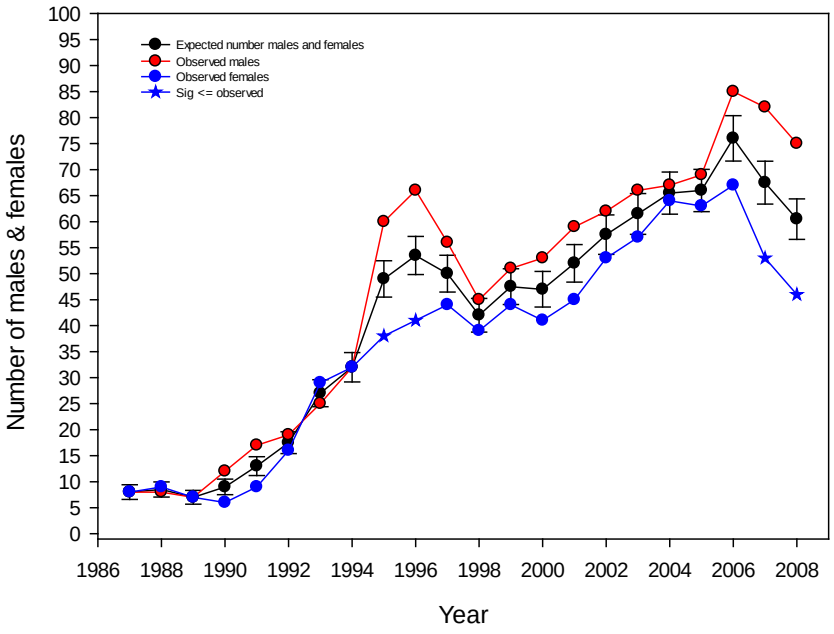
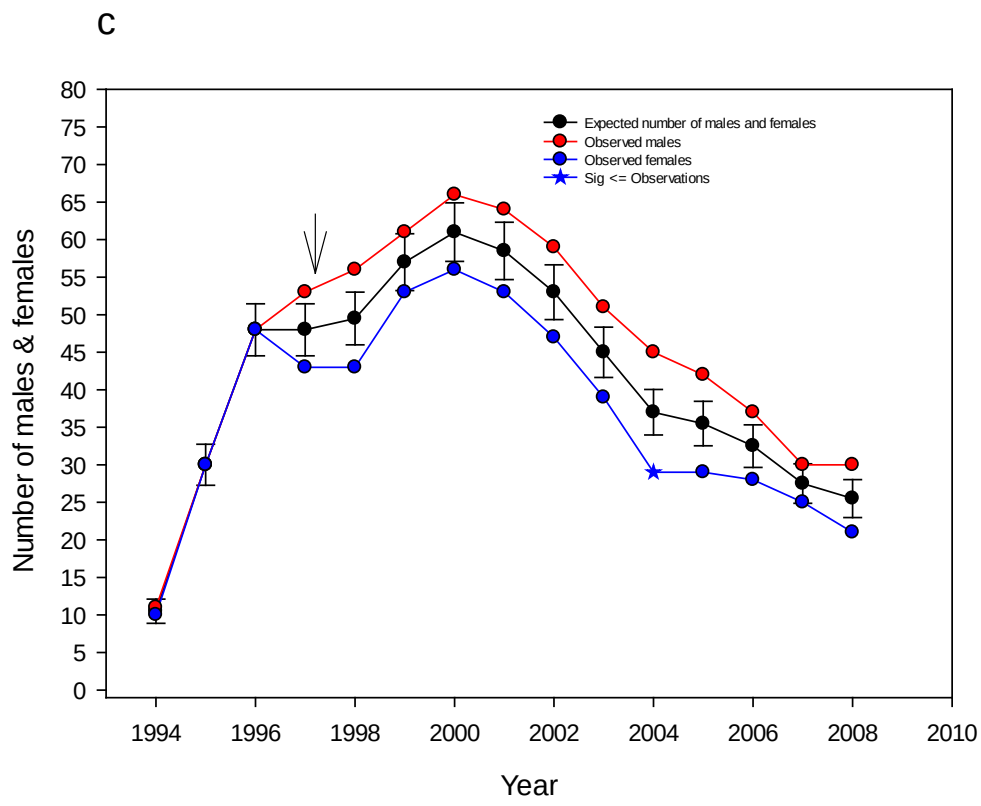


Figure 2.5 a-e Changes in adult sex ratio in each subpopulation. Expected number ((number of males + number of females)* 0.5) ± (SE) of adult females and males compared to observed number in each subpopulation between 1987 and 2008, (a) observed female number in Pigeon Wood was significantly < expected number in 1999, 2000, 2001, 2002, 2006, 2007 and 2008, (b) observed female number was significantly < expected number in Plaine Lievre in 1995, 1996 and 2007, (d) observed female number was significantly < expected number in Ile aux Aigrettes in 1999. Observed female numbers in both Bel Ombre (c) and Combo (e) did not differ significantly from the expected number across the period studied. Arrows indicate end of the releases.



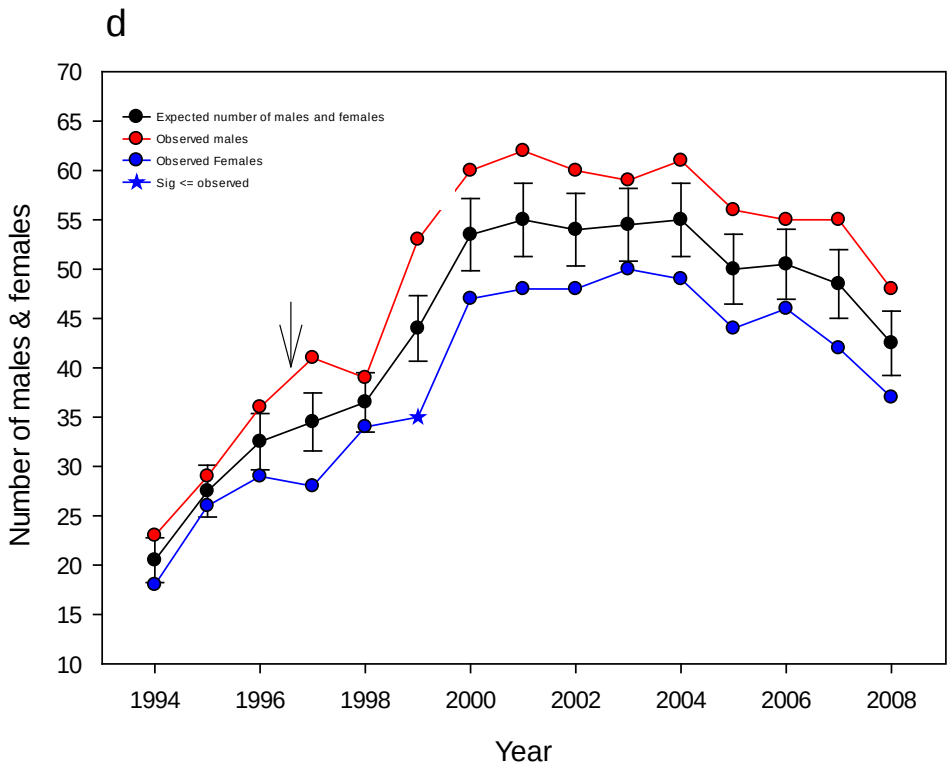


Figure 2.5 a-e. Continued

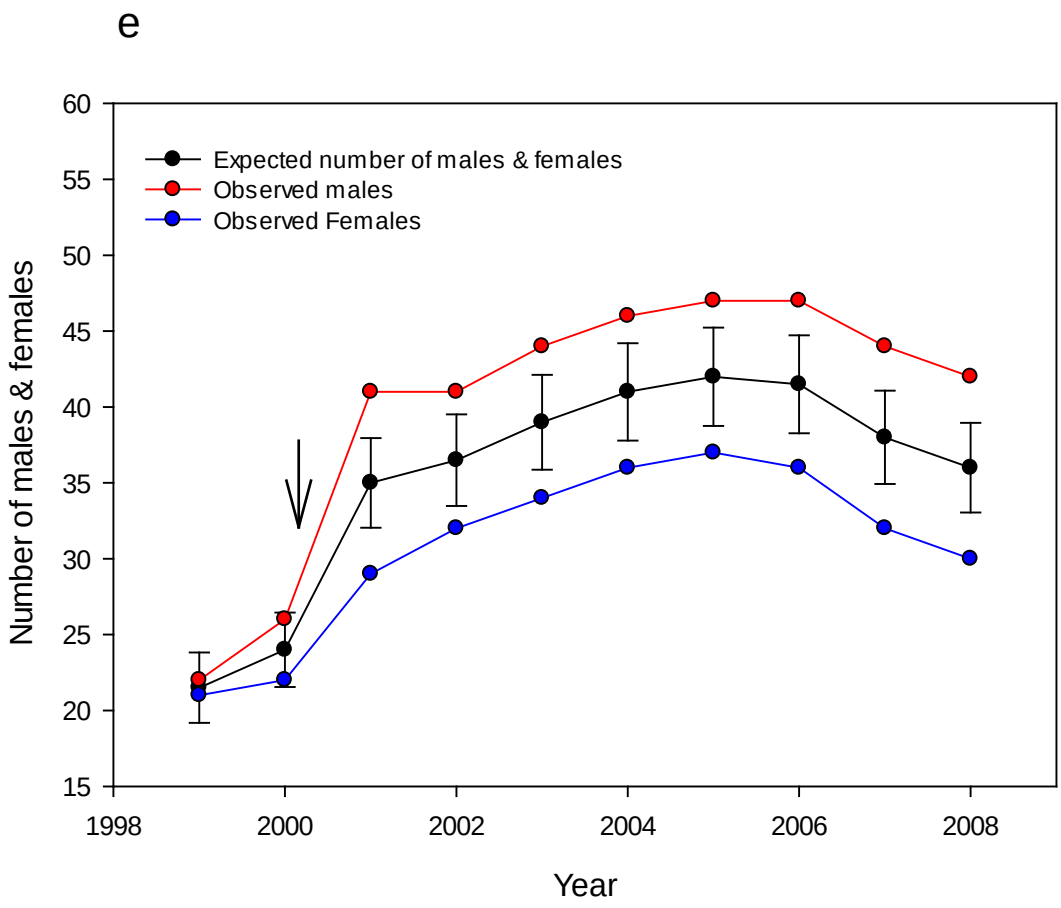


Figure 2.5 a-e Continued

2.3.2 Sex ratio in adult mortality

Fisher's exact test was performed to examine whether the overall annual mortality rate in adults was associated with sex. I found no evidence of an association between the annual mortality rate and sex in adult birds (p-v > 0.05) in the period between 1987 and 2008 (Figures 2.6 and 2.7 a-e and 2.8).

The association between the annual mortality rate in adults and sex ratio was not significant (p-v > 0.5) over the period between 1987 and 2008 in any sub-population.

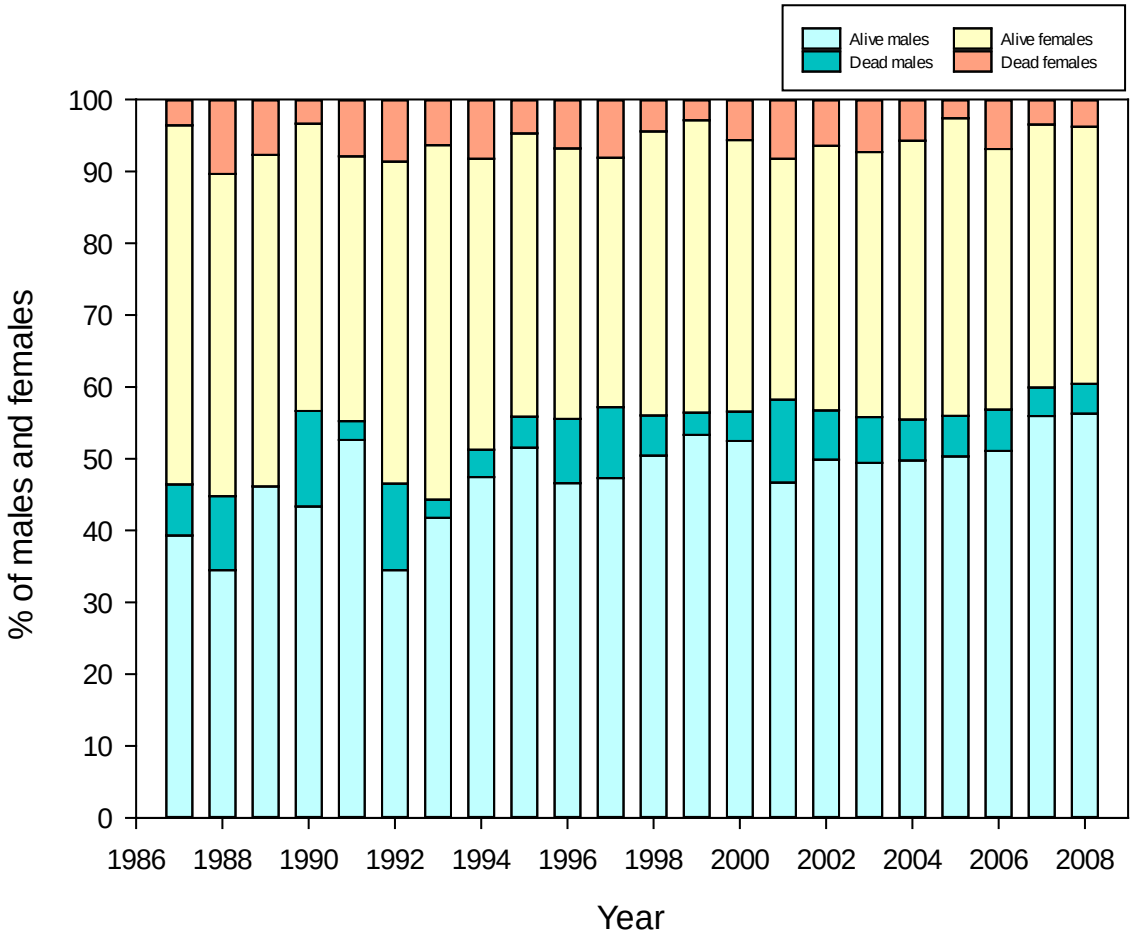


Figure 2.6 Sex ratio in adult mortality. Percentage of males and females alive and dying in all sub-populations between 1987 and 2008.

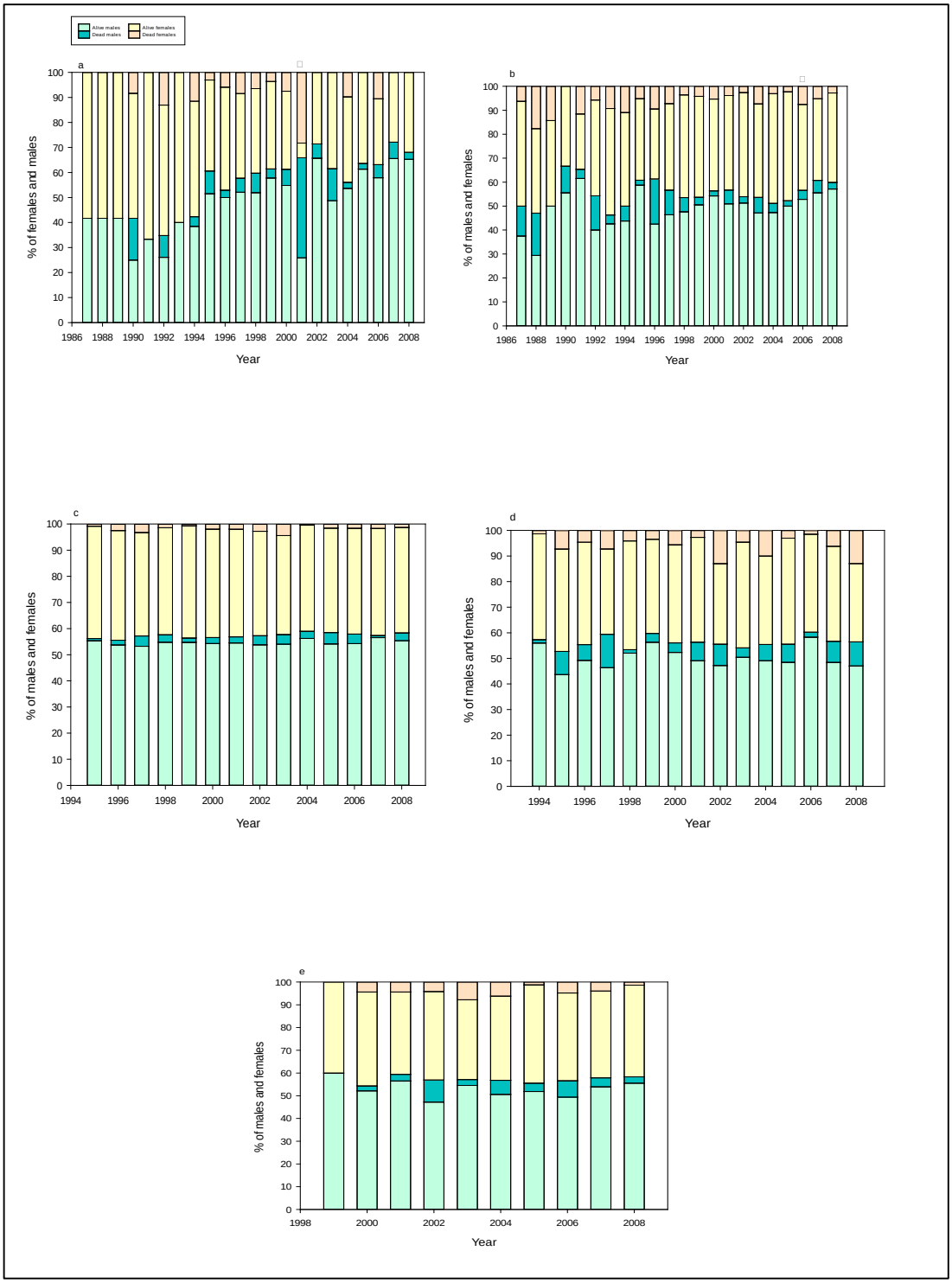


Figure 2.7 a-e. Sex ratio in adult mortality. Percentage of alive and dead males and females in each single sub-population between 1987 and 2008. (a) in Pigeon Wood, (b) in Plaine Lievre, (c) in Bel Ombre, (d) in Ile aux Aigrettes and (e) in Combo.

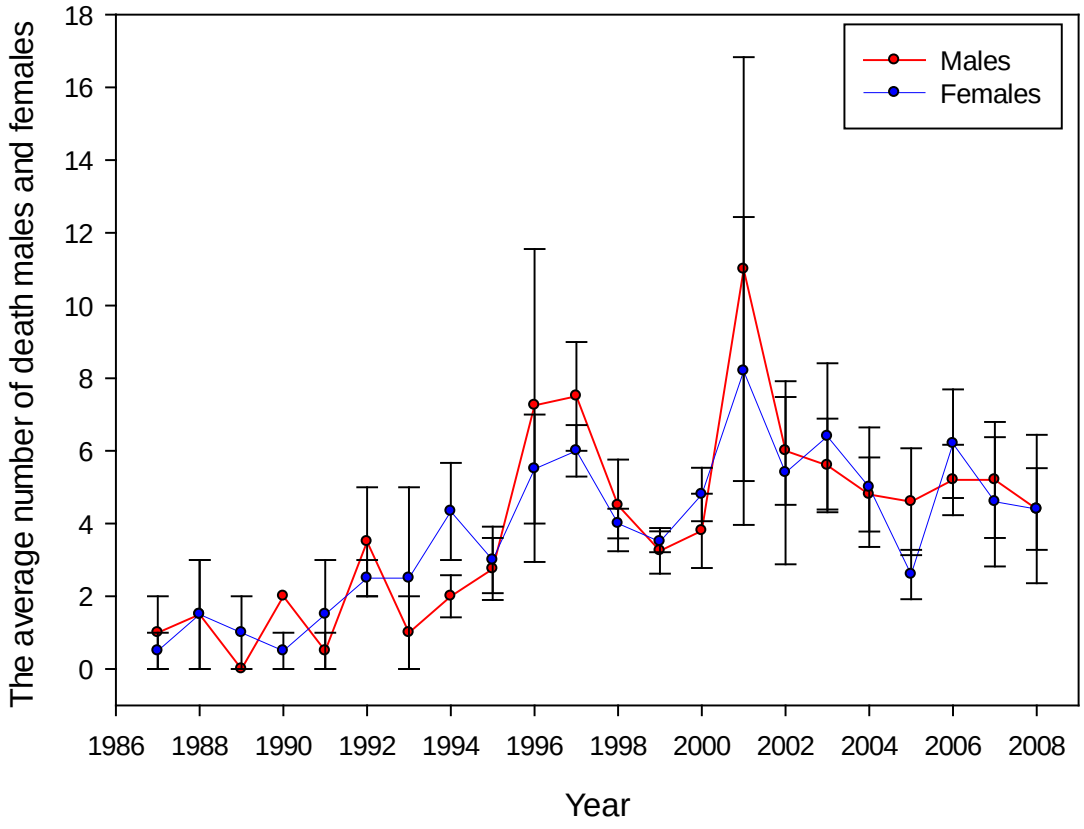


Figure 2.8 Changes in sex ratio in adult mortality in all subpopulations. Mean (±SE) of deaths in females and males in all subpopulations between 1987 and 2008.

2.3.3 Sex ratio and longevity

There was no significant difference in longevity between either sex or sub-populations over the 22 years between 1987 and 2008 (Table 1 and Figure 2.9).

Table 2.1 GLM with longevity as response variable and site and sex as random factors.

Factor	DF	MS	F-Value	P
Site	4	1.77303	1.93	0.103
Sex	1	0.00293	0.00	0.955
Error	778	0.91729		
Total	783			

DF: the degrees of freedom, MS: mean squares, F: the statistic

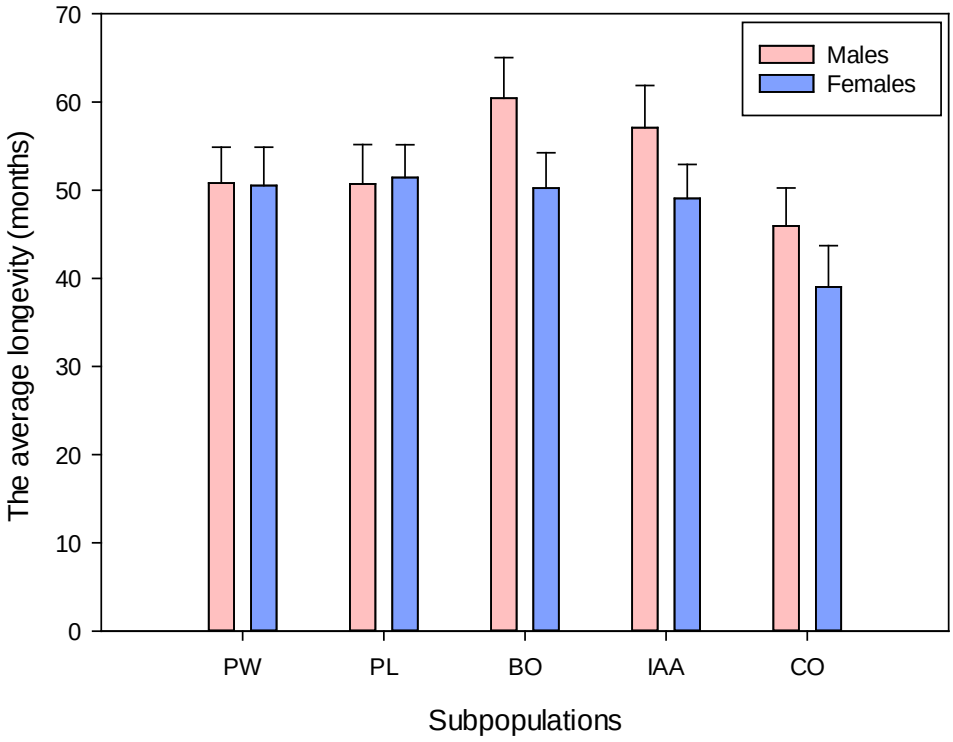


Figure 2.9 Longevity sex ratio in all subpopulations. The mean (±SE) longevity of females and males in the five subpopulations, Pigeon Wood, Plaine Lievre, Bel Ombre, and Combo. Longevity did not diver significantly neither between sexes nor between subpopulations between 1987 and 2008.

2.3.4 Sex ratio in fledglings

The fledgling sex ratio was calculated using a BINOMDIST function of all sub-populations with a probability (p) of female = 0.5. The sex ratio of fledglings (including those birds that hatched in captivity before being released) showed that the number of males significantly outweighed the numbers of females in five years: namely, 1995, 1997, 2001, 2006 and 2007 (Figure 2.10). The relationship between numbers of fledgling males and females and the sex ratio in adults was examined and found that number of fledgling males is strongly associated with male-biased adults (Pearson correlation = -0.549, P-Value = 0.008).

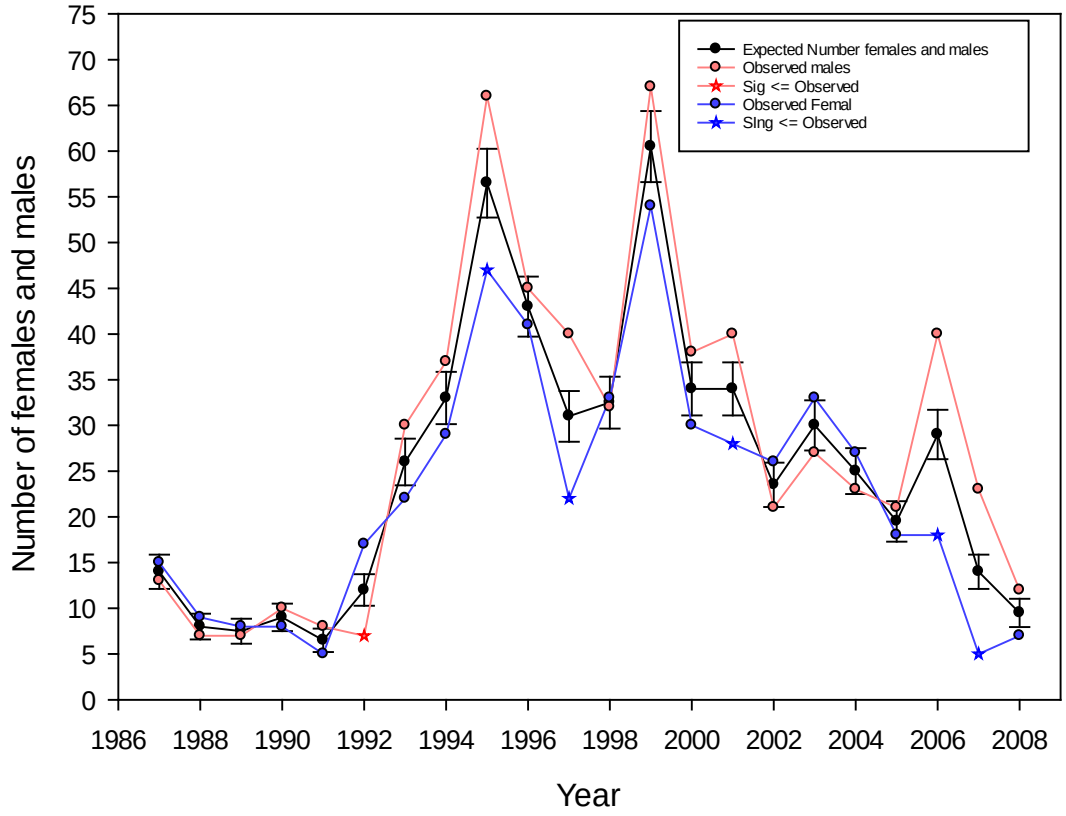


Figure 2.10 Changes in fledgling sex ratios across all sub-populations. The expected numbers ((observed fledgling males number + observed fledgling females number) * 0.5) ± (SE) of fledgling females and males compared to the observed numbers in all sub-populations between 1987 and 2008.

Analyzing the fledgling sex ratio in each sub-population over the study period showed a significant male bias in three sub-populations: Pigeon Wood (1994, 2001 and 2007), Plaine Lievre (1995 and 2006), and Ile aux Aigrettes (1997 and 1999) (Figures 2.11 and 2.12 a-e). One sub-population (Pigeon Wood) showed an overall significant male bias over 22 years (p-v = 0.007) (mean females ± SE = 4.84 ± 0.551; mean males ± SE = 6.79 ± 0.726) (Figure 2.13).

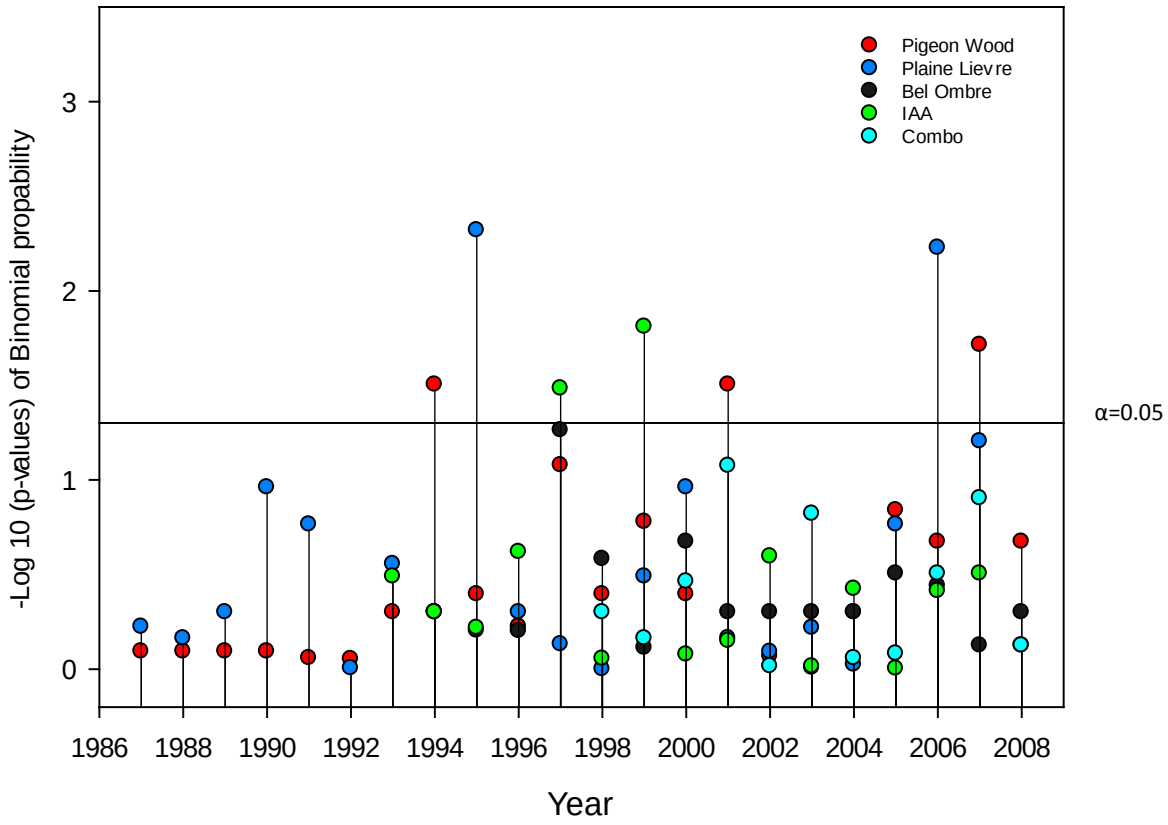


Figure 2.11 Changes in fledgling sex ratios in each sub-population. The probability (-log10 transformed) that observed fledgling female numbers would be equal to or less than expected fledgling numbers, calculated using a BINOMDIST function in each sub-population with a probability (p) of female = 0.5. Three sub-populations showed significant male biases among fledglings: Pigeon Wood (1994, 2003 and 2007), Plaine Lievre (1995 and 2006) and Ile aux Aigrettes (1997 and 1999).

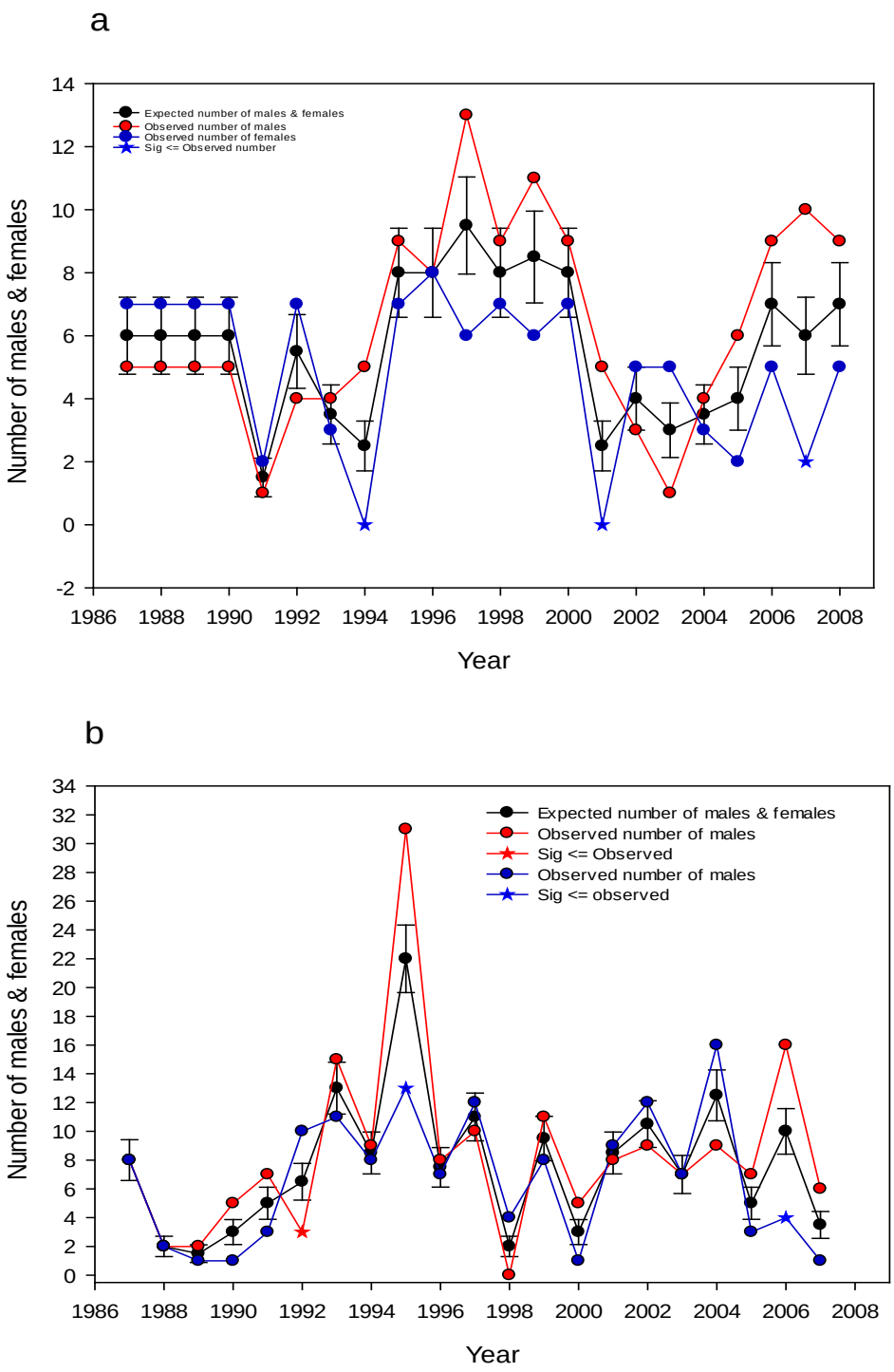
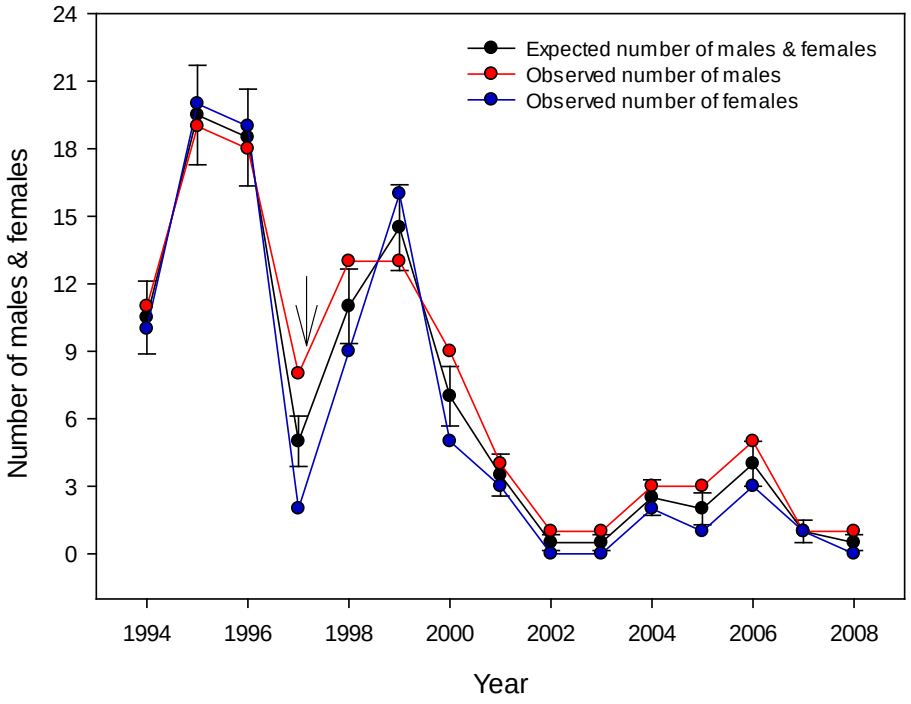


Figure 2.12 a-e. Changes in fledgling sex ratio in each subpopulation. Expected number (number of males + number of females)* 0.5) ± (SE) of fledgling female and male adults compared to observed number in each subpopulation between 1987 and 2008, (a) observed female numbers in Pigeon Wood were significantly < expected in 1994, 2003 and 2007, (b) observed fledgling female numbers were significantly < expected in Plaine Lievre in 1995 and 2006, (d) observed fledgling female numbers were significantly < expected in Ile aux Aigrettes in 1997 and 1999. Observed fledgling female numbers in both Bel Ombre (c) and Combo (e) did not differ significantly from the expected number across the period studied. Arrows indicate end of the releases.

c



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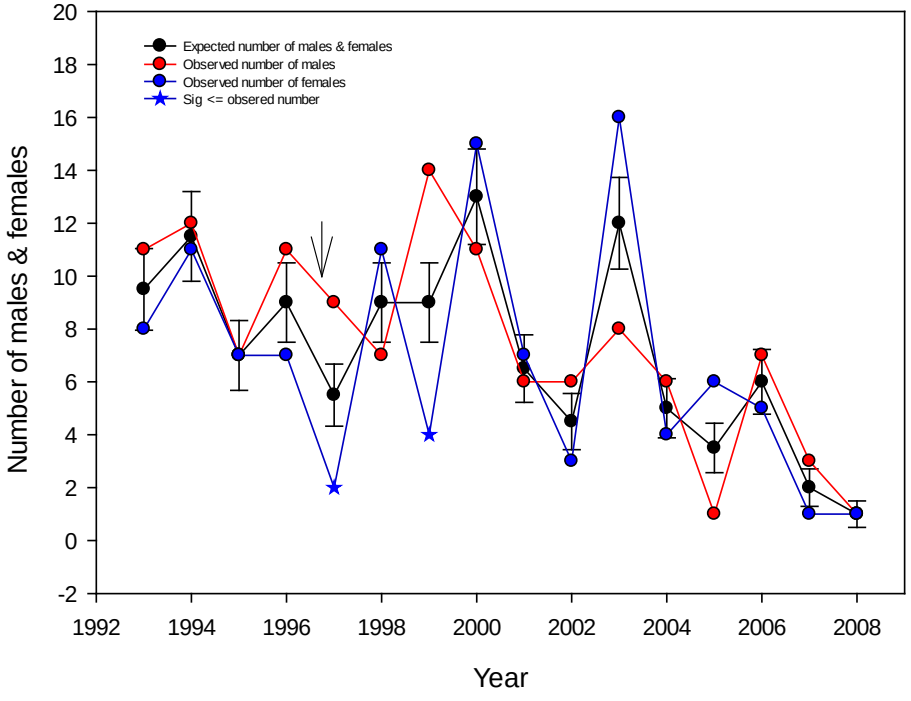
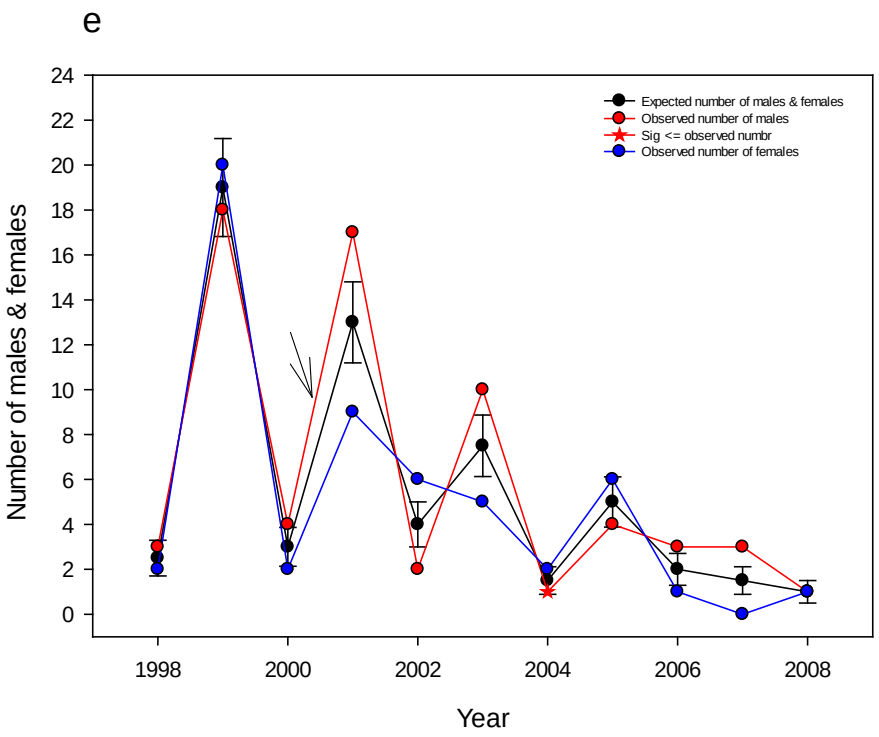


Figure 2.12 a-e. Continued



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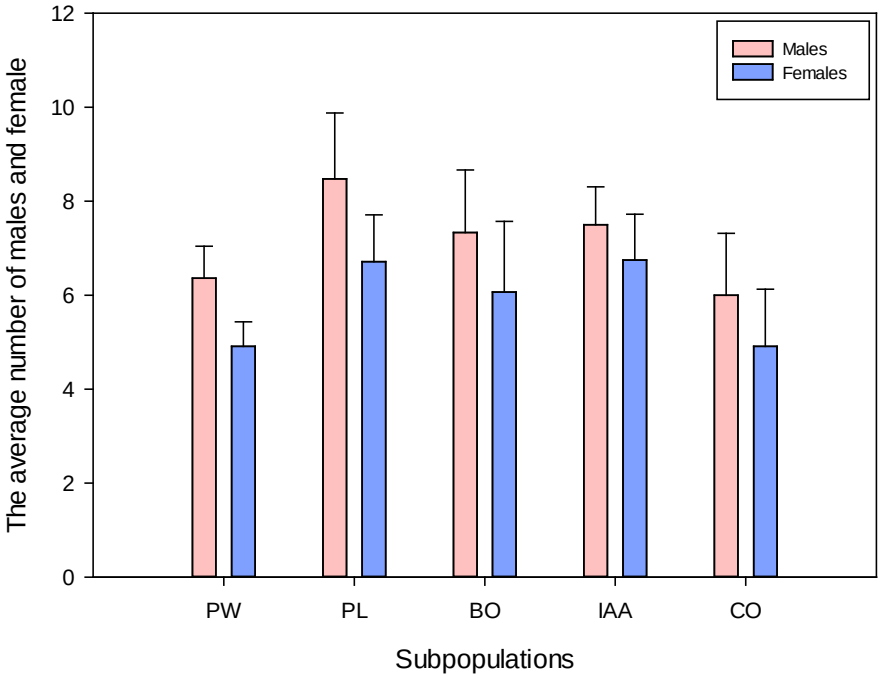

Figure 2.13 The Mean (±SE) of fledged males and females in the five subpopulations between 1987 and 2008. (PW) Pigeon Wood, (PL) Plaine Lievre, (BO) Bel Ombre. (IAA) Ile aux Aigrettes and (CO) Combo.

2.3.5 Hatchling sex ratio in the Ile aux Aigrettes sub-population

The sex ratio at hatching in 322 squabs hatched between 2004 and 2008 in the Ile aux Aigrettes sub-population was examined. I found no evidence that the sex ratio deviated from parity across that period of time; that is, p-values were always greater than 0.05 (Figure 2.14). I also calculated the probability that the proportion of males or females in broods within 49 families in Ile aux Aigrettes was equal to or less than the numbers observed. There was no evidence that male or female numbers deviated from parity; that is, p-values ranged between 1.00 and 0.063 for male and between 1.00 and 0.073 for females (Figures 2.15).

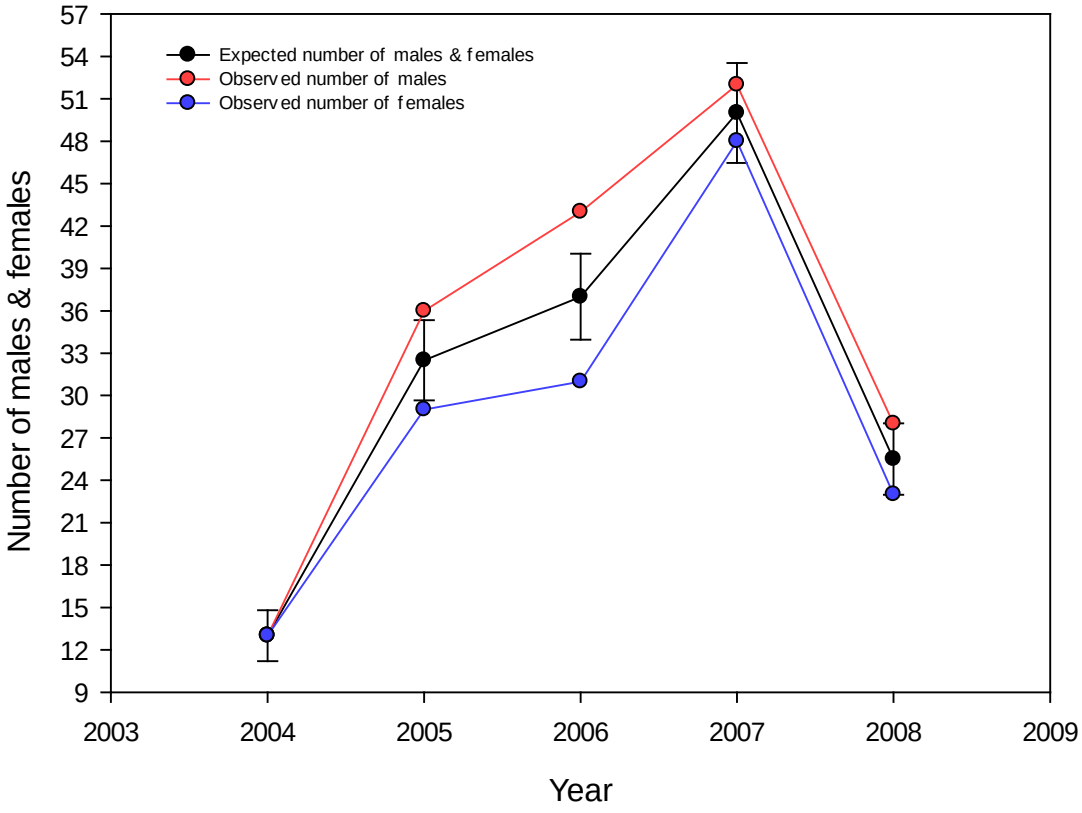

Figure 2.14 Changes in squab (hatchling) sex ratios in the Ile aux Aigrettes sub-population between 2004 and 2008. The expected hatchling numbers ((number of males + number of females) * 0.5) ± (SE) compared to the observed hatchling numbers.

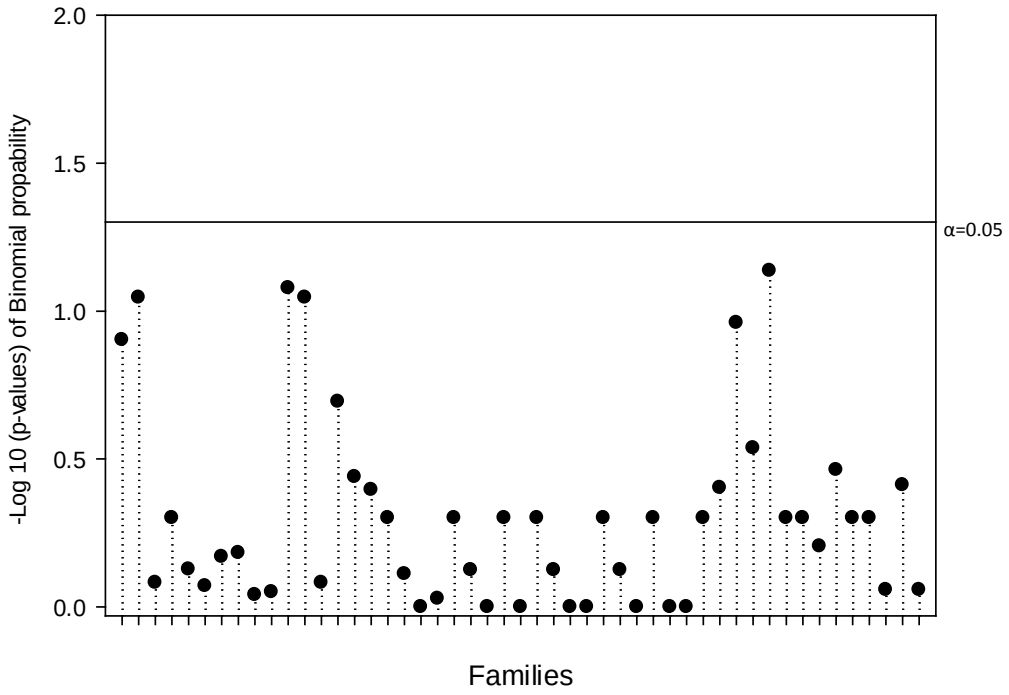

Figure 2.15 Brood sex ratio. The probability (-log10 transformed) that observed female numbers in broods in 49 pink pigeon families in Ile aux Aigrettes subpopulation varies from parity, calculated using a BINOMDIST function with probability (p) = 0.5. There is no significant difference between observed and expected hatchling female numbers.

2.4 Discussion

2.4.1 Adult sex ratio

The present study was conducted to assess the sex ratio in the pink pigeon population between 1987 and 2008. Specifically, it sought to determine whether mortality and longevity varied significantly between adult males and females and whether the numbers of hatchling and fledgling males and females deviated significantly from equality (1:1). In the present study, it has been found that the overall sex ratio in the adult pink pigeon population has been significantly biased towards males since 1995. This finding supports the results of other studies that have shown that the sex ratios in many bird species are male-biased (reviewed in Donald, 2007; Taylor and Parkin, 2008). It also supports the observations of earlier studies by Bunbury (2006) and Concannon (2014). However, Bunbury (2006) findings were based on cumulative data of 931 birds over a period of 17 years. That study reported that, in early periods, when the

population size was small, the sex ratio was female-biased. I agree with the Bunbury (2006) study that females outnumbered males in the early periods, including the periods from 1987 to 1989 and from 1991 to 1994 (see figures 2.2 and 2.3). However, we also show that there was no significant sex bias in any year between 1987 and 1994. Concannon (2014) did not test whether the skew of sex ratio from parity was significant. In order to identify which sub-population(s) contributed to the male bias in the pink pigeon population, the sex ratio was analysed within each sub-population. In this context, I agree with Bunbury (2006) study, which found that the sex ratios of all sub-populations were male-biased following the end of releases of captive bred birds. However, our results showed that the deviation in sex ratio is significantly biased towards males in four sub-populations at different times, including Plaine Lievre (in 1995, 1996, 2007 and 2008), Pigeon Wood (in 1999, 2000, 2001, 2002, 2005, 2006, 2007 and 2008), Bel Ombre and Ile aux Aigrettes (in 2004 and 1999 respectively). It might be that this bias in the adult sex ratio was driven by variety in the mortality rate and/or longevity between adult males and females and/or by biases in the fledgling and/or hatchling stages.

There is no evidence of an association between the mortality rate and sex in adult birds in the period between 1987 and 2008, either in the entire population or in each individual sub-population ($p > 0.05$). The highest average number of adult deaths across sub-populations occurred in 2001 (M ± SE = 11 ± 5.830 males and 8.2 ± 4.235 females). Natural factors (e.g. disease and predation), can influence the parity of the sex ratio by increasing mortality more in one sex than in the other (Safford and Jones, 1998). In the case of the pink pigeon, predators, including monkeys, mongoose, rats and cats, generally feed on the birds' nests (Bunbury, 2006) thus their influence on the adult sex ratio may be marginal. Males incubate during the day and females incubate during the night and early morning (Bunbury, 2006). Although sex-biased predation can affect the sex ratio (Christe *et al.,* 2006), it is challenging to quantify the role of predation during incubation on sex ratio in mainland pink pigeon populations although installation of camera traps at nest sites may shed useful light on this. Bunbury et al. (2008) study of infection by *Trichomonas gallinae* showed that there were no significant differences between sexes in infection prevalence over time in *T gallinae* (see chapter 3). As mentioned in the literature review, the adult sex ratio in most bird species is male-biased

(e. g Greene and Fraser, 1998), which may be caused by a lower survival rate (i.e., the reverse of the mortality rate) in females than in males (Trivers, 1972; Stenzel *et al.,* 2011). The sex differences in survival rate may be explained by the fact that homogametic sex (which is female in birds) is more prone to recessive deleterious mutations (Maklakov and Lummaa, 2013). However, in the case of the pink pigeon, the underlying reason might be different, since the survival rate/mortality rate did not differ significantly between adult females and males. This finding seems to be consistent with other species. For example, Dobson (1987) compared differences between the survival of males and females of fifteen species of common British birds and found no significant differences between sexes in the majority of species. Studying survival for one pink pigeon sub-population, Ile aux Aigrettes, revealed no significant differences in survival between sexes in adults (Concannon, 2014). Studies on the role of longevity effects on adult sex ratio are rare because this requires long-term population studies. However a few studies on other taxa have shown that sexes do differ in longevity. For example, Litzgus, (2006) found that female Spotted Turtles *Clemmys guttata* lived significantly longer than males. An evolution study performed by Chen and Maklakov (2014) showed that male roundworms *Caenorhabditis remanei* live longer than females as a result of evolution in longevity which is driven by sex-specific interactions between environmental factors and internal conditions of organism. Various studies of humans have indicated that the difference in longevity between sexes is related to the loss of telomere chromosome caps (e.g. Aviv et al., 2005; Honig et al., 2006). This relationship has been found in other taxa. For example, Barrett et al., (2013) and, Barrett and Richardson (2011) reviewed recent studies that claimed a relationship between telomeres loss and lifespan in different species and concluded that a relationship cannot be generalized relying on just one taxa. In this thesis, the lowest difference between sexes in average longevity over 22 years was found in the Pigeon Wood and Plaine Lievre sub-populations, with longevities of M±SE=50.53 ±4.34 months in females and 50.81 ±4.08 months in males for Pigeon Wood and of 51.44 ±3.72 months in females and 50.71 ±4.48 months in males for Plaine Lievre. If the pink pigeon sex ratio was linked to longevity, It would be that these two sub-populations to have lower male biases than other sub-populations; however, they were found (as it has been shown previously) to

have significant male biases during more than one period, particularly in comparison to other sub-populations, which were found to have equality in the sex ratio at all times.

Adult population sex ratios can deviate from parity if there is a bias in the primary or secondary sex ratio or if there are differences in age, including survival/mortality and longevity, between adult males and adult females (Gibbons and Gibbons, 1990). Because there is no evidence found that the male bias in pink pigeons emerged at the adult stage, the fledgling sex ratio across all sub-populations and in each individual sub-population was examined.

The fledgling sex ratio across all subpopulations was significantly male-biased in four different years, namely 1995, 1997, 2006 and 2007. Moreover, it was found that the number of young fledglings was strongly associated with the existence of a male-bias among adults (Pearson correlation = -0.549, P-Value = 0.008). This means that adult male bias in the pink pigeon population could be related to male bias at the secondary level (after hatching) or primary level (before hatching) rather than a mortality-biased sex ratio in adults. Findings showed a significant male bias in fledglings in three subpopulations, including Pigeon Wood (in 1994, 2001 and 2007), Plaine Lievre (in 1995 and 2006) and Ile aux Aigrettes (in 1997 and 1999). These deviations over the 22-year study period may accelerate the overall male bias in fledglings in the total population. Sex bias at the secondary level occurs if there is a mortality bias prior to the hatchling stage (Ewen *et al.,* 2001; Benito and GonzÁLez-SolÍS, 2007), in post-hatch mortality (Clutton-Brock *et al.,* 1985; Pérez *et al.,* 2006) or if there is a sex bias at the primary level (Rutkowska and Cichoń, 2006).

Female-biased mortality in embryos or squabs might be the cause of sex ratio bias in the fledgling sex ratio in these periods especially in the Pigeon Wood sub-population. Inbreeding may affect offspring survival during the embryo and nestling periods due to size dimorphism (Brekke *et al.,* 2010), although a study by Frankham and Wilcken (2006) concluded that directional distortions in the sex ratio are not a consistent signal of inbreeding depression. However, a significant variation in the proportion of *T. galinae* infection among sub-populations has been found (Bunbury 2006 and chapter three in this thesis), and, interestingly, Pigeon Wood was the only sub-population of five that showed significant variation in the proportion of infection between males and females. Hence, differences in susceptibility/resistance to disease between sub-populations or

between sexes might the cause for a potential mortality bias in embryos or squabs in Pigeon Wood. Ongoing work is investigating disease-resistance-related SNPs in order to compare and contrast the RAD-seq genotypes between individuals and sexes.

2.4.2 Hatchling sex ratio

The sex allocation theory predicts that parents will produce an approximately equal proportion of both sexes, as long as each sex is equally costly for parents to produce (Fisher, 1930). However, this parity may be disrupted when the primary sex ratio is adjusted to achieve fitness benefits (Trivers and Willard, 1973) or when differential offspring mortality occurs (Lee *et al.,* 2010; Griffiths, 1992; Bradbury and Griffiths, 1999). No evidence was found of bias in primary sex ratio at the one population, IAA, where all newly hatched squabs could be accessed due to the reduced height of the lowland vegetation. However, the fledgling sex ratio was male-biased in three sub-populations in different years, including Pigeon Wood (in 1994, 2001 and 2007), Plaine Lievre (in 1995 and 2006), and IAA (1997 and 1999) (Figure 2.12 a-e). Introduced predators including the black rat (*Rattus rattus*) and the crab-eating macaque (*Macaca fascicularis*) have been reported as the most common predator affecting eggs and young of pink pigeon (Jones, 1987; Jones *et al.,* 1992). However, IAA has been cleared from all exotic mammalian predators compared to mainland subpopulations which is an important difference to consider.

In order to investigate whether the sex ratio is secondary-biased (e.g., as a result of mortality bias) or primary-biased, it would be ideal to determine the sex ratio of hatchlings in each sub-population. Hence, I investigated the prediction that the sex ratio in adults would be unbiased if there was no adult mortality bias and if the fledgling and hatching sex ratios were also unbiased. In order to carry out this analysis, the sex of 322 squabs that hatched between 2004 and 2008 in Ile aux Aigrettes was identified. The sex ratio was not biased toward either males or females between 2004 and 2008, and the p-values were always greater than 0.05. There was male fledgling bias in 1997 and 1999; however, these two periods fall outside the test periods, which began in 2004. The year 1999 was the only year in which the Ile aux Aigrettes population showed significant male bias. However, it also showed a significantly high male mortality bias during this year, which means that the male mortality brought the sex ratio back to a non-significant difference in the following years. As a result, in the absence of mortality bias and

hatchling bias, the adult sex ratio is consistent with the hatchling sex ratio. In order to confirm this finding, I tested brood sex ratios within 49 pink pigeon families. Brood sex ratios are related to various factors including paternal condition (e. g. Alonso-Alvarez and Velando, 2003), the fitness gains from producing sons and daughters (e. g. Øigarden and Lifjeld, 2013) and environmental conditions (e. g. Clutton-Brock *et al.,* 1985). I expect that the influences of these factors will differ between families, which may lead to bias in the brood sex ratio. Interestingly, the brood sex ratio was unbiased in all broods, a finding that confirmed our previous results.

2.5 Implications for Conservation of sex ratio of the pink pigeon

A primary aim in the recovery programmes of most threated and endangered species is to minimize extinction risk. This can be achieved by addressing the direct and indirect reasons for population reduction. The pink pigeon population has recovered from a very few birds to approximately 400 individuals now. The conservation management aims for this species are to expand the population size to over 600 individuals. However, several factors including habitat loss, predation, inbreeding depression and biased sex ratio are potential factors limiting population growth. Sex ratio in pink pigeon has been investigated in this chapter to examine the significance of bias in sex ratio and to identify causes behind this bias. The result showed significant bias in sex ratio towards male across time with male bias in fledglings in two subpopulations namely, Pigeon Wood and Plaine Lievre, resulting in the potential cause of skew in adult sex ratio.

Sex ratio is one of demographic stochastics that are used to estimate population viability (Gilpin and Soule', 1986). This is because biased-male sex ratio can increase the risk of extinction in vulnerable populations (Grayson *et al.,* 2014) via increase male-male competition and decrease reproductive success which affect population growth and viability (Ginsberg and Milner-Gulland, 1994).

One of the immediate interventions is to introduce more females into the mainland subpopulations to equalise the sex ratio in these subpopulations. However, long-term conservation management needs to diagnose the causes for male –bias before fledgling, especially in the Pigeon Wood and Plaine Lievre subpopulations that showed significant male-bias in fledglings. Data on reproductive success need to be recorded for each pair including fertility, number of eggs hatched, mortality before fledgling and number of

young fledged. This is difficult at the current time in mainland subpopulations due to the difficulty in accessing nests built in tall trees.

2.6 Conclusion

This study confirms that the adult sex ratio of the pink pigeon population is significantly male-biased. No evidence has been found that a mortality or longevity bias in adults was responsible for this skew in the sex ratio. Fledgling male bias was found to be significant in different years and subpopulations, especially in Pigeon Wood, which showed significant male-bias in three different periods. This may indicate a male bias in the primary or secondary sex ratios, causing bias in the fledglings. Investigations into hatchling and brood sex ratios in one subpopulation, the Ile aux Aigrettes, between 2004 and 2008 did not reveal any skew in the sex ratio of this sub-population.

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Chapter 3

Trichomonas gallinae infection in five endangered subpopulations of the pink pigeon (*Nesoenas mayeri*)

Summary

Infectious diseases caused by bacteria, viruses, protozoa and fungi may negatively influence the dynamics of wild populations, particularly when the immunogenetic variation in such populations has been compromised by inbreeding and genetic drift. The pink pigeon (*Nesoenas mayeri*) of Mauritius is one of the most endangered pigeons in the world (IUCN, 2015); it experienced a severe population bottleneck of only around 10 birds in 1975, but the population recovered to approximately 400 birds in seven subpopulations by 2014. Parasitic diseases, especially Trichomonosis caused by protozoa, are potential factors that can reduce squab and adult survivorship of the pink pigeon. Two aspects of Trichomonosis infections in pink pigeons are analysed in this chapter. First, the spatial and temporal variation in infection incidence of individual birds recaptured bimonthly was investigated between September 2002 and April 2004. A total of 418 individually ringed birds were captured and screened for *Trichomonas gallinae* across five subpopulations. Individuals were caught up to 10 times during this period which allowed the identification of individual birds with significantly low and high infection incidence. Some of these individuals were sequenced using Restriction site Associated DNA Sequencing (RAD-Seq).

Overall, the infection incidence differed significantly among subpopulations, but there was no significant difference between the sexes. In addition, there was a significant temporal variation in the proportion of infected birds which varied between sites. This significant site x period interaction shows that *T. gallinae* infection rates in the five subpopulations are out of synchrony. This implies that social interactions between individuals within each subpopulation could potentially impact infection dynamics within subpopulations.

I also analysed data on bird longevity and its relationship with *T. gallinae* infection. I performed statistical analysis for 251 birds which died before February 2010. All these birds had *T. gallinae* infection data that was examined between 2002 and 2004.

Longevity between birds that were found positive to infection in at least three bimonthly screening occasions was not significantly different from these that were found to be consistently negative to infection in at least three screening times.

3.1 Introduction

3.1.1 Disease in wild populations

Wild species are exposed to many parasitic diseases that include a wide range of both macroparasites (e.g. helminths, nematodes and arthropods), and microparasites (e.g. bacteria, viruses, protozoa, oomycetes and fungi) (Tompkins *et al.,* 2011). In small populations, which often suffer from low immunogenetic diversity (Eimes *et al.,* 2011), infectious diseases can have a significant impact on the population dynamics of natural populations by reducing fecundity and nestling survival, and/or by increasing mortality (Scott, 1988; Gulland, 1995).

The response to pathogens in wild populations is highly complex and is often influenced by the existence of different pathogenic strains, co-infection with other parasites (Munson *et al.,* 2010), host genetics (Smith *et al.,* 2009), variation in host demography (e.g. age, sex and sexual maturity), variation in density of populations and by the presence of different species within communities (Benskin *et al.,* 2009; Pedersen and Babayan, 2011). Moreover, such responses are subject to interactions with other drivers, such as habitat loss and climate change (Schelkle *et al.,* 2012).

It is becoming increasingly difficult to ignore the role of host density in infection prevalence among individuals (Johnson *et al.,* 2011; McCallum *et al.,* 2001). Parasite epidemics can occur when the reproductive ratio (R_0) is greater than unity so that the parasite can spread through a host population. In the case of directly transmitted parasites, R_0 is a function of the transmission efficiency, the contact rate and the duration that an infected host is contagious (Dietz, 1993). In frequency-dependent transmission, the contact rate (e. g. sexual contact) is independent of host population density, which results in constant infection despite the decline in host population density. Unlike the density-dependent transmission, frequency-dependent transmission can make hosts more prone to population extinction due to infectious diseases (Ryder *et al.,* 2007; Johnson *et al.,* 2011).

3.1.2 Immune defence and reproduction

It is assumed that the immune system of the host would defend against a variety of infectious diseases. However, according to life history theory, evolutionary and/or physiological trade-offs are predicted to occur when investing in immune defence as a life-history trait, as well as other life-history traits (Sheldon and Verhulst, 1996; Gustafsson *et al.,* 1997; Møller *et al.,* 1997; Lochmiller and Deerenberg, 2000; Zuk and Stoehr, 2002). Although there are inherent difficulties in capturing and screening individuals in free-living populations, there is evidence from experimental studies that reduction in immunity is linked to an increase in investment in reproductive effort in a variety of species (Hanssen *et al.,* 2005; Knowles *et al.,* 2009).

Studies of the relationship between reproduction and longevity in birds are rare, and most of these studies have only been conducted on mammals, reptiles and insects. In mammals, longevity is negatively linked to an increase in reproductive effort in *Homo Sapiens* (Meeûs, 2000), and the same has been found in Asian elephants (*Elephas maximus*) (Hayward *et al.,* 2014). In insects (*Asobara tabida*), the number of eggs has been found to be negatively correlated to longevity (Ellers, 1996), and there have been trade-offs between reproductive effort and survival in fruit flies (*D. melanogaster*) (Flatt, 2011). In birds, longevity was found to be lower in both sexes of infected cowbirds than that of two uninfected blackbird species (Hahn and Smith, 2011).

3.1.3 Pink pigeons

The pink pigeon (*Nesoenas mayeri*) of Mauritius is one of the most endangered pigeons in the world, having recovered from a severe population bottleneck (Jones *et al.,* 1992). It has been estimated that the number of birds in the wild in 1975 was around 10 birds (Jones *et al.,* 1992; Jones *et al.,* 1999). However, after the recovery programme (established on Mauritius and in Jersey Zoo in 1976 and 1977, respectively) and following the reintroduction programme started in 1987, the number of free-living birds increased dramatically from just a few birds to 297 birds in 1998, 355 birds in 2003 (in five sub-populations (Swinnerton *et al.,* 2004; Swinnerton *et al.,* 2005b), 380 birds in 2008 (Bunbury *et al.,* 2009) and the number stabilized to around 400 birds in 2014. The decline in the pink pigeon population was caused by habitat destruction, predation by introduced mammals, competition (Jones *et al.,* 1992), a lack of food sources (Edmunds

et al., 2008) and probably introduced diseases (Jones, 1987; Greenwood, 1996; Swinnerton, 2001). Infection parasitic disease that are socially transmitted, such as Trichomonosis and Leucocytozoonosis, are considered to be a major factor in the reduction of squab and adult survivorship (Swinnerton *et al.,* 2005a; Swinnerton *et al.,* 2005b; Bunbury *et al.,* 2007a; Bunbury *et al.,* 2007b; Bunbury *et al.,* 2008).

3.1.4 Disease in pink pigeons

Trichomonosis and Lecucocytozoonosis are caused by the protozoan parasites *Trichomonas gallinae* and *Leucocytozoon marchouxi*, respectively (Swinnerton *et al.,* 2005a; Swinnerton *et al.,* 2005b; Bunbury *et al.,* 2007a; Bunbury *et al.,* 2008; Bunbury *et al.,* 2009). Trichomonosis is a widespread disease affecting several bird species throughout the world, including Columbiformes (e. g. Bunbury, 2011; Stimmelmayr *et al.,* 2012), Psittacines (e. g. McKeon *et al.,* 1997), Galliformes (e. g. Mantini *et al.,* 2009), raptors (e. g. Sansano-Maestre *et al.,* 2009) and Passerines (e. g. Lehikoinen *et al.,* 2013), and it generally affects the upper respiratory and digestive systems (Park, 2011). It has been found that this disease is present in Columbid species such as the Seychelles blue pigeon (*Alectroenas pulcherrima*), Madagascar turtle-dove (*Streptopelia picturata*), barred ground dove (*Geopelia striata*), spotted dove (*Streptopelia chinensis*), zebra dove (*Geopelia striata*) and Senegal dove (*Streptopelia senegalensis*) (Bunbury *et al.,* 2007b; Park, 2011). An investigation of 90% of fledgled birds in the entire free-living pink pigeon population showed that 84.3% of them were culture positive with *T. gallinae* in at least one screening. 2991 culture based screenings were performed every 2 months between 2002 and 2004 (Bunbury *et al.,* 2008). The average prevalence of *T. gallinae* infection by culture in other dove species in Mauritius (spotted Dove, zebra Dove and Senegal doves) was 44.3% (Bunbury *et al.,* 2007b). The cause of mortality was investigated for 43 wild pink pigeons (≥6 months of age) between 2002 and 2005, by Bunbury et al. (2008) and *T. gallinae* infection was found to be the most common cause of mortality in 22 birds (51.2%). Infection can occur directly when the infected adult birds feed their squabs, or indirectly by sharing food and water sources (Swinnerton *et al.,* 2005a; Bunbury *et al.,* 2008). Lesions in the digestive and upper respiratory tracts can cause starvation or asphyxiation, which usually leads to the death of the infected birds (Narcisi *et al.,* 1991).

Although prevalence of *T. gallinae* in the pink pigeon population was studied extensively by Bunbury (2006) and Bunbury et al. (2008), it is nevertheless important to assess the incidence of infection in individual birds over time. This gives a better indication of resistance (or susceptibility) than single point measurements, and it allows for an analysis of the relationship between infection data and genome wide genetic variation (See Chapter 5). In addition, the new analysis incorporated additional data on the sex, births and deaths which enabled a comprehensive analysis on the effects of *T. gallinae* infection incidence (observed over time) and lifetime reproductive fitness. This chapter focuses on two aspects of Trichomonosis infections in pink pigeons; first, the spatial and temporal variation in proportion of *Trichomonas gallinae* infection incidence through study of records for 418 ringed and known-sex birds in five subpopulations sampled from 2002 to 2004. In particular, I performed statistical analysis to investigate: (a) whether there were differences in the proportion of infection between sub-populations and sexes; (b) if differences were found, whether the population density of the pink pigeon within subpopulations could explain the variation observed; (c) whether there was temporal variation in infection, and whether this differed between males and females; (d) if there was spatiotemporal variation in the proportion of infected birds; and (e) I also identified individuals that were significantly less infected than expected based on the site and sex average, with the aim of exploring whether there may be (immuno) genetic variation present in the population that could be subjected to selection, in order to examine those birds using restriction-site-associated DNA sequencing (RAD-seq) analysis in an ongoing genome study of the pink pigeon.

Secondly**,** I investigated longevity and its relationship with Trichomonosis; by undertaking a statistical analysis of infection for 251 birds which died before Feb 2010. In particular, I tested: (a) whether there were significant differences in the proportion of infection incidence between subpopulations and between sexes; (b) whether there was a significant difference in the longevity birds found to be 100% positive to infection and birds that were found to be consistently negative to infection; and (c) whether there was any correlation between the proportion of infection incidence in adult birds and their number of young fledglings.

3.2 Methods

3.2.1 Capture and Screening

Sampling was conducted bimonthly from September 2002 to April 2004 by Bunbury (2006), and the capture and swabbing procedure is described in detail in (Bunbury 2006; Bunbury *et al.,* 2008). To detect *Trichomonas gallinae*, an average of 90% of all ringed birds across the five subpopulations (at Plaine Lievre, Pigeon Wood, Bel Ombre, Ile aux Aigrettes and Combo) were swabbed in the mouth, oropharynx, oesophagus and crop with a sterile cotton bud which was used for incubation in an InPouch TF culture pack (Biomed Diagnostics). The cultures were incubated at 38°C for at least 72 h before searching for the parasite using a microscope (Bunbury *et al.,* 2005; Bunbury *et al.,* 2008).

3.2.2 Data analysis

3.2.2.1 Infection incidence in 418 birds

The aim of this analysis was to find out whether there were differences in infection incidence between between subpopulations, time periods, and sexes. Although the data on the proportion of infected birds is not normally distributed (Shapiro-Wilk test: $R=0.991$, $p<0.01$), the variances are homogeneously distributed (Levene's Test statistic = 0.698, $p= 0.71$), and hence, a General Linear Model (GLM) was conducted. A General Linear Model (GLM) was conducted with the proportion of infected birds as the response variable, and 'Sex' as a fixed factor crossed with 'Site' as the random factor. To examine if the variation in the proportion of infected birds was explained by a variation in subpopulation density, a General Linear Model (GLM) was run with the proportion of infected birds as the response variable and subpopulation density as a covariate. To examine whether there was significant temporal variation in infection incidence, the GLM was also run with 'Time' as a covariate crossed with 'Site'. The interaction term tests whether the infection incidence differs between populations across time, i.e. whether the spatial variation in infection is temporally in synchrony.

To identify individuals that are significantly less infected than expected based on the site and sex average, I calculated the probability of finding an individual less often infected

than by chance using Binomial mass function. The probability values (p) were transformed using ($-\log_{10}$) to visualise the individual variation in infection incidence.

All analysis tests were conducted in Minitab 17 and figures were drawn using SigmaPlot 13.

3.2.2.2 Longevity and infection data of 251 birds

This statistical analysis was performed for 251 birds that died before February 2010. All of these birds have *T. galinae* infection data that were examined between 2002 and 2004.

3.2.2.2.1 Longevity

Because the data on longevity were not normally distributed (Ryan Joiner, similar to Shapiro-Wilk, test: R=0.990, p<0.010), the data were transformed by taking the square root of each observation to achieve normality (RJ=0.995, p>0.10). The variances were homogeneously distributed (Bartlett's Test statistic = 13.12, p = 0.157), and hence, a General Linear Model (GLM) was performed to test whether there were differences in longevity data between sites, sexes and infection incidence of individual birds. In this GLM, the longevity data for birds was run as a response variable, and infection incidence of individual birds as a covariant factor; sex was a fixed factor crossed with site as the random factor. As the interaction site x sex was non-significant ($F_{4, 241}$ =0.85, p=0.497), the model was run without the interaction.

3.2.2.2.2 Infection incidence

Because the data of infection incidence of 251 dead birds were not normally distributed (Ryan Joiner (similar to Shapiro-Wilk) test: R=0.991, p<0.010), the data were transformed by taking arcsine of the square root of each observation (Ryan Joiner test: R=0.996, p>0.100). The variances were homogeneously distributed (Bartlett's Test statistic = 2.30, p=0.986), hence a General Linear Model (GLM) was performed to test whether there were differences in proportion of infection data between subpopulations and sexes. In this GLM, the infection incidence data was run as a response variable, sex was a fixed factor crossed with site as the random factor. As the interaction site x sex was non-significant ($F_{4, 258}$ =1.41, p=0.230), the model was run without the interaction site x sex.

3.2.2.2.3 Difference in longevity between birds that were found to be 100% positive to infection and birds with 100% negative to infection.

A two sample T-test was performed to test if there was a significant difference in longevity between those birds that are 100% positive to infection across screening times and those that are 100% negative to infection across screening times.

3.2.2.3 Relationship between infection prevalence in adults and fledglings

Extensive data collected on fledglings and pedigrees are available for the Ile aux Aigrettes subpopulation (site 4). Hence, data of 66 individuals were used to correlate incidence of infections in fledglings to the proportion of infection incidence in the adult birds.

3.3 Results

3.3.1 Infection incidence in 418 birds

3.3.1.1 Site, sex and time differences

The average number of fledgled and adult birds infected with Trichomonosis in the whole population was high (mean ±SE = 0.86 ± 0.02 of 418 screened birds). The average infection incidence (proportion of positive screenings) across 10 bimonthly screenings of these birds was 50.42 % (±SE 0.01). There was significant variation in the proportion of infection among sites ($P<0.001$). Site 5 (Combo) showed the lowest infection incidence rate (mean ±SE = 0.3097 ± 0.0380), and the highest infection incidence rate was observed at site 3 (Bel Ombre) (mean ±SE = 0.6620 ± 0.0382), (Figure 3.1 and Table 3.1). The interaction 'sex' x 'site' was non-significant ($F_{4, 408}=1.47$, $p=0.209$), and the GLM was run without the interaction. Overall, there were no significant differences in infection incidence rates between the sexes over time (Figures 3.1 and 3.2, and Tables 3.1 and 3.2). However, one sub-population, Pigeon Wood, showed significant variation in the proportion of infection between males and females (mean ±SE = 0.663± 0.0805 and 0.4304± 0.0688 respectively), (Figure 3.1) Density also failed to show significant differences between the sites ($F_{1, 8}=0.61$, $P= 0.456$) and thus could not explain the variation in the proportion of infected birds between sites (Table 3.3).

There was a significant difference in the proportion of birds infected with *T. gallinae* between the 10 bimonthly sampling periods (Table 3.2 and Figure 3.2).

Table 3.1 GLM for difference in proportion of infection between sites and sexes. Proportion of infection as response variable, site as a random factor and sex as a fixed factor.

Factor	DF	MS	F	P
Site	4	1.2048	10.44	<0.001
Sex	1	0.1840	1.59	0.207
Error	412	0.1154		
Total	417			

DF: the degrees of freedom, MS: mean squares, F: the statistic, P: the probability.

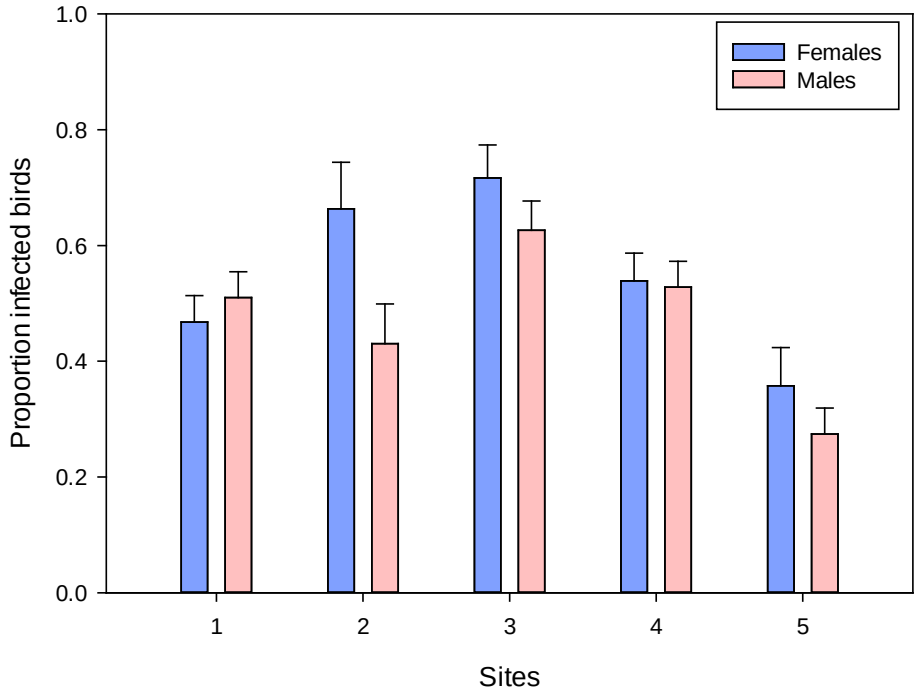

Figure 3.1 Bar chart showing the mean and standard error of the proportion of infected adult male and female birds across the five sites. (1= Plaine Lievre, 2= Pigeon Wood, 3= Bel Ombre, 4= IAA, 5= Combo).

Table 3.2 GLM for difference in proportion of infection between sexes and times. Proportion of infection as response variable, sex as fixed factor and time as covariate.

Factor	DF	MS	F	P
Sex	1	0.000531	0.36	0.555
Time	1	0.045052	31.71	<0.001
Error	17	0.001467		
Total	19			

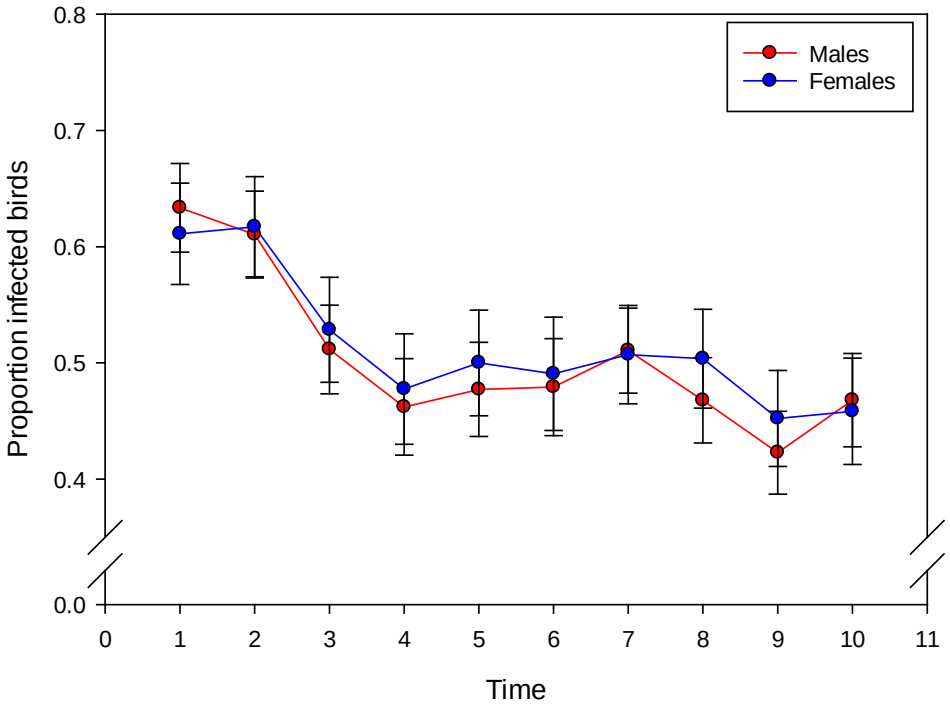

Figure 3.2 Temporal variation in the proportion of infected adult male and female birds.

Table 3.3 GLM with the proportion of infection as the response variable, site as a random factor and density as a covariate.

Factor	DF	MS	F	P
Site	4	1.2019	10.38	<0.001
Density	1	0.0181	0.16	0.693
Error	412	0.1158		
Total	417			

3.3.1.2 Spatial-temporal differences

There were significant differences in (infection) incidence rates between the sites, but there was no significant difference in the proportion of birds infected with *T. gallinae* between the 10 bimonthly sampling periods (Table 3.4 and Figure 3.3). Interestingly, there is a significant sites x time interaction, which indicates that the temporal variation in the proportion of infected individuals differs significantly between the sites. In other words, some sites may show a relatively high infection rate at one time and a low rate at another time, which indicates that the infection is out of synchrony across the five subpopulations. As a consequence, temporal variation is not significant, given that a high

infection incidence at time *t* in one population is offset by a low infection incidence at different time somewhere else.

Table 3.4 GLM for difference of proportion of infection between sites and times. Infection as response variable, site as a random factor crossed with time as a covariate.

Factor	DF	MS	F	P
Site	4	0.045911	6.37	<0.001
Time	1	0.028898	4.01	0.052
Site x Time	4	0.054050	7.49	0.000
Error	40	0.007213		
Total	49			

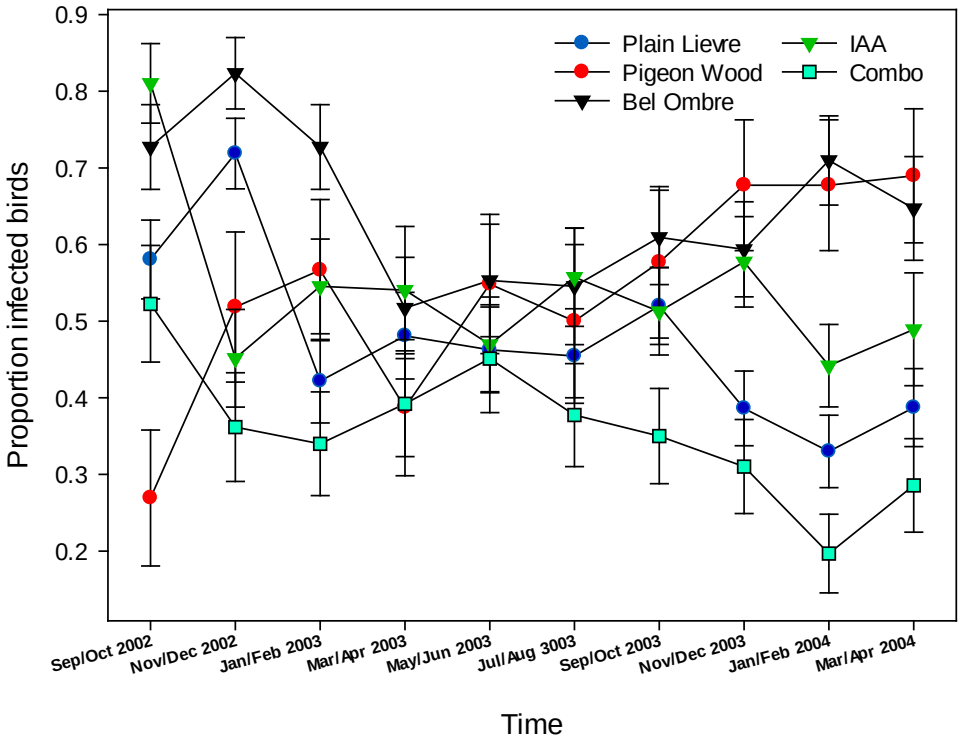

Figure 3.3 Spatial-temporal variation in the proportion of infected birds across subpopulations, given the significant site x time interactions.

3.3.1.3 Persistence of infection

The majority of birds (96.2 % of 418) were expected to have persistent infection and just 16 birds (3.8 %) had the probability of being significantly less infected than expected by chance using a critical value α=0.05, and two birds of these were significant using a

critical value α=0.01 (indicated by *) which were located at site 3 (Bel Ombre) (Figure 3.4).

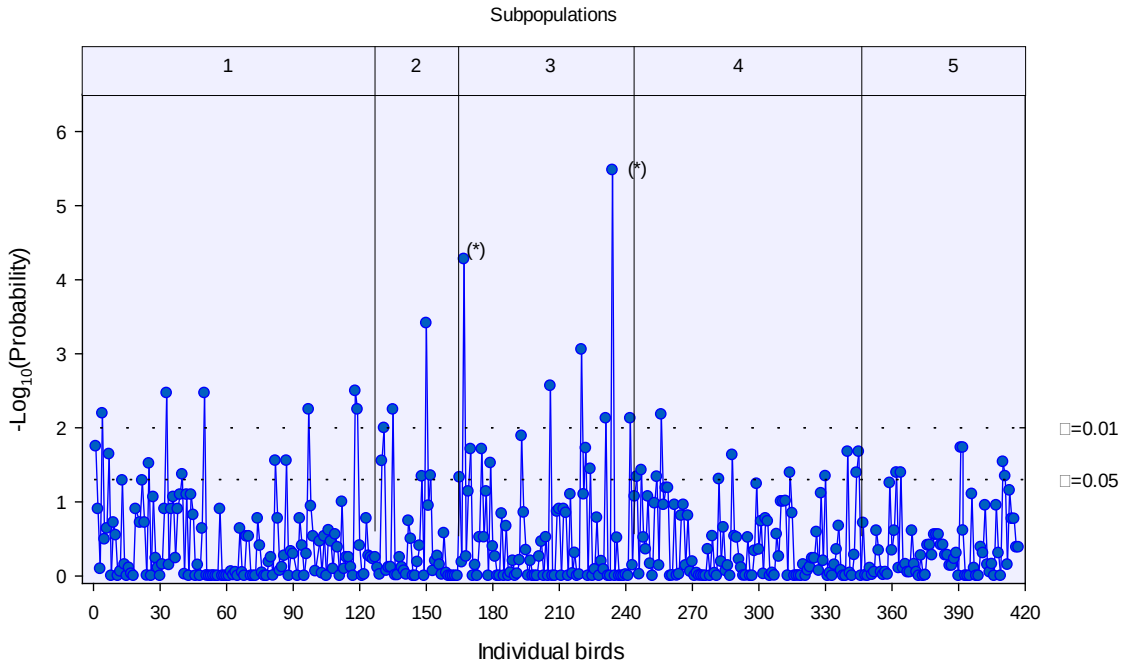

Figure 3.4 The probability (-log10 transformed) of infection incidence calculated using a Binomial mass function using all N=418 birds. The number at the top are subpopulation: 1= Plaine Lievre, 2= Pigeon Wood, 3= Bel Ombre, 4= IAA, 5= Combo).

3.3.2 Longevity and infection data of 251 dead birds

3.3.2.1 Longevity

A GLM was performed to examine whether longevity is different between sexes and sites. Test was run as longevity as response variable, infection incidence of individual birds as continuous variable; sex was a fixed factor and site as random factor. There was significant variation in the longevity of birds between subpopulations (Figure 3.5 and Table 3.5). However, there is no significant variation in the longevity of birds between sexes (Table 3.5). When a t-test was performed to find out if there is a difference in longevity among sexes at each site, the longevity did not differ significantly between males and females at each site including Plaine Lievre, Pigeon Wood, Bel Ombre, IAA and Combo) (T-Value, d.f and P-Value = 1.70, 62 and 0.095; 0.72, 21 and 0.482; -0.04, 43 and 0.969; 1.41, 63 and 0.164; -0.56, 24 and 0.578, respectively). Birds at Site 3 (Bel

Ombre) live longest (mean ±SE = 100.66 ± 4.93 months) and site 5 (Combo) lived shortest (mean ±SE = 59.64 ± 5.93 months).

Table 3.5 GLM for difference in longevity between sexes and sites in 251 dead birds with proportion infection as covariant factor; sex was a fixed factor and site as the random factor.

Factor	DF	MS	F	P
Site	4	24.620	4.62	<0.001
Sex	1	16.805	3.15	0.077
Proportion positive	1	5.248	0.98	0.322
Error	244	5.329		
Total	250			

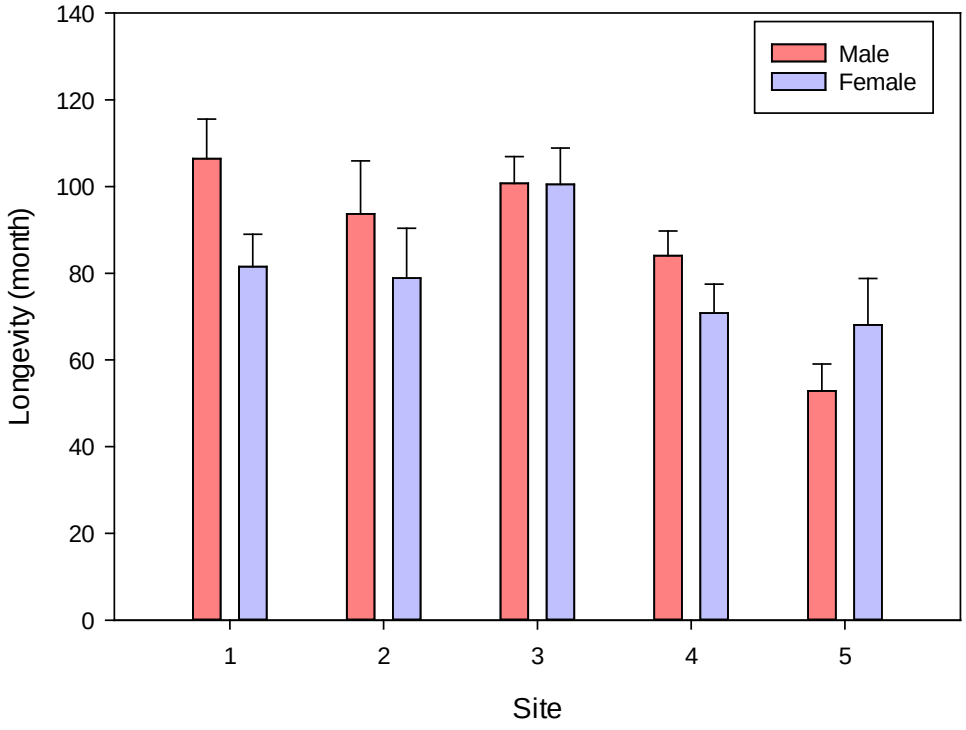

Figure 3.5 Bar chart showing the mean and standard error of the longevity of male and female birds across the five sites. (1= Plaine Lievre, 2= Pigeon Wood, 3= Bel Ombre, 4= IAA, 5= Combo).

3.3.2.2 Infection incidence

There was a significant difference in infection incidence between subpopulations. However, there was no significant difference between sexes (Figure 3.6 and Table 3.6). The highest infection incidence rate was observed at site 3 (Bel Ombre) (mean ±SE =

0.67 ± 0.04), and site 5 (Combo) showed the lowest infection incidence rate (mean ±SE = 0.37 ± 0.059) (Figure 3.6).

Table 3.6 GLM for proportion of infection, of 251 dead birds, between sexes and sites. Proportion of infection as a response variable; sex was a fixed factor crossed with site as the random factor.

Factor	DF	MS	F	P
Site	4	1.51	5.84	<0.001
Sex	1	0.2228	0.86	0.354
Error	245	0.2584		
Total	250			

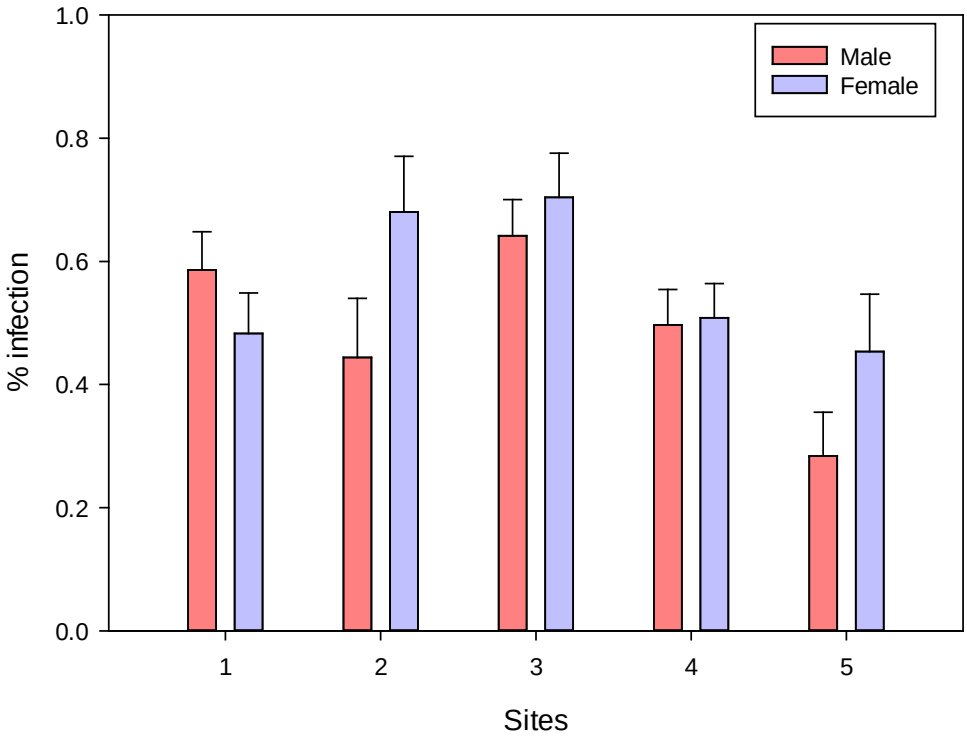

Figure 3.6 Bar chart showing the mean and standard error of the proportion of infection in male and female birds across the five subpopulations. (1= Plaine Lievre, 2= Pigeon Wood, 3= Bel Ombre, 4= IAA, 5= Combo).

3.3.2.3 The difference in longevity between infected and uninfected birds.

In order to assess whether infection with *T. galinae* affects the survival of individuals, the difference in longevity (month) was examined between 73 adult dead birds that had been found 100% positive to infection (M±SE= 90.06±5.53 months) in at least three screenin, and birds that has been found 100% negative to infection in at least three screenings (M±SE= 89.7±11.4). Examining the difference between two groups using a 2 sample T-test found no significant differences (T-Value= -0.03, d.f = 32, P-Value = 0.977).

3.3.3 The relationship between the infection in 66 birds and number of fledglings that they produced

In order to examine whether there is association between infection with *T. gallinae* and fledgling success, number of young fledgled from 66 birds (33 parents) in one subpopulation (IAA) was regressed against their infection data. No significant relationship was found between these variables (regression: $F_{1,64} = 3.57$, p = 0.063, R-Sq = 5.3%) (Figure 3.7).

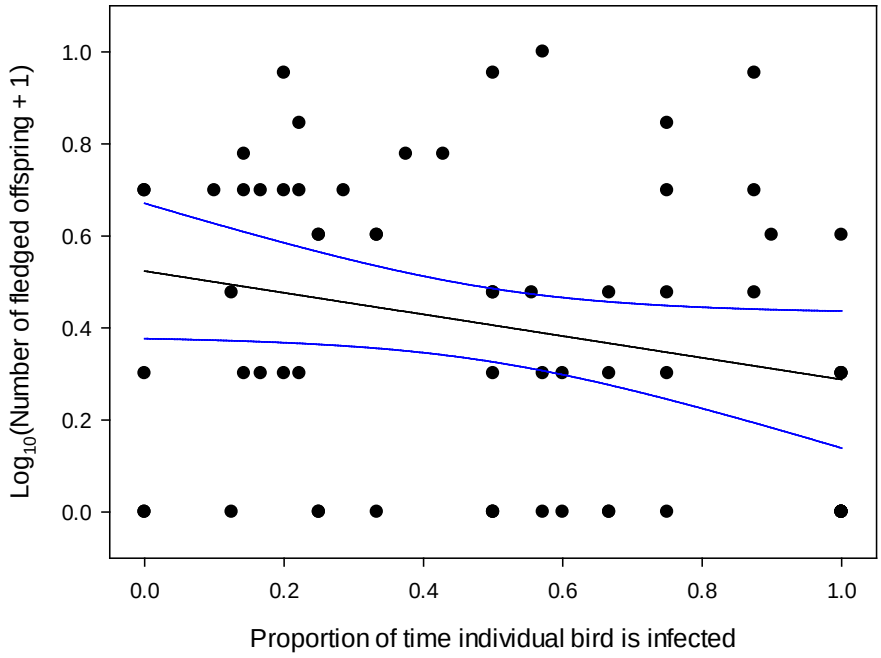

Figure 3.7 Linear regression of the proportion infection with *T. gallinae* in 66 adult birds against number of their young fledged."

3.4 Discussion

In this study, the percentage of adult pink pigeons (*Nesoenas mayeri*) (N=418) culturing positive for *T. gallinae* was high (85.65%) and the average percentage of adult birds found infected during bimonthly sampling over 20 months was 50.42%. A high infection rate of *T. gallinae* has been found in other Columbids such as the Seychelles blue pigeon (Bunbury, 2011), wood pigeons (*Columba palumbus*)(VillanÚA et al., 2006), European turtle doves (Streptopelia turtur), Eurasian collared doves and stock doves (*Columba oenas*) (Lennon et al., 2013).

Table 3.7 Infection rate of *T. gallinae* in different Columbids species in different countries.

Species	% Prevalence	Country	Author
Pink pigeon (*Nesoenas mayeri*)	50.42 50.30	Mauritius	This thesis (Bunbury, 2006)
Seychelles blue pigeon *Alectroenas pulcherrima*	47.10	Seychelles	(Bunbury, 2011)
Common Wood Pigeon *Columba palumbus*	34.2 47	Spain UK	(VillanÚA et al., 2006) (Lennon et al., 2013).
European Turtle Dove *Streptopelia turtur*	86	UK	(Lennon et al., 2013).
Eurasian Collared Doves *Streptopelia decaocto*	86	UK	(Lennon et al., 2013).
Stock Doves *Columba oenas*.	40	UK	(Lennon et al., 2013).

In this thesis, the proportion of infected birds differed significantly among the five subpopulations in Mauritius. The highest proportion of infection was at site 3 (Bel Ombre) and the lowest was at site 5 (Combo). These findings support the analysis of the same screening data conducted by Bunbury et al. (2008) using the Chi-square test. Although there is evidence that older hosts develop rapid resistance to pathogens (Gay *et al.,* 2006), I found, by analysing the longevity data of 251 dead birds across the five subpopulations, that birds at the Bel Ombre site lived longer than those at the other four sites, despite having the highest infection rate of infection. Likewise, (Bunbury, 2006) found that the mean age varied significantly among sites during the same period with birds at Bel Ombre being the oldest site. From these findings, it can be inferred that the presence of parasitic infection does not always lead to visible symptoms of disease or influence the long-term survival of the host if it has the ability to tolerate the presence of pathogens

(Horns and Hood, 2012). Tolerance, a concept that has only recently been brought to the field of animal immunity (e. g. Schneider and Ayres, 2008; Råberg *et al.,* 2009), is one type of the three defence mechanisms including avoidance (disease susceptibility to infection), resistance (reduces the pathogen burden after the infection has occurred) and tolerance (decreases death rate once infected by reducing susceptibility to tissue damage) (Blanchet *et al.,* 2010; Medzhitov *et al.,* 2012). Hence, tolerance might be the defence strategy that can explain the situation at site 3 (Bel Ombre) that had both the highest average age in the birds and the highest infection prevalence. Generally, in most vertebrates, males tend to be more susceptible to infectious diseases than females as a result of cost associated to mating system (for example, sexual displays) (Zuk, 1990). However, this was not the case with the pink pigeon. The rate of infection did not significantly differ between the sexes, and the infection trend of males and females mirrored each other closely across the 10 bimonthly sampling periods. This may be due to bi-parental care when *T. gallinae* may transmit between sexes via feeding of squabs, or it may be due to the behaviour of food interchange between females and males during the breeding seasons. This could support the aide that social contact rather than host density determines the infection dynamics of *T. gallinae.* Furthermore, this suggests that infection with *T. gallinae* is not a factor for skewing the adult sex ratio in the pink pigeon population. Nevertheless, *T. gallinae* could potentially bias the sex ratio if it was responsible for sex-dependent mortality before adulthood in squabs before fledging (Bunbury *et al.,* 2009), but based on the present data, it appears that both sexes are equally prone to contract a *T. gallinae* parasite infection as adults.

Significant differences in the proportion of birds infected between the 10 periods of time were detected. In addition, when the site x time interaction was included, a highly significant temporal variation in the proportion of infection was detected which indicates spatiotemporal variation. Here, I aimed to delineate the spatial and temporal components of the infection dynamics, adding to the analyses by Bunbury et al. (2008) carried out on the same data. I have shown that the infection dynamics between subpopulations are out of synchrony, as indicated by the highly significant site x time interaction. This suggests that *T. gallinae* is either entirely more stochastic, and that temporal waves of infections percolate through the subpopulations, or that prevalence of *T. gallinae* is driven by social interaction. This supported by result in Bunbury et al.

(2008) how found that birds aged 3-12 months were significantly less likely to be infected with *T. gallinae* than those older than 12 months. Interestingly the contact rate in birds aged 3-12 months seems to be lower than in those birds aged more than 12 months because juveniles (i.e. birds older than 6 months) are no longer dependent on their parents for feeding and most have not yet begun to demonstrate breeding behaviours such as food interchange between the sexes. However, it might be difficult to ignore the effect of species diversity within the community (Keesing *et al.,* 2006). The presence of different species within a community, including other dove species that have a relatively high prevalence of *T. gallinae* infections, may increase the contact rate when they share the same sources of water and food (Bunbury *et al.,* 2007b).

I identified a small number of birds that were consistently free of *T. gallinae* infections and calculated the probability of not detecting an infection in a bird relying on the sex and site average of the infection incidence. In addition to 16 birds were found to be significantly less infected, two birds were found to be significantly less infected than predicted by chance. There are various potential explanations that could explain this observation, such as, for example, that these birds are more socially isolated from other birds in their subpopulation. However, both birds were found to live longer, and there is no evidence to suggest that they had less probability of contracting an infection due to fewer social contacts. The alternative explanation which I favour is therefore that despite a potential severe inbreeding and loss of genetic variation, immunogenetic variation continues to segregate in the wild pink pigeon population. This genetic variation could be pivotal for the long-term survival and viability of the pink pigeon, and the RAD sequencing 175 birds is currently available (chapter 4 and 5 in this thesis) with the aim to assess the genome wide genetic variation in immune genes that are associated to potential resistance to *T. gallinae* infection.

There was no relationship between the proportion of adult birds infected and the number of fledglings. Although the relationship is close to being considered significant, with $p = 0.063$, it is inconsistent with several studies that found that immune functions were reduced when engaging in increased reproductive efforts (Zuk and Stoehr, 2002). In birds, male zebra finches (*Taeniopygia guttata*) showed a higher prevalence of parasites with increasing clutch size than females (Deerenberg *et al.,* 1997). The

reproductive effort of female collared flycatchers (*Ficedula albicollis*) elevated the intensity of protozoan parasite infections (Nordling *et al.,* 1998). The immune response is negatively influenced by increases in brood size in barn swallows (Saino *et al.,* 1997). Immune functions were reduced with large clutches in female common eiders (S. mollissima) (Hanssen *et al.,* 2005). Reproductive effort increased blood parasite infection levels in different bird species (Knowles *et al.,* 2009). Parasite infection increased with both increasing age and increasing clutch size in great tits (*Parus major*) (Norris *et al.,* 1994). This reduction in immune defence against reproduction function not only arises due to limited resources, but also to minimize the risk of undesirable immune reactions (Zuk and Stoehr, 2002). On the other hand, reliance on fledgling alone may not be enough to understand the relationship between the immune system and reproductive functions, especially with high mortality in squabs before fledging. Perhaps more data on numbers of eggs laid and hatchability are required to clarify this relationship.

3.5 Conclusion

The results of this study are consistent in a number of aspects with a previous study conducted by Bunbury et al. (2008) in regard to the existence of a variation in detectable Trichomonas infections among subpopulations but not between sexes. However, highly significant spatiotemporal variation in the proportion of infected birds has been detected here. Social contact might be the most important factors for prevalence of *T. gallinae.* Infection within subpopulations. The fact that there were few birds consistently free of *T. gallinae* infections may indicate that immunogenetic variation continues to segregate in the wild pink pigeon population, which was examined in the next two chapters using genome wide SNPs markers that were generated using RAD-seq .

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Chapter 4

Genome-wide variation in the Mauritian pink pigeon, *Neseonas mayeri*

Summary

Small, fragmented populations can lose genetic variability through random genetic drift and inbreeding. Inbreeding tends to have a negative impact on fitness of individuals through the fixation of recessive deleterious alleles in homozygous state, which result in inbreeding depression. In addition, the loss of genetic variation in small populations also reduces the essential elements available to natural selection, thereby reducing the evolutionary potential and ability to respond to environmental change. The pink pigeon, *Nesoenas mayeri* (Prévost, 1843), has been recovered to around 400 individuals from a bottleneck of 10-12. Conservation aims to increase the number of pink pigeons to 600 birds. The high mortality of young birds (prior to reaching breeding age) may indicate to inbreeding depression and is likely to accelerate the loss of genetic variability. This chapter examines genetic variability in the pink pigeon population using high-throughput genomic markers generated using a new approach, Restriction-site Associated DNA Sequencing (RAD-seq). A total of 175 birds from five subpopulations were analysed using 45,840 Single Nucleotide Polymorphisms (SNPs). The average observed heterozygosity (Ho) and expected heterozygosity (He) was 0.27 and 0.28, respectively, suggesting there is little evidence of contemporary inbreeding. When separating immune genes from non-immune genes, one subpopulation (Bel Ombre) showed a highly significant excess of observed heterozygotes at the immune genes, which is consistent with balancing selection counteracting drift. However, a more detailed temporal genetic analysis revealed that the Ile aux Aigrettes (IAA), Combo (CO) and Bel Ombre (BO) showed significant negative correlations between genome wide heterozygosity and the date an individual was hatched over the period from 1994 to 2008. Regression analyses revealed that time explained between 17.4 and 47.6% of the variation in heterozygosity, which suggests that these three subpopulations are continuing to lose genetic variation despite their apparent recovery. Furthermore, when comparing the gene diversity of parents directly with their offspring, 6.15% of heterozygosity was lost in a single generation in the IAA subpopulation. This alarming loss in genetic polymorphism is equivalent to an effective pop-

ulation size of Ne=7.625. These results highlight the urgent need for genetic supplementation of captive bred birds from any remaining zoo populations, and other conservation management interventions in order to stop the continued loss of genetic variation due to inbreeding and drift.

4.1 Introduction

4.1.1 Genetic variation in threatened populations

The assessment of genetic variation in endangered and threatened species has high priority in the field of conservation biology. One of the issues of greatest concern that can most influence the maintenance of wild populations is the loss of genetic diversity. Small or isolated populations can lose genetic variability through two processes: a long-term process caused by direct random genetic drift and a short-term process caused by inbreeding which accelerate genetic drift (Amos and Balmford, 2001). Random genetic drift refers to random fluctuations in allele frequencies from generation to generation due to the random sampling of gametes during Mendelian inheritance (Keller *et al.,* 2012). The influence of the random sampling of gametes is expected to be marginal in large populations whereas this is a major evolutionary force in small populations (Hallatschek *et al.,* 2007). Genetic drift is particularly pronounced in fragmented populations or metapopulations, as in the absence of gene flow, drift tends to increase the genetic divergence between subpopulations whilst reducing the gene diversity within each subpopulation (Van Oosterhout *et al.,* 2004; Méndez *et al.,* 2011). Population bottlenecks and founder effects also rapidly reduced genetic variation, and the effects of genetic drift can be observed many generations after the bottleneck event (Saccheri *et al.,* 1999; Hundertmark and Van Daele, 2010). The interaction between inbreeding and other stochastic factors, including environmental, demographic and catastrophic factors, can accelerate the loss of genetic variation within a population (Frankham *et al.,* 2010). In conservation genetics this is known as the "extinction vortex", in which the loss of genetic variation increases a population's vulnerability to extrinsic stochastic events, which in turn reduces the effective population size resulting in a further loss of genetic variation (Gilpin and Soule, 1986). This self-reinforcing process can drive an endangered inbred population rapidly to extinction (Frankham *et al.,* 2010). Genetic drift is likely to increase both homozygosity and the frequency of deleterious alleles, leading to a decline in population fitness due to inbreeding depression (Neaves

et al., 2015). In addition, the consequences of a reduction in genetic variability can make a population less resistant to environmental stress and predation (Reed *et al.,* 2002), and it may elevate susceptibility to infection and disease (Trinkel *et al.,* 2011). The loss of genetic variation has been documented in several wild species, such as the Mauritius kestrel, *Falco punctatus* (Groombridge *et al.,* 2000); the black stilt, *Himantopus novaezelandiae* (Hagen *et al.,* 2011), and the Hawaiian goose, *Branta sandvicensis* (Paxinos *et al.,* 2002; Veillet *et al.,* 2008). However, research on the subject has mostly been carried out based on a few limited markers, such as microsatellites, and conservation genomic analysis are still relatively rare, but see (Allendorf *et al.,* 2010; Hecht *et al.,* 2013). The loss of genetic variation can cause differentiation between populations of a given species. Population differentiation occurs when there is reduction in the rate of swapping individuals and genes between populations or subpopulations of species (a reduction in gene flow) (Holderegger and Di Giulio, 2010). Habitat fragmentation by human and environmental barriers, such as distance barriers, is a process that is expected to block gene flow between populations that live in a variety of environmental conditions and increase the degree of genetic differentiation among these populations via genetic drift or local adaptation (Pérez-Espona *et al.,* 2008). However, differentiation can act faster when populations or subpopulations are small or have recently experienced bottleneck events (Forstmeier *et al.,* 2007). Alleles within these populations are likely to be fixed at fast rates rather than differentiated via random genetic drift or the selection process.

More recently, conservation genomics approaches such as restriction-site-associated DNA sequencing (RAD-seq) have become available, providing accurate interpretations of differentiation through a large number of markers. For example, Emerson et al. (2010) examined genetic divergence in the pitcher plant mosquito, *Wyeomyia smithii,* a species that is geographically distributed into southern and northern groups. Although these groups can be distinguished genetically using classical markers, the patterns of genetic differentiation between populations within these two groups cannot be detected using such markers. Alternatively, RAD-seq was applied to generate a large number of SNPs that were found to vary between the populations within these two groups (Emerson *et al.,* 2010). Similarly, the RAD-seq technique has been used by Guo et al. (2015) to study differentiation between ten populations of three-spined stickleback *Gasterosteus*

aculeatus that live in different degrees of salinity and different temperatures. Differentiation was discovered among these populations, allowing the researchers to conclude that local adaptation gives rise to heterogenic genomic divergence, despite gene flow (Guo *et al.,* 2015).

4.1.2 Pink pigeon

The Mauritian pink pigeon is an endangered species that has successfully recovered from a low of ten to twenty wild individuals in one small area, Pigeon Wood, in 1975 (Jones *et al.,* 1992) to reach a relatively stable number of around 400 individuals in seven subpopulations today (Mauritius Wildlife Foundation, 2012).

Little is known about genetic variation in the pink pigeon population, and it is not clear what genetic factors affect conservation efforts for this species. Only two previous studies have investigated genetic aspects of the pink pigeon population. The inbreeding coefficient for captive birds was calculated in the early stage of a recovery programme using pedigree data in Jones et al. (1989), which found high inbreeding coefficients between pairs. Swinnerton et al. (2004) investigated mitochondrial DNA (mDNA) sequence variation in the d-loop region in a pink pigeon founder and discovered a low level of diversity. However, these studies were restricted to captive individuals and limited genetic resources. In this study, a new approach, the restriction-site associated DNA sequencing technique (RAD-seq), has been applied to identify and genotype thousands of markers across the genome, which were utilised to study genome-wide genetic variation in the pink pigeon population.

The major aim of this chapter is to assess several aspects of the genome-wide genetic variation and genetic structure of the pink pigeon population. The particular aims are to:

1) Estimate the genome-wide observed and expected heterozygosity for each subpopulation and the total population.

2) Compare the genetic variation at putative immune genes with variation across the rest of the genome.

3) Assess the loss of genetic variation within subpopulations over time.

3) Calculate the rate of inbreeding in the pedigreed Ile aux Aigrettes subpopulation within a single generation.

4) Calculate the genetic differentiation (Fst) between pink pigeon subpopulations.

4.2 Materials and methods

4.2.1 Study sites:

At the time of the population bottleneck the remnant birds occurred in a single sub-population called Pigeon Wood from which eggs and adults were taken to establish a captive breeding facility in Black River (Jones and Owadally, 1988). Six further pink pigeon sub-populations on Mauritius have since been established from the founder stock (Mauritius Wildlife Foundation, 2012)

The Pigeon Wood, Bel Ombre, Plaine Lievre (also called Camp) and Combo subpopulations are located in the Black River Gorges National Park. Another was established on Ile aux Aigrettes, a small islet off the eastern coast of Mauritius from which introduced mammalian predators had been eradicated (Jones *et al.,* 1992; Jones, 1987) (Figure 4.1). An additional two subpopulations in the Lower Black River Gorges and in the Pétrin were set up in 2007 and 2012, respectively (Mauritius Wildlife Foundation, 2012), and are not included in the present study.

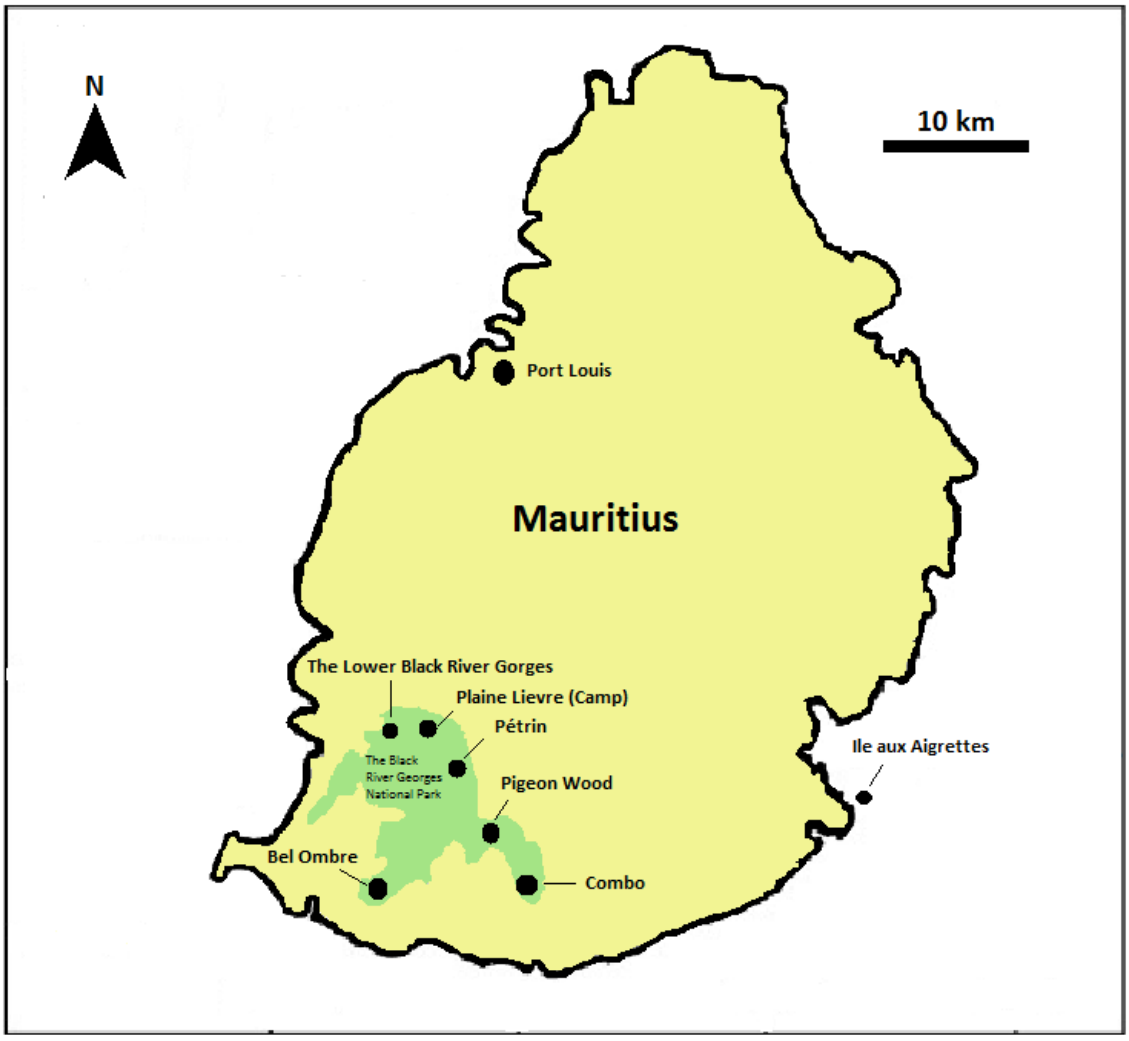


Figure 4.1. Map showing the location of seven subpopulations on Black River Gorges National Park and the Ile aux Aigrettes on Mauritius (Adapted from: Swinnerton *et al.,* 2005b).

4.2.2 Blood sampling and birds data collection

Blood samples were collected from birds between October 2004 and September 2008. The capture procedure and blood collection methods have been previously described elsewhere (Bunbury, 2006; Bunbury *et al.,* 2007).

Hatchling dates and subpopulation data were obtained from Mauritian Wildlife Foundation data bases from Jun 1976 to Oct 2010.

4.2.3 DNA extraction, library preparation and sequencing

This work has been done **in** collaboration with The Genome Analysis Centre (TGAC), Norwich. For more details, see appendix 1 and 2.

- DNA extraction and quality control: DNA was extracted from blood samples using the Agencourt® GenFind™ V2 Blood & Serum Genomic DNA Isolation Kit in accordance with the manufacturer's instructions. DNA from muscle tissue was extracted using a phenol/chloroform extraction method. DNA yield was assessed using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) to measure DNA concentration and the E-Gel electrophoresis system (Life Technologies, Carlsbad, CA) before viewing and photographing using UVIdoc HD2 (UVitec, Cambridge, UK).

- Library preparation and sequencing

a) RAD-Seq library preparation

Genomic DNAs, from individual blood samples, were digested high fidelity HF-**Sbf**I (New England Biolabs) and an adapter P1 (a modified Illumina adaptor) was ligated to the fragments' compatible ends. Samples were then pooled and sheared to an average size of 500 bp using a Covaris S2 acoustic sonicator (Covaris Inc.). The P2 Adapter, a modified Illumina adapter, was then added and ligated to the fragments. 10 µL of this product was used in a PCR amplification with 25 µL Phusion Master Mix (NEB), 1.5 µL of 10 µM Illumina amplification primer mix (P1-forward primer: 5'-AATGATACGGCGACCACCGA-3', and P2-reverse primer: 5'-CAAGCAGAAGACGGCATACGA-3'). RAD-seq libraries were then sequenced using paired-end sequencing on an Illumina HiSeq 2500 in rapid run mode.

b) Whole genome sequencing library preparation

For whole genome sequencing, three types of libraries were prepared from leg muscle tissue, including Amplification-free Illumina libraries, DISCOVAR libraries, and long mate-pair libraries. These libraries were purified and QC checked with the Bioanalyzer DNA HS assay (Agilent Technologies, Inc, CA) and further quantified by both Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) before pooling before they were sequenced using paired-end sequencing (2 x 250 bp for DISCOVAR and 2 x 150 bp for Long Mate Pair libraries) with a 1% PhiX spike, on an Illumina HiSeq 2500 in rapid run mode.

-Bioinformatics

The pink pigeon reference was assembled using DISCOVAR (Weisenfeld, et al. 2015) and scaffolded with long mate pair libraries using SOAPdenovo (Li et al., 2010). Analysis of

the draft pink pigeon genome assembly using Abyss-fac revealed the N50 to be 8 Mbp. Assessment using CEGMA (Parra, et al 2009) shows the assembly to be 60% complete (90% partial), using a set of 248 highly conserved protein family genes.

RAD-seq reads were demultiplexed using RADplex (Leggett, 2013) with a mismatch of 0. Read filtering using FastQC (Andrews, 2010) and trimming using the "fastq-mcf" program from the ea-utils package (Aronesty. 2011). Mapping to the reference sequence was performed using the Burrows-Wheeler Aligner (BWA-mem) tool (Li, et al. 2013). Filtering and indexing were performed using SAMtools (Li, et al. 2009). Sequence depth calculating and SNPs calling were performed using SAMtools (Li, 2011). For immune genes, a python script was written using the BioPython library to retrieve nucleotide identities from gene identities and subsequently the FASTA sequences. Locations for these genes were found by blasting the FASTA files to a local blast database of the Pink Pigeon reference. Python scripts were then used to identify marker sites that fell within 0.1 Mb of these regions. F_{st} statistics was obtained using the method of Weir and Cockerham using VCFtools **--weir-fst-pop** and the F_{is} of individuals were obtained using VCFtools **–het.** PLINK (.ped and .map) files were made using VCFtools –plink. Association tests and Identity-by-state (Gibson and Baker) distances were performed using PLINK. Heterozygosities were calculated from the PLINK (pedigree and map) files using a bespoke pyth on script.

4.2.4 Statistical analysis

Because the data on observed heterozygosity (Ho), expected heterozygosity (He) homozygosity, and F_{IS} in both immune and non-immune genes are not normally distributed, a non-parametric test (Kruskal-Wallis test) was used to assess adaptive genetic variation. In addition, rather than the mean and the standard error, I have reported the median and the first and third quartile (Q1 and Q3).

To calculate genetic loss in each subpopulation, the genome wide observed heterozygosity, and inbreeding coefficient (F_{IS}) were calculated and regressed against the date an individual was hatched. Heterozygosity and inbreeding coefficient were calculated after excluding the Z-chromosome because this would introduce a sex bias with females being the heterogametic sex (WZ). In order to draw the graph and run the

regression analysis, the date of the first hatched bird was equated to zero, and the number of days that elapsed for subsequent hatchings was calculated.

To estimate the effective population sizes (*Ne*) and loss in genetic variation across two generations in the IAA population, the genome wide heterozygosity of 20 parents and 44 offspring which hatched between 2004 and 2008 was determined. The difference between two groups were tested using a nonparametric test (Kruskal-Wallis test). Based on this, the effective population size (*Ne*) was collected with the following formula:

$$H_{\text{offspring}} = H_{\text{parents}}(1 - 1/2Ne),$$

where *H* is the observed heterozygosity and *Ne* is the effective population size (Frankham et al. 2010). To estimate inbreeding coefficient (F_{IS}) for the IAA subpopulation across time, I calculated the mean of inbreeding coefficient (F_{IS}) within three groups of birds, including birds that hatched between 1994 and 1999, 2000 and 2004, and 2005 and 2008. Then, I performed a one-sample t-test with a hypothesised mean of zero, which represents random mating in a panmictic population without inbreeding. In order to estimate genetic distance between subpopulations, F_{ST} pairwise was calculated using VCF tools (the variant call format) with the Weir & Cockerham F_{ST} based on all SNP data (45k variants). Linear regression was run to examine the relationship between the Pairwise F_{ST} and geographical distances between subpopulations. Statistical analysis was run using Minitab 17 and graphs were produced using SigmaPlot 13.

4.3 Results

4.3.1 Population statistics

In order to quantify genome wide genetic diversity of the pink pigeon population, 175 birds were sequenced using RAD-seq techniques including 116 individuals from Ile aux Aigrettes (IAA), 12 individuals from Plaine Lievre (PL), 12 individuals from Bel Ombre (BO) , 10 individuals from Pigeon Wood (PW) and 25 individuals from Combo (CO). The average observed heterozygosity (Ho) and expected heterozygosity (He) over 45,840 loci were very similar across subpopulations ($0.27 \leq Ho \leq 0.29$ and $0.27 \leq He \leq 0.28$) (Table 4.1). This suggests that the subpopulations are in approximate panmixia, and that there is no evidence of severe inbreeding.

Table 4.1 Average observed and expected Heterozygosity in each pink pigeon subpopulation and in the total population across 45,840 polymorphic loci.

Subpopulation	Observed heterozygosity (Ho)	Expected heterozygosity (He)
IAA	0.27	0.27
PW	0.28	0.27
BO	0.27	0.27
PL	0.27	0.27
CO	0.29	0.27
Total population	0.27	0.28

4.3.2 Assessment of adaptive genome genetic variation

To assess adaptive genetic variation in the pink pigeon population, the heterozygosity was calculated separately for immune-related genes (Appendix 2) within each subpopulation and compared with that of non-immune genes. This analysis was performed on adult birds and hence, squabs were removed from IAA list.

Ile aux Aigrettes (IAA):

Observed heterozygosity was calculated across 69 adult IAA individuals. There was no significant difference in observed heterozygosity (Ho) between immune genes (Q1, Median and Q3 = 0.10145, 0.27536 and 0.40580), and non-immune genes (Q1, Median and Q3 = -0.11594, 0.27536 and 0.42029), (Kruskal-Wallis test: H = 1.60; d.f. = 1; P = 0.206). Also there was no significant difference in expected heterozygosity (He) between immune genes (Q1, Median and Q3 = 0.09764, 0.27778 and 0.42344) and non-immune genes (Q1, Median and Q3 = 0.12193, 0.28733 and 0.43242), (Kruskal-Wallis: H = 1.55, d.f. = 1, P = 0.213). In addition, F_{IS} did not differ significantly between immune genes and non-immune genes (Q1, Median and Q3 = -0.08296, -0.02178 and 0.06441) and (-0.08967, -0.02222 and 0.06441), respectively), (Kruskal-Wallis: H = 0.48, d.f. = 1, P = 0.488) (Appendix 3).

Pigeon Wood:

Observed heterozygosity was calculated across 10 adult Pigeon Wood individuals. There was no significant difference in observed heterozygosity (Ho) between immune genes (Q1, Median and Q3 = 0.10000, 0.30000 and 0.40000), and non-immune genes (Q1, Median and Q3 = 0.10000, 0.30000 and 0.40000), after Bonferroni correction for testing

5 subpopulations (alpha = 0.05/5 = 0.01): (Kruskal-Wallis test: H = 5.79, d.f. = 1; P = 0.016). Also there was no significant difference in expected heterozygosity (He) between immune genes (Q1, Median and Q3 = 0.09500, 0.32000 and 0.45500) and non-immune genes (Q1, Median and Q3 = 0.09500, 0.25500 and 0.42000) after Bonferroni correction (Kruskal-Wallis test: H = 4.80, d.f. = 1, P = 0.028). In addition, F_{IS} did not differ significantly between immune genes and non-immune genes (Q1, Median and Q3 = (-0.20000, -0.11111 and 0.04762) and (0.04762, -0.09890 and -0.09890)), respectively, (Kruskal-Wallis test: H = 2.11, d.f. = 1, P = 0.146, respectively) (Appendix 4).

Bel Ombre:

Observed heterozygosity was calculated across 10 adult Bel Ombre individuals. There was no significant difference in observed heterozygosity (Ho) between immune genes (Q1, Median and Q3 = 0.083330, 25000 and 0.41667), and non-immune genes (Q1, Median and Q3 = 0.08333, 0.25000 and 0.41667), (Kruskal-Wallis test: H = 2.22, d.f. = 1; P = 0.136). Also there was no significant difference in expected heterozygosity (He) between immune genes (Q1, Median and Q3 = 0.15278, 0.27778 and 0.44444) and non-immune genes (Q1, Median and Q3 = 0.08678, 0.27778 and 0.43388), (Kruskal-Wallis test H = 1.87, d.f. = 1, P = 0.172). However, there is a highly significant difference in F_{is} between immune genes and non-immune genes (Q1, Median and Q3 = -0.19314, -0.06140 and 0.11111) and (-0.14286, -0.04348 and 0.13818)), respectively, (Kruskal-Wallis test: H = H = 10.41, d.f. = 1, P = 0.001, respectively), (Figure 4.2), (Appendix 5).

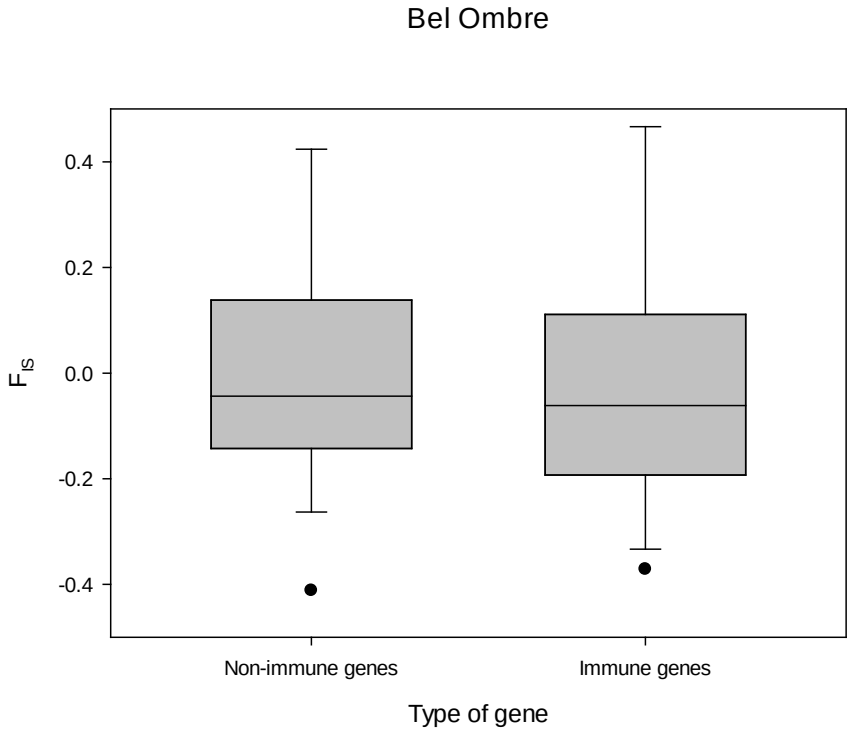


Figure 4.2. Box plot showing the median and first and third quartile of heterozygosity of the group of immune genes and non-immune genes in the Bel Ombre population.

Combo:

Observed heterozygosity was calculated across 24 adult Combo individuals. There was no significant difference in observed heterozygosity (Ho) between immune genes (Q1, Median and Q3 = 0.12500, 0.29167 and 0.45833), and non-immune genes (Q1, Median and Q3 = 0.12500, 0.29167 and 0.45833), (Kruskal-Wallis test: H = 1.81, d.f. = 1, P = 0.178). Also there was no significant difference in expected heterozygosity (He) between immune genes (Q1, Median and Q3 = 0.11719, 0.30469 and 0.44444) and non-immune genes (Q1, Median and Q3 = 0.11719, 0.27778 and 0.42969), (Kruskal-Wallis: H = 1.00, d.f. = 1, P = 0.218). In addition, F_{IS} did not differ significantly between immune genes and non-immune genes (Q1, Median and Q3 = -0.17073, -0.06667 and -0.00840) and -0.17073, -0.06667 and 0.00000), respectively), (Kruskal-Wallis: H = 2.25, d.f. = 1, P = 0.133) (Appendix 6).

Plaine Lievre:

Observed heterozygosity was calculated across 12 adult Plaine Lievre individuals. There was no significant difference in observed heterozygosity (Ho) between immune genes

(Q1, Median and Q3 = 0.08333, 0.25000 and 0.41667), and non-immune genes (Q1, Median and Q3 = 0.08333, 0.25000 and 0.41667), (Kruskal-Wallis test: H = 0.02, d.f. = 1, P = 0.900). Also there was no significant difference in expected heterozygosity (He) between immune genes (Q1, Median and Q3 = 0.15278, 0.29752 and 0.44444) and non-immune genes (Q1, Median and Q3 = 0.08678, 0.27778 and 0.44444), (Kruskal-Wallis test H = 1.54, d.f. = 1, P = 0.214). In addition, F_{IS} did not differ significantly between immune genes and non-immune genes (Q1, Median and Q3 = -0.14286, -0.04348 and 0.16084) and (-0.17483, -0.04348 and 0.11111), respectively), after Bonferroni correction for testing 5 subpopulations (alpha = 0.05/5 = 0.01): (Kruskal-Wallis test: H = 6.45, d.f. = 1, P = 0.011) (Appendix 7).

4.3.3 Estimating loss of genetic variation in the pink pigeon

In order to quantify the levels of heterozygosity in each subpopulation across time, regression analyses were performed to examine the relationship between the genome wide heterozygosity data and the date an individual was hatched for (133) birds across five subpopulations including Ile aux Aigrettes (IAA), Pigeon Wood (PW), Combo (CO), Plaine Lievre (PL) and Bel Ombre (BO), over the period between 1994 and 2008. The analysis showed significant correlations between genome wide heterozygosity and inbreeding coefficient (F_{IS}), and the date an individual was hatched among three subpopulations, Ile aux Aigrettes (IAA), Combo and Bel Ombre (BO) (Table 4.2 and Figure 4.3 a-e). This result suggests genetic drift across the time between 2004 and 2008 in these three subpopulations.

Table 4.2. Analysis of Variance (ANOVA) for estimating loss of genetic variation in the pink pigeon. The genome wide heterozygosity as response variable and the date of hatching from 16/01/1994 (the date of the first hatched bird in the dataset) as covariate. The ANOVAs were calculated for each of the five subpopulations separately (i.e. Ile aux Aigrettes (IAA), Pigeon Wood, Combo, Plaine Lievre, and Bel Ombre).

Subpopulation	R-Sq %	Source	df	SS	MS	F	P
IAA	22.7	Regression	1	0.008439	0.008439	21.41	<0.001
		Residual Error	73	0.028775	0.000394		
		Total	74	0.037214			
Pigeon Wood	10.2	Regression	1	0.000471	0.000471	0.91	0.368
		Residual Error	8	0.004146	0.000518		
		Total	9	0.004617			
Combo	17.4	Regression	1	0.001091	0.001091	4.62	0.043
		Residual Error	22	0.005188	0.000236		
		Total	23	0.006278			
Plaine Lievre	12.5	Regression	1	0.000596	0.000596	1.42	0.26
		Residual Error	10	0.004188	0.004784		
		Total	11	0.004784			
Bel Ombre	47.6	Regression	1	0.006518	0.006518	9.08	0.013
		Residual Error	10	0.007181	0.000718		
		Total	11	0.013698			

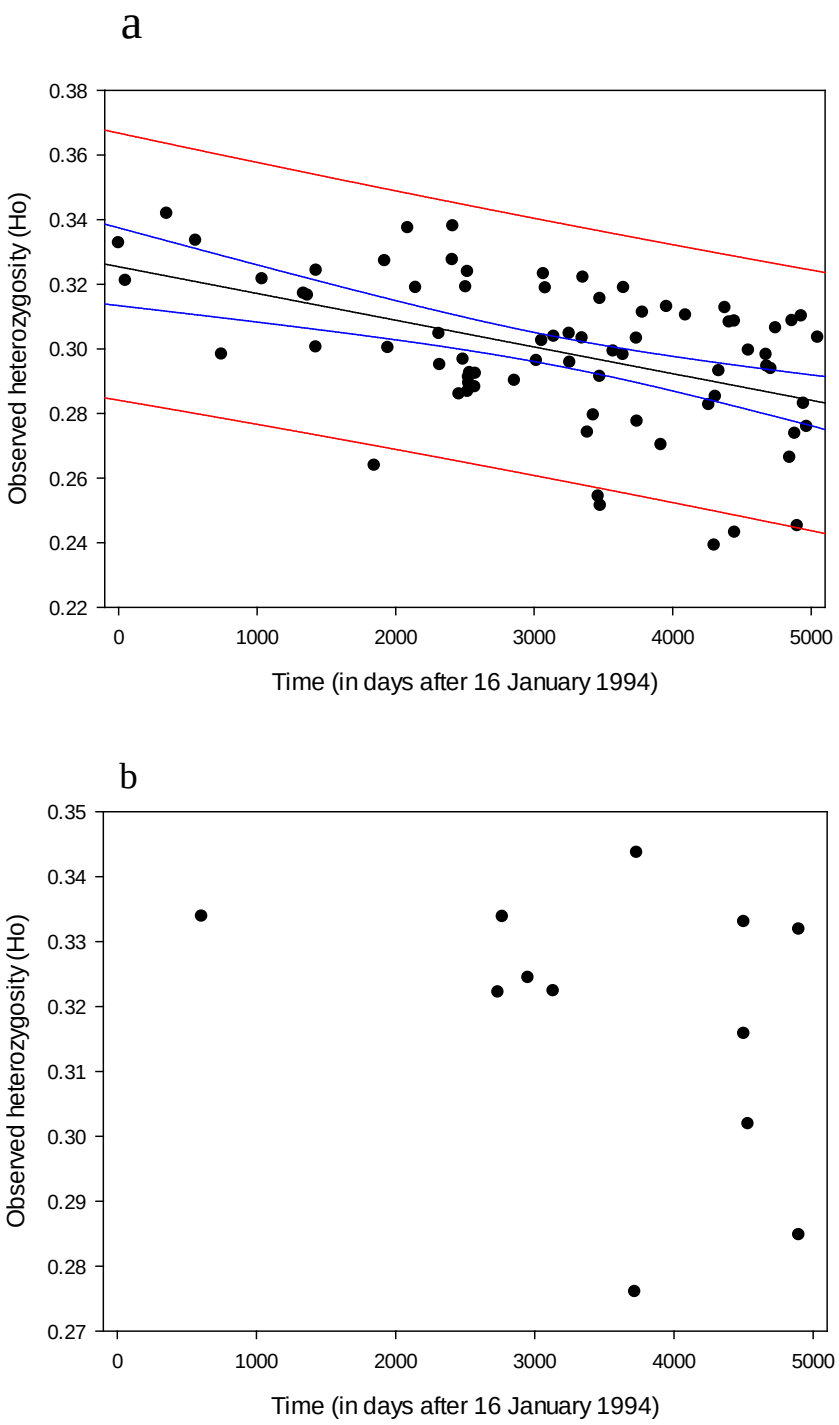
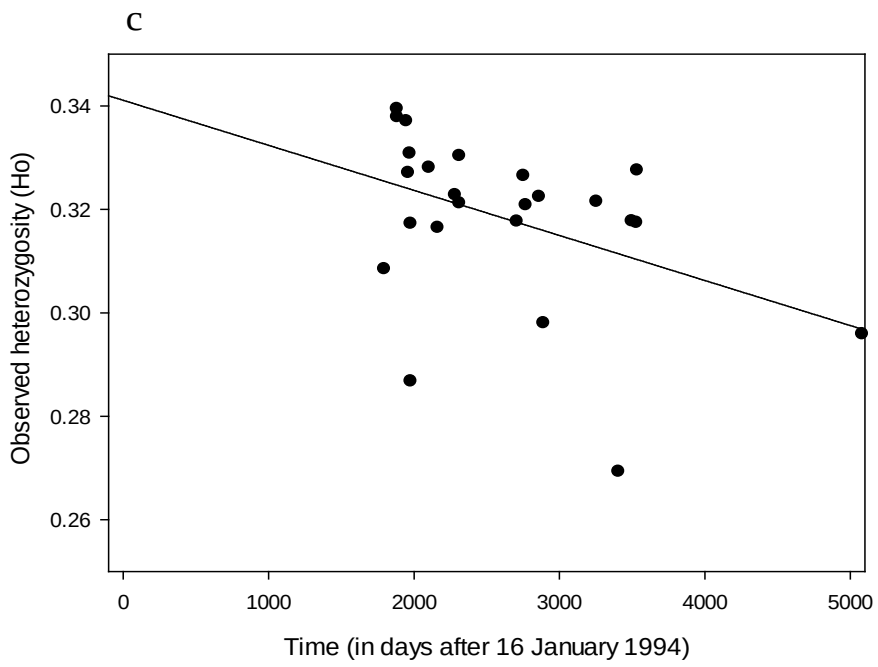


Figure 4.3 a-e. Linear regression of observed heterozygosity against the day of hatching after 16/01/1994 (the date of the first hatched bird in the dataset) in the a)IAA, b)Pigeon Wood, c)Combo, d) Plaine Lievre, and e) the Bel Ombre sub-population, respectively. Shown are the regression line, and for the IAA population the 95% confidence interval of the regression line (in blue) and 95% predictor interval of all data points (in red).



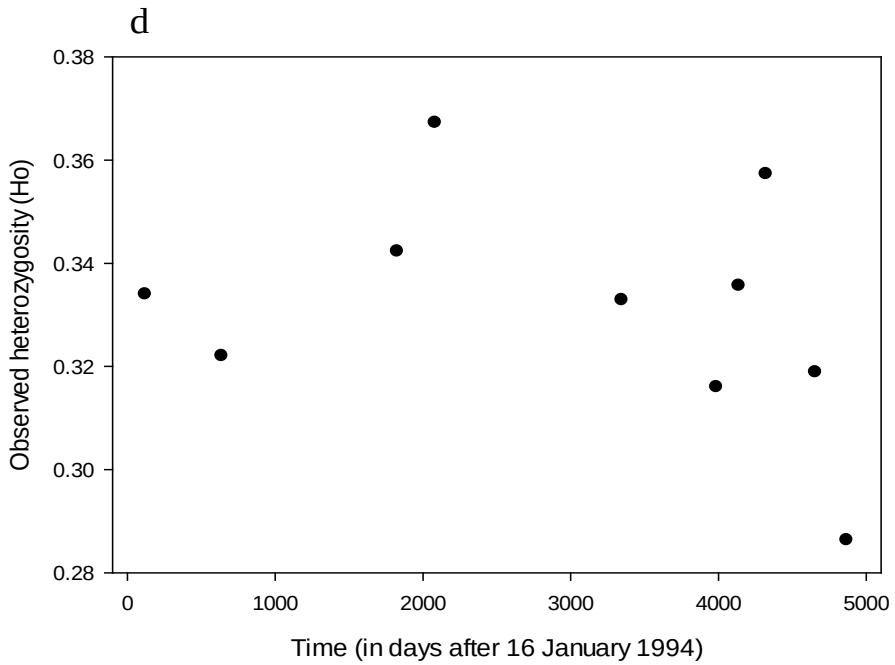


Figure 4.3 a-e. Continued

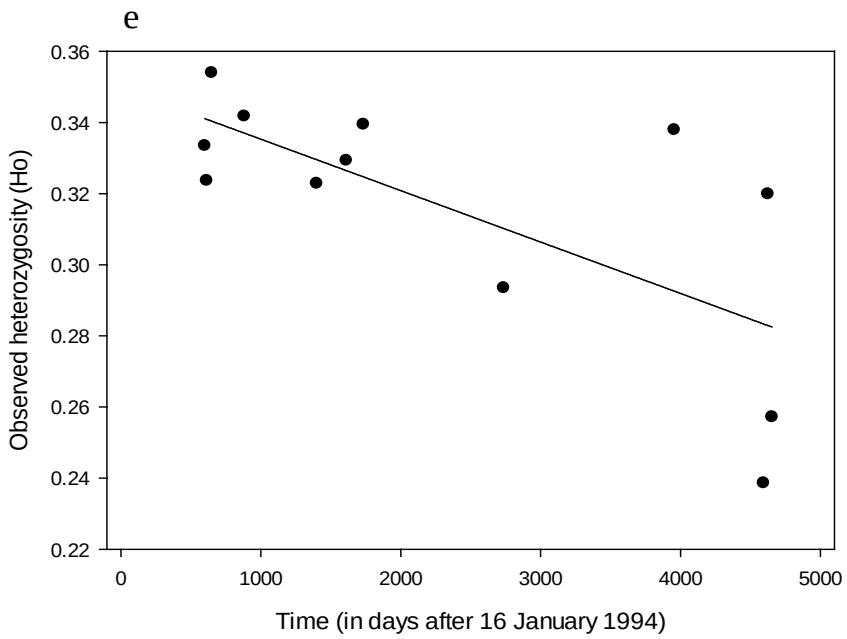


Figure 4.3 a-e. Continued

4.3.4 Estimating of effective population sizes

In order to estimate the effective population sizes (Ne) and loss in gene diversity across two generations in the IAA subpopulation, the genome wide heterozygosity of parents and offspring hatched between 2004 and 2008 was calculated. There was a significant reduction (6.15%) in heterozygosity within a single generation (Kruskal-Wallis test: H=10.72; d.f. =1; p=0.001) in observed heterozygosity for parents and offspring generation (Q1, Median and Q3 = (0.29230, 0.31420 and 0.32401) and (0.27676, 0.29381 and 0.30330), respectively) (Appendix 8), (Figure 4.4).

Based on this, the effective population size (*Ne*) was collected with the following formula:

$H_{offspring} = H_{parents}(1 - 1/2Ne) = 7.625$.

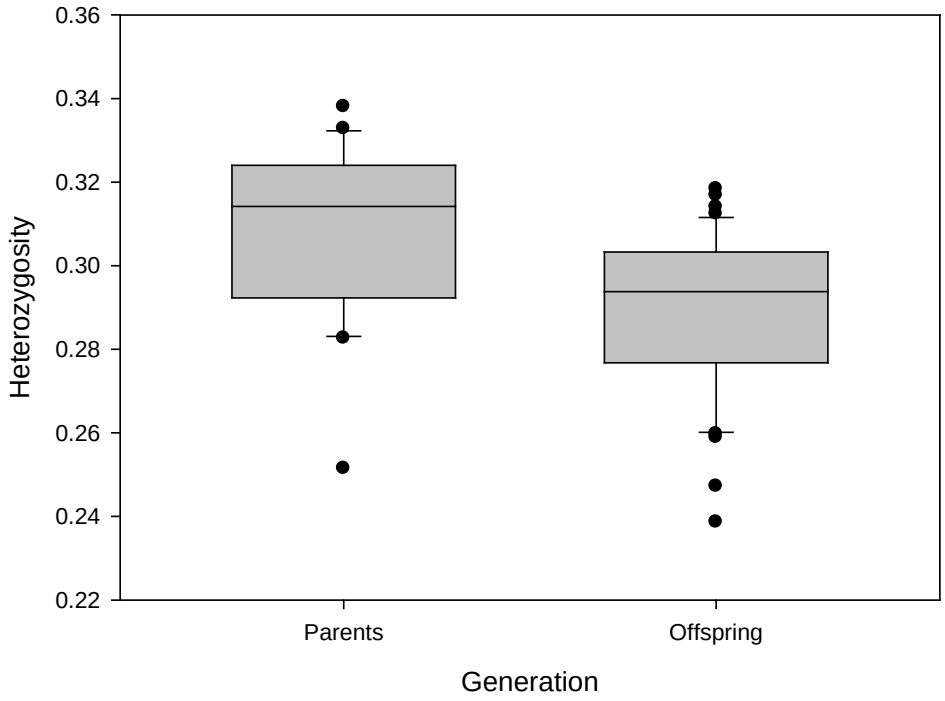


Figure 4.4. Box plot showing the median and first and third quartile of observed heterozygosity of the group of parents and offspring in the IAA population.

4.3.5 Calculating inbreeding coefficient (F_{IS}) for the IAA subpopulation

The mean inbreeding coefficient (F_{IS}) for the IAA subpopulation was calculated across time. Besides random genetic drift, there was also strong evidence for increased inbreeding in the IAA population over time. A one-sample t-test was used with a hypothesised mean of zero. Whereas the first individuals hatched between 1994 and 1999 are significantly less inbred than expected based on random mating in a panmictic population (T= -61.23, p< 0.0001), (mean ±SE) Fis = -0.07059 ±0.00115), birds hatched between 2005 and 2008 are relatively more inbred than expected based on random mating population (T= 8.25, p< 0.0001), (mean ±SEM) Fis = 0.00857 ±0.00104)) (Figures 4.5 and 4.6 a-c). These results indicate that inbreeding forms an increasingly serious threat to the genetic diversity in the IAA subpopulation, with an increasingly smaller number of individuals contributing to the gene pool of the next generation.

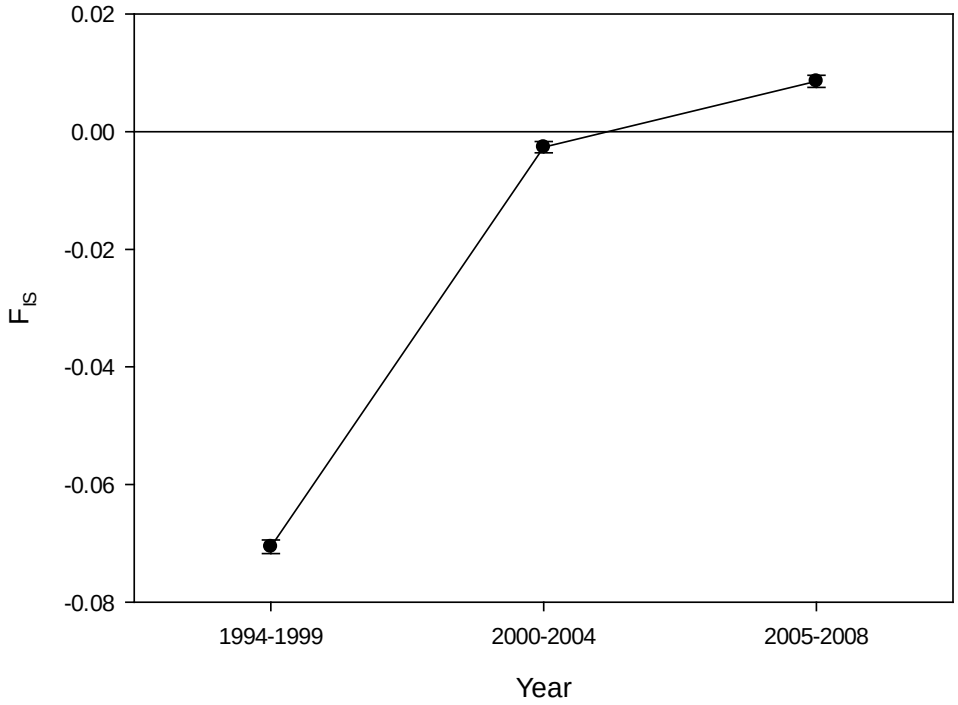


Figure 4.5 The mean (±SE) inbreeding coefficient (F_{IS}) calculated based on the observed and expected genome wide heterozygosity in the IAA population across time. Birds hatched between 1994 and 1999 are significantly less inbred than expected, birds born hatched between 2005 and 2008 are relatively more inbred than expected based on random mating.

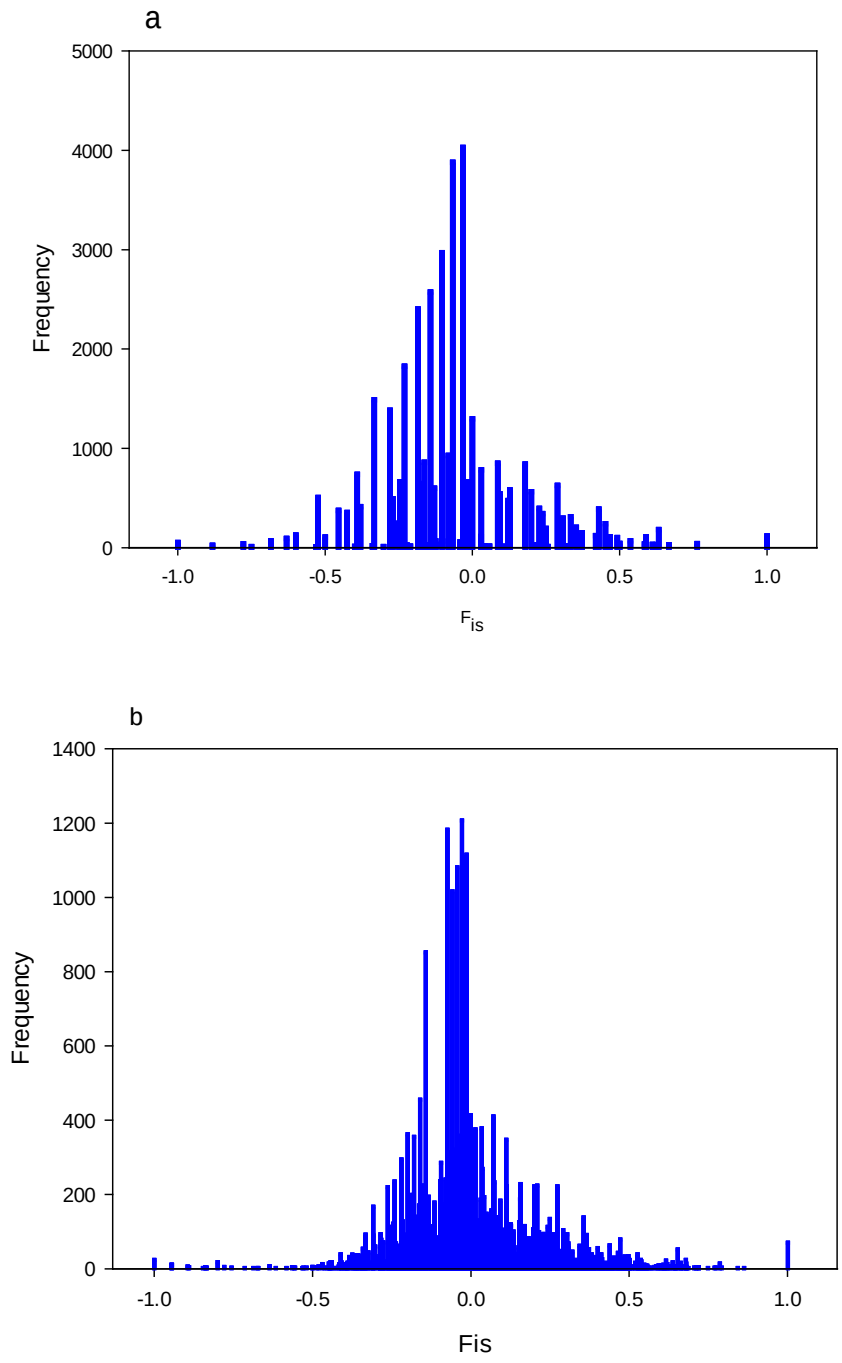


Figure 4.6 a-c. Distribution of F_{IS} IAA subpopulation across time. a) F_{IS} between 1994 and 2000, b) between 2001 and 2004 and c) between 2005 and 2008.

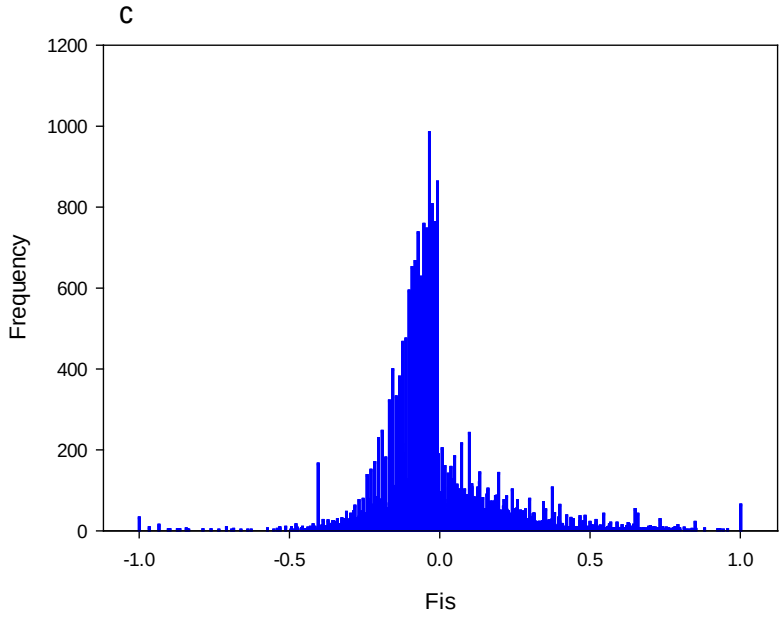


Figure 4.6 a-c. Continued

4.3.6 Calculating genetic distance between pink pigeon subpopulations

To estimate the genetic distances between pink pigeon subpopulations, the pairwise F_{ST} values between the subpopulations were calculated. Ile aux Aigrettes (IAA) was found to be genetically most distinct from the other subpopulations. The pairwise F_{ST} values ranged from 0.009 between Bel Ombre and Pigeon Wood, and 0.063 between IAA and Plaine Lievre. (Table 4.3). The Mantel test was performed to examine the relationship between Pairwise F_{ST} and geographical distances between subpopulations. The correlation between Pairwise F_{ST} and geographical distances is positive and statistically significant (R= 0.923, p= 0.050). (Table 4.4 and Figure 4.7). This result suggests genetic isolation by distance between pink pigeon subpopulations is driven by genetic drift

Table 4.3. Genetic distance (Fst/(1-Fst)) matrix of five pink pigeon subpopulations.

IAA	BO	CO	PW	PL	
0					IAA
0.049318	0				BO
0.053741	0.017294	0			CO
0.059322	0.009082	0.014199	0		PW
0.06383	0.01626	0.031992	0.025641	0	PL

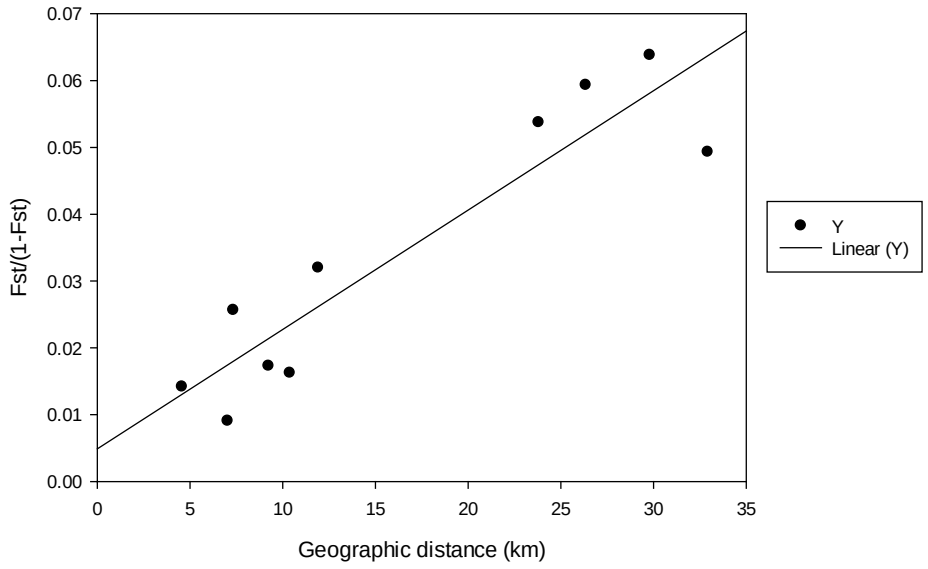


Figure 4.7. Mantel test of the relationship between population pairwise as genetic distance and geographic distance for five pink pigeon subpopulations. The equation is Y=0.0018X+0.0049, R^2=0.8525, R= 0.923.

Given the strong effects of inbreeding over time it is likely that random genetic drift increases genetic Differentiation between subpopulations when analysed over a temporal scale. In other words, drift is expected to increase the genetic differentiation expressed as F_{ST} over time. A temporal F_{ST} analysis was conducted and compared the level of genetic differentiation of the IAA relative to the other subpopulations. For this analysis, samples of subpopulations were separated into two time slots, before and after the year 2000 for Bel Ombre and Pigeon Wood, and before and after 2004 for Plaine Lievre to 2008. For the Combo subpopulation, the temporal sampling was sufficient to divide the samples into three time slots (before 2000, between 2000 and 2002, and after 2002). Then, pairwise F_{ST} values were calculated between the IAA population and the four remaining populations for their respective time slots. Figure 4.8 shows that the level of genetic differentiation increased almost linearly over time, with the IAA becoming increasingly differentiated from all other subpopulations

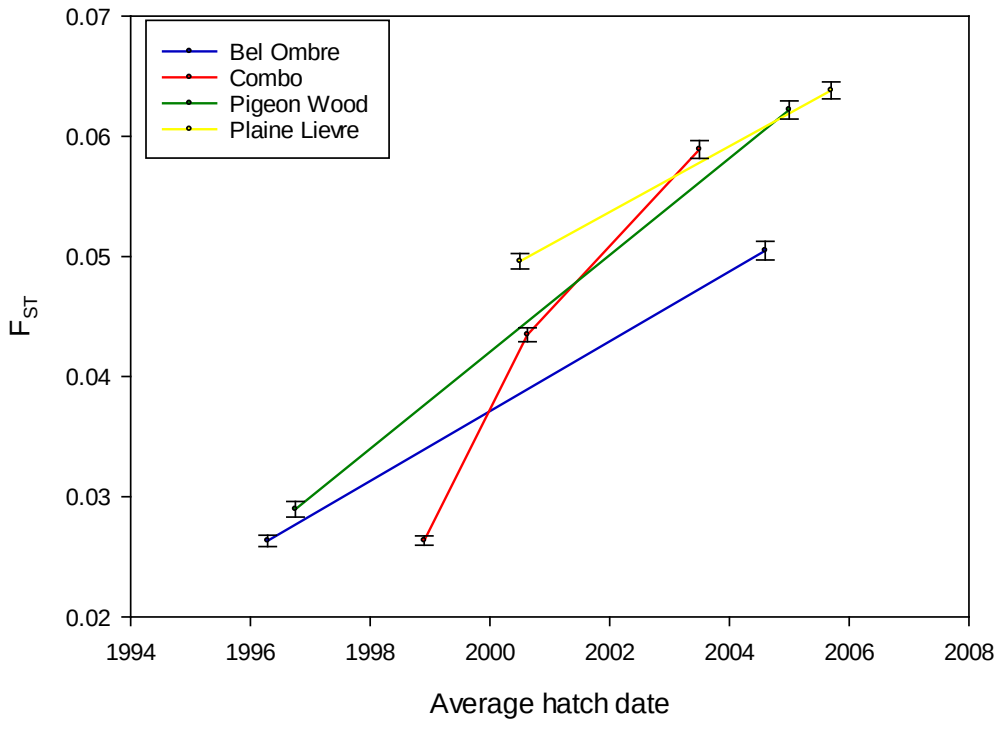


Figure 4.8 The temporal F_{ST} shows the genetic differentiation between the IAA subpopulation and the four remaining subpopulations (Bel Ombre (blue), Combo (red), Pigeon Wood (green) and Plaine Lievre (yellow)). Shown are the mean±SE of pairwise F_{ST} between the IAA subpopulation and another subpopulation sampled during the same period. The level of genetic differentiation increases over time, which shows that genetic drift causes the subpopulations to increasingly diverge.

4.4 Discussion

The main objective of this chapter was to assess the genome-wide genetic variation and genetic differentiation between five subpopulations of the pink pigeon (*Nesoenas mayeri*) between 1994 and 2008. Population genetic statistics were calculated including the observed and expected heterozygosity, the inbreeding coefficient (F_{IS}) and the level of population genetic differentiation (F_{ST}). These analyses allow us to assess the effects of genetic drift and inbreeding, and to estimate the effective population size. No information is available in the literature on the genetic effective population size of the pink pigeon which is of crucial importance to assess the effectiveness of the conservation management practices employed. By using a genome wide approach covering 45,840 polymorphic loci (single nucleotide polymorphisms, SNPs), this study reveals that despite intensive conservation management, the wild pink pigeon populations have experienced a rapid loss of heterozygosity over a short period of time which inflates the genetic differentiation between the subpopulations. The effective population size in one of the populations (IAA) appears to be one order of magnitude lower than the census population size, and this alarmingly low Ne/N ratio in a managed population highlights the need for urgent intervention. In particular, we suggest that augmenting gene flow between the (increasingly more) genetically differentiated subpopulations may help to mitigate the loss of heterozygosity and ameliorate inbreeding depression. In addition, genetic supplementation from captive bred populations of pink pigeons present in zoos may prove a valuable injection of novel genetic variation that can help to counteract the loss by drift and inbreeding.

4.4.1 Genome wide genetic variation

The first question in this chapter seeks to determine the level of heterozygosity across the pink pigeon genome. The results of this study showed that genome-wide genetic diversity is very low for this species. The average (Ho) and (He) values over 45,840 loci were very similar in the total population: 0.27 and 0.28, respectively. The average observed heterozygosity in the subpopulations ranged from 0.27 in IAA, Bel Ombre and Plaine Lievre to 0.29 in Combo. The average expected heterozygosity was identical in all these subpopulations (0.27).

Genetic diversity has been found to be low in most threatened species using classical methods such as microsatellite markers (Evans and Sheldon, 2008; Spielman *et al.,* 2004). Although this has also been demonstrated by utilising genome-wide genetic markers such as single nucleotide polymorphisms (SNPs), which detected different heterozygosity levels between endangered and vulnerable species and non-endangered species (Li *et al.,* 2014), SNPs have several important advantages over microsatellites. Firstly, an important difference between microsatellites and SNPs is that microsatellite loci have a high mutation rate more than that of single nucleotide mutations (Schlötterer, 2004). As a consequence, microsatellite markers tend to remain polymorphic with high levels of heterozygosity even in small and endangered populations (Ouborg *et al.,* 2010). For example, the level of genetic differentiation measured by microsatellite markers tends to underestimate that of other markers such as SNPs (Gautier *et al.,* 2013). Importantly, because microsatellites tend to be in non-coding DNA whereas SNPs can occur both in coding and non-coding DNA, the latter are more relevant for functional genetic variation (Kohn *et al.,* 2006). In addition, SNP markers allow for the estimation of the effective population size because the loss in heterozygosity can be used to calculate the level of inbreeding and hence, the effective population size (Pujolar *et al.,* 2013). Finally, another important advantage of SNPs over microsatellites is that the reduction in heterozygosity (and hence, the increase in homozygosity) is directly related to inbreeding depression that is caused by the fixation of recessive deleterious alleles in homozygous condition (Hoffman *et al.,* 2014), and the rate with which this happens can be accurately estimated using a large number of SNPs as analysed in this study.

4.4.2 Genetic variation of immune related genes

Heterozygosity was calculated separately for 167 immune-related genes within each subpopulation and compared with non-immune genes (other autosomal genes), which were used as a control group. The findings of this investigation showed that there were no significant differences in Ho, He and the main inbreeding coefficient (F_{IS}) between immune genes and non-immune genes in four of five subpopulations. However, in one subpopulation (Bel Ombre) there was a significant difference in (F_{IS}) between immune genes and non-immune genes. The F_{is} of the immune genes was significantly smaller than zero, indicating a relative heterozygous excess which is consistent with balancing selection.

Balancing selection has been reported as a major force shaping the evolution of immune genes (Ferrer-Admetlla et al., 2008), and parasite-mediated selection has often been implicated as a source of balancing selection (Charbonnel and Pemberton, 2005). In pink pigeon, it is likely that the high Trichomonosis-related mortality in young pink pigeons acts as a strong selective pressure. In the case of the Bel Ombre subpopulation, which showed higher genetic variation in the immune genes, it was shown in chapter 3 that this subpopulation had the highest number of infected birds, which may indicate to selection pressure. In addition, this subpopulation contains birds that were found to live longer with this high infection compared to the other subpopulations. This suggests that the Bel Ombre subpopulation may have developed a resistance to *T. gallinae* infection; however, selection in this subpopulation may be weak because of small population, and genetic drift may continue to affect population size (Frankham *et al.,* 2010).

4.4.3 Estimating genetic loss in pink pigeon

Small and endangered populations are expected to experience losses in genetic variation (Amos and Balmford, 2001; Charlesworth, 2003). In order to assess genetic losses in the pink pigeon population, the relationship between the genome-wide heterozygosity data and the date individual birds were hatched (133 birds) was examined across five subpopulations, including IAA, Pigeon Wood, Combo, Plaine Lievre and Bel Ombre, over the period between 1994 and 2008. The results revealed a significant loss of genome-wide heterozygosity in three subpopulations: IAA, Combo and Bel Ombre. Such loss in heterozygosity can be driven by interrelated factors including chance effects, reduction in population size and increased inbreeding (Frankham, 2005). The influence of these factors on heterozygosity was examined.

First, the genome-wide heterozygosity of parents and offspring was examined to calculate effective population size (Ne). Even though only 44 offspring and 20 parents were analysed, the effective population size could theoretically be infinite (which would have been the case if the heterozygosity of the parents and offspring was identical). In other words, this estimate of effective population size is not biased by the small sample size, but reflects the fact that the effect of drift is still present in the current pink pigeon population. This analysis showed that the effective population size was alarmingly low with only Ne=7.625 birds effectively contributing to the gene pool of the next-

generation. **Second**, the F_{IS} for the IAA subpopulation was calculated across time. Besides random genetic drift, which is a stochastic process, there is also strong evidence for increased inbreeding in the IAA population over time. It has been found that birds hatched between 2005 and 2008 are relatively more inbred than would be expected based on random mating. This suggests that relatively related birds breed and reproduce offspring that are more inbred than expected based on random mating. It seems that effects of inbreeding accelerate genetic drift, causing a faster than expected diminishing of genome-wide heterozygosity. Although the current population size is significantly larger than 7.625 birds, drift and inbreeding are still operating in a so-called 'drift depth'. This takes place when the equilibrium level of heterozygosity has not been reached, and when the population is not yet in a drift mutation balance (Reed, 2007).

4.4.4 Genetic distance between pink pigeon subpopulations

In addition to the previous analysis, which showed a decrease in heterozygosity over time in three of five subpopulations, we calculated the Weir & Cockerham F_{ST} for genetic distance between subpopulations.

Local adaption occurs within isolated populations as a response to environmental conditions, which increases the degree of genetic differentiation among these populations, even with the existence of gene flow (Guo *et al.,* 2015). It can also occur as a result of genetic drift (Palo *et al.,* 2003; Leinonen *et al.,* 2006). Movement between pink pigeon subpopulations is limited (Jones and Swinnerton, 1997) suggesting low or negligent amounts of gene flow. However the significance positive relationship between the pairwise F_{ST} and geographic distances between pink pigeon subpopulations suggests that genetic drift in combination with low levels of gene flow is operating in this fragmented population. However, with an effective population size less than 10 birds in the IAA population, natural selection is incapable of changing allele frequencies due to environmental or demographic processes because there are too few birds to select from. In summary, we propose that the pattern of isolation by distance observed in this study is explained by strong genetic drift in combination with low levels of gene flow between adjacent populations, and we reject the hypothesis of natural selection increasing genetic differentiation in this system due to the very small effective population size.

4.5. Implications for conservation genetics of the pink pigeon

The goals of conservation management of pink pigeon species are to decrease the risk of extinction and address issues limiting the growth of the population to a viable size (Jones, 2004). The conservation management process includes estimating and maintaining genetic diversity and minimizing factors that reduce productivity and survival, such as genetic loss, breeding, predation and disease (Jones, 2004). This has included continuing control programmes of introduced mammalian predators in BRGNP, and their complete eradication from the offshore islet IAA (Jones, 2004). Strong evidence suggests that disease is the most common cause of mortality in young pink pigeons (Swinnerton *et al.,* 2005a; Bunbury *et al.,* 2008). A severe conservation problem that emerges from the findings of this chapter is that the genome-wide genetic variation within the pink pigeon population is low compared to that of non-endangered species such as Little egret *Egretta garzetta*, Great black cormorant *Phalacrocoraxcarbo*, Budgerigar *Melopsittacus undulates*, Turkey vulture *Cathartes aura* (Li et al., 2014),, and that there is a continued, sharp decline in genetic diversity. This issue appears to be driven by the very small Ne, which means that genetic drift and inbreeding can still have an effect until the population reaches a drift mutation balance. This problem might be responsible for a reduction in the fitness of pink pigeons, including high infertility rates of eggs laid (> 50%) and mortality in squabs as a result of Trichomoniasis (Bunbury, 2006). Intensive genetic conservation management is vital to avoid the risk of extinction.

There are some management options to elevate genetic variability and minimise inbreeding depression. One strategy is to translocate birds from one subpopulation to another. The pink pigeon metapopulation consists of subpopulations that are increasingly genetically differentiated. This genetic differentiation can be converted into heterozygosity and novel substrate for selection by enhancing gene flow between subpopulations. In addition, the findings in this chapter demonstrate a higher level of adaptive variation in the Bel Ombre subpopulation than in other subpopulations, supporting the findings in Chapter 3, which showed that few birds in this subpopulation were identified to be significantly less infected with *T. gallinae* than expected. However, alarmingly, this population also shows the fastest rate of loss of genetic variation, with 47.6% of the variation in heterozygosity being explained by time. This might be related

to the extreme high infection with trichomoniasis in this population, which exacerbates the effects of drift, which could push this population towards an irreversible extinction vortex.

Using the genomic data presented in this study, future work is needed to identify genomic regions or genes responsible for adaptive variations, including immune genes associated to increased resistance to disease. Another conservation strategy for managing genetic variability is to promote gene flow between the subpopulations, and release birds from different captive pink pigeon populations in zoos around the world in an effort to genetically supplement the wild population. Hence, additional future work should assess genome-wide genetic variations in captive pink pigeons (in zoos) compared to free-living populations to investigate the viability of introducing zoo birds.

4.6. Conclusion

By using a RAD-seq approach and genotyping 45,840 polymorphic loci, this study reveals that despite intensive conservation management, the wild pink pigeon population has experienced a rapid loss of heterozygosity over a short period of time which has inflated the genetic differentiation between the subpopulations. The effective population size appears to be considerably lower than the census population size, highlighting the need for urgent intervention. In particular, we suggest that augmenting gene flow between the (increasingly more) genetically differentiated subpopulations may help to mitigate the loss of heterozygosity and ameliorate inbreeding depression. In addition, genetic supplementation from captive bred populations of pink pigeons present in zoos may prove a valuable injection of novel genetic variation that can help to counteract the loss by drift and inbreeding. Given the evidence of balancing selection acting on immune-related genes in one of the subpopulations, further research in Chapter 5 is dedicated to identifying genomes regions associated with increased disease resistance. By finding genetic variants associated to disease resistance and/or tolerance in the wild and screening the captive bred stock for those variants, this research will be able to inform conservation genetic management by identifying the most suitable individuals for genetic supplementation.

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Chapter 5

Assessment of inbreeding depression in the Mauritian pink pigeon population

Summary

Inbreeding depression is a major subject of interest within the fields of conservation biology and population genetics. It has been identified as one of the main factors contributing to the increase of extinction risk in small populations. The Mauritian pink pigeon (*Nesoenas mayeri*) (Prévost, 1843) is a bottlenecked species that has recovered from about 10 to 20 in 1975 to approximately 400 birds today. The major conservation issues preventing the growth of the population include the high squab mortality and continued infections with parasitic diseases resulting in severe pathology and death. Questions have been addressed about whether this loss in fitness is related to genetic factors. To answer this question, the association between genome-wide heterozygosity and fitness-related traits was analysed using 45,841 single nucleotide polymorphisms (SNPs) generated using restriction-site associated DNA sequencing (RAD-seq). A significantly negative association was found between genome-wide heterozygosity and infection with *Trichomonas gallinae*. Longevity of adult birds and fledgling success showed a significant positive relationship with the level of genome-wide heterozygosity. Reproductive success in terms of the number of nests, eggs laid and hatchlings which died, however, did not show a significant relationship with the level of genome-wide heterozygosity. I hypothesised that reproductive success might not be a polygenic trait, but rather affected by single or few genes with major effect. To test this hypothesis, we performed a genome-wide association study (GWAS) to identify the genomic region that could explain the variation in the number of eggs laid by 24 females. A genomic region was found approximately 0.4 Mb away from the progesterone receptor gene (PRG) previously identified as a potential gene affecting egg laying in other taxa. This result is promising for additional research using quantitative trait loci (QTL) analyses or GWAS to identify genes affecting reproductive traits. Importantly, this provides notable evidence that this small genomic region needs to be prioritised and its variation needs to be conserved in captive breeding programs. These findings suggest that reduction in reproduction success in pink pigeon might be affected by the fixation of a small number

of detrimental alleles at particular loci resulting in severe inbreeding depression. I propose that conservation efforts of the pink pigeon should be focused on genetic supplementation of the endangered wild population with captive bred birds harbouring favourable genetic variants at this PGR region to restore fitness and breeding success in the wild.

5.1 Introduction

An early observation by Darwin (Darwin, 1868) was a warning that progeny produced by mating between closely related individuals tend to be less viable (in Allendorf *et al.,* 2012). This fact, known now as inbreeding depression (Charlesworth and Charlesworth, 1999), leads to a reduction in population fitness, such as survival, reproductive success and individual health, as well as resistance to infectious diseases (Crnokrak and Roff, 1999; Oosterhout *et al.,* 2000b; Frankham *et al.,* 2010). The most possible explanation for inbreeding depression is the fixation of recessive deleterious mutations in homozygous genotypes, and this is known as the dominance model of inbreeding (Charlesworth and Charlesworth, 1987; Charlesworth and Charlesworth, 1990; Charlesworth and Willis, 2009). According to this model, the loss of fitness is due to the expression of the recessive deleterious effects of two copies of the same allele that are brought together by consanguineous mating in a homozygous genotype in the offspring. The alternative model is known as the overdominance model of inbreeding depression, and this suggests that the loss of fitness due to inbreeding is caused by the reduction in heterozygosity in itself (Charlesworth and Charlesworth, 1987; Charlesworth and Charlesworth, 1990; Charlesworth and Willis, 2009). According to this model, heterozygous genotypes are fitter than homozygous genotypes, a phenomenon known as overdominance (Charlesworth and Willis, 2009; Charlesworth and Charlesworth, 1987; Charlesworth and Charlesworth, 1990). Currently, the consensus is that most inbreeding depression is, however, due to dominance effects of recessive deleterious mutations, which is consistent with the "Nearly Neutral theory" of molecular evolution (Ohta, 1992; Ohta and Gillespie, 1996). According to this model, recessive deleterious alleles are continuously added to the gene pool by mutations as well as by gene flow from other populations. Because these alleles are recessive and not (or only partially) expressed in heterozygous state, their allele frequency can stochastically increase in the population similar to that of neutral alleles due to genetic drift (Hedrick, 2001). As a consequence,

most outbreeding species have a substantial genetic load of recessive deleterious alleles segregating in the genome of all individuals in the population (Bataillon and Kirkpatrick, 2000). However, these recessive deleterious alleles will remain typically at low frequency, and estimates of this genetic load have been made for various species across a wide range of taxa (Frankham, 2005).

The fitness effects of these recessive deleterious alleles are typically minor in large populations because of two reasons. Firstly, the equilibrium frequency of recessive deleterious mutations will be low in large populations because natural selection will operate against these alleles once their frequency increases so that they may occur in homozygous condition, revealing their deleterious fitness effects (Kohn *et al.,* 2006). Secondly, in large populations the rate of consanguineous mating (inbreeding) is generally low because the probability of two genetically related individuals mating tends to be small (Frankham *et al.,* 2010). However, detrimental mutations can be found at high frequency, or even fixed, in small populations because natural selection is inefficient in small populations; it's effects being overshadowed by random genetic drift (Kohn *et al.,* 2006). Indeed, deleterious alleles with selection coefficients $s \leq (2Ne)^{-1}$, where Ne is the effective population size, behave similar to neutral alleles (Hedrick, 2001). In other words, a deleterious allele that reduces the fitness has a similar chance to go to fixation in the population with a small effective population size.

The evidence of the severity of inbreeding depression threatening the survival of endangered species has been found in several studies. For example, as a result of being isolated and having small population size, endangered bird species endemic to islands, including the Mauritius kestrel (*Falco punctatus*) (Ewing *et al.,* 2008), the black stilt (*Himantopus novaezelandiae*) (Hagen *et al.,* 2011) and the Hawaiian goose (*Branta sandvicensis*) (Paxinos *et al.,* 2002; Veillet *et al.,* 2008), have suffered from inbreeding and consequently experience low genetic diversity.

Inbreeding depression is one of the "extinction vortices" (Gilpin, 1986) that can interact with other stochastic factors, including environmental, demographic and genetic stochasticity, to hasten the extinction process in small populations (Frankham *et al.,* 2010). It often leads to a reduction in adaptive variation (Hedrick, 2001) and heritable changes in amino-acid substitution in response to natural selection (Ouborg *et al.,* 2010).

A consequence is a decline in population fitness (Oosterhout *et al.,* 2000b; Avdi and Banos, 2008), including decreases in resistance to environmental stress (Reed *et al.,* 2002), and elevated susceptibility to infection and diseases (Trinkel *et al.,* 2011).

5.1.1 Association between fitness-related traits and genetic variation

Inbreeding depression has been reported in a wide range of domestic (e. g. Santana Jr *et al.,* 2010; Santana *et al.,* 2012) and wild populations (e. g. Walling *et al.,* 2011). It can affect many fitness-related traits of individuals within a population, such as clutch size (Ortego *et al.,* 2007), sperm quality (Fitzpatrick and Evans, 2009), birth weight and survival (Walling *et al.,* 2011; Townsend and Jamieson, 2013), egg production (Pooley, 2013), hatching rates (Heber and Briskie, 2010), body condition (Fareed and Afzal, 2014), sexual ornamentation and courtship behaviour (Van Oosterhout *et al.,* 2003), sterility and fecundity (Van Oosterhout *et al.,* 2007) and susceptibility to infectious diseases (Lyons *et al.,* 2009). Assessing the relationship between individual genetic variations and individual life history characteristics, such as morphological and physiological measures, is known as heterozygosity–fitness correlations (HFCs) (Chapman *et al.,* 2009), in which a significant HFC indicates inbreeding depression (Ruiz-López *et al.,* 2012). Estimating heterozygosity and fitness-related traits can be achieved using the pedigree data for individuals within a population (Oosterhout *et al.,* 2000a). However, this method has been applied to few wild populations because of data being unavailable for both pedigree and life history (Grueber *et al.,* 2008). Microsatellite markers have become a popular choice for assessing HFC in many organisms. This is because microsatellites are selectively neutral loci found across the whole genome (Grueber *et al.,* 2008). However, there is controversy about the feasibility of using a low number of microsatellite markers (which is typically restricted to ~20 markers being screened) to estimate HFCs, because they do not have the power to infer genome-wide heterozygosity (Chapman *et al.,* 2009) and this can increase the error variance in the assessment of genome-wide heterozygosity and thus increase the correlation (Hoffman *et al.,* 2014). In addition, microsatellite loci tend to have many alleles due to their high mutation rate, and hence, the level of homozygosity tends to be low even in relatively small inbred populations, further limiting their application in HFC studies particularly when only a limited panel of loci is being genotyped.

However, with the advent of next-generation sequencing techniques, it has become possible to discover, sequence and genotype large numbers of markers across the genomes of many individuals within a population (Davey *et al.,* 2011). For example, restriction-site associated DNA sequencing (RAD-seq) increases the power to obtain HFCs and to identify loci that influence related fitness traits in a population (Miller *et al.,* 2014). This technique was used by Hoffman *et al.* (2014), who found a significant negative correlation between genome-wide heterozygosity generated from thousands of markers and the inbreeding coefficient in oldfield mice (*Peromyscus polionotus*) compared to heterozygosity obtained using a smaller number of microsatellite markers in the presence of pedigree. Moreover, these authors also applied this comparison to a wild population of harbour seals (*Phoca vitulina*) based on 27 microsatellites and 14,585 single nucleotide polymorphisms (SNPs), and they found a significant HFC for parasite infection with SNPs compared to a weak correlation with microsatellites in the absence of a pedigree (Hoffman *et al.,* 2014). This is evidence that genome-wide heterozygosity obtained from large numbers of genome-wide genetic markers is superior to the screening of a limited number of polymorphic microsatellite loci in studies that aim to analyse the correlation of fitness-related traits and inbreeding in endangered populations and species.

5.1.2 Fitness-related traits in the Mauritian pink pigeon population

The Mauritian pink pigeon (*Nesoenas mayeri*) (Prévost, 1843) is an endangered bottlenecked species (Jones *et al.,* 1992). It has been recovered from 10 to 20 birds in 1975 (Jones *et al.,* 1992; Jones *et al.,* 1999) to a stabilised number of approximately 400 birds now.

The current aim of conservation programmes for the pink pigeon is to increase the number to 600 free-living bird (Mauritius Wildlife Foundation, 2012). However, there have been potential factors limiting the increase in size of the pink pigeon population, including habitat destruction, predation, competition, lack of food sources, bias in sex ratio, parasitic diseases, egg infertility and squab mortality.

Trichomonosis is a parasitic disease caused by the protozoan parasite *Trichomonas gallinae*, which has been investigated in the pink pigeon (Bunbury, 2006; Bunbury *et al.,* 2008). Bunbury *et al.* (2008) studied almost the entire population (426 adult birds) of

pink pigeons between 2002 and 2004 and showed that more than 84% of screened pink pigeons were infected with *T. gallinae* (Bunbury *et al.,* 2008). In chapter 3 of this thesis, the same data were analysed for 418 adult birds and it was found that 86% of these pink pigeons were infected with *T. gallinae*.

Reproductive success of the pink pigeon population on Ile aux Aigrettes (IAA) was studied for 12 months in 2004 by Bunbury (2006), who found high losses in the numbers of hatchlings and fledglings. Of 443 eggs laid that year, fertility was 49.4%, hatchability was low with 160 of 219 fertile eggs and 66% of nestlings died from Trichomonosis, while only 12.5% of nestlings fledged.

Reduced survival and productivity and high susceptibility to parasitic infection in the pink pigeon population are likely related to inbreeding. The inbreeding coefficient for captive birds was calculated in the early stages of the recovery programme for captive birds using pedigree data by Jones et al. (1989), who found low levels of egg productivity, hatchability and fledging in pairs which showed a high inbreeding coefficient. The inbreeding analysis was repeated later for reproductive success, relying on data that were collected in the 1980s and 1990s (Swinnerton *et al.,* 2004). Authors estimated the effect of inbreeding on demographic parameters in both captive and released pink pigeons on IAA using pedigree data, which showed significant effects of inbreeding on egg fertility and survival rates of squab, juvenile and adult birds. However, the relatedness between founders of the entire pink pigeon population is not known, which generates uncertainty when calculating inbreeding coefficients based on pedigree records. The pedigrees of 180 birds sequenced in this thesis were examined using the genomic data and few birds were identified as unrelated genetically to their recorded parents. Theoretically, relying on inaccurate pedigree data for an inbreeding-fitness correlation analysis may significantly bias the results. Hence, I used genomic methods to estimate inbreeding depression to examine the link between genetic parameters, as well as the high prevalence of infection with parasitic disease and low productivity and high mortality in young birds. In particular, it was not previously known whether these traits are affected by multiple loci or by a single deleterious allele. In light of parasitic disease results in chapter 3 and findings on reproductive success in Bunbury's (2006) in the pink pigeon, in this chapter, I have applied RAD-seq techniques to genotype

thousands of SNPs across the whole genome to identify whether there is a relation between fitness-related traits and genetic factors in the pink pigeon population.

The major aim of this chapter is to assess the relationship between genome-wide genetic heterozygosity and fitness-related traits in the pink pigeon population, in particular the proportion of screenings where birds tested positive *T. gallinae*, reproductive success, survival and longevity.

5.2 Materials and Methods

5.2.1 Study sites

The pink pigeon population currently consists of seven subpopulations (Mauritian Wildlife Foundation, 2012) of which five , including Pigeon Wood (PW), Bel Ombre (BO), Plaine Lievre (PL) (also called Camp), Combo (Co) and IAA, are included in this study. The subpopulations, PW, BO, PL and CO are located in the Black River Gorges National Park. However, the IAA population is located on a small islet off the eastern coast of Mauritius (Jones, 1987; Jones *et al.,* 1992) (Figure 5.1). An additional two subpopulations in the Lower Black River Gorges and at Pétrin were set up in 2007 and 2012, respectively (Mauritian Wildlife Foundation, 2012), and are not included in the present study.

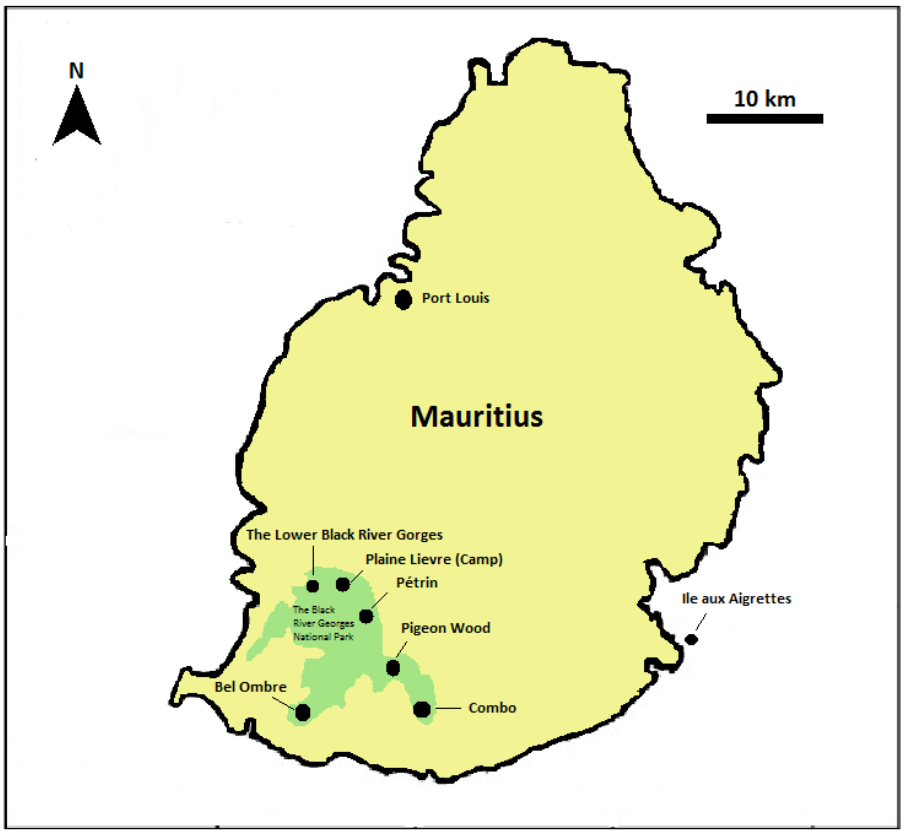

Figure 5.1. Map showing the locations of the seven subpopulations in Black River Gorges National Park and IAA on Mauritius (Adapted from: Swinnerton *et al.,* 2005).

5.2.2 Blood collection, infection screening and reproductive data

The screening of birds for *T. gallinae* infection was conducted bimonthly from September 2002 to April 2004 by Bunbury (2006), and the capture and swabbing procedure is described in detail in Bunbury (2006) and Bunbury et al. (2008). To detect *T. gallinae*, birds were swabbed in the mouth, oropharynx, oesophagus and crop with a sterile cotton bud, which was then used for incubation of the the InPouch TF culture pack (Biomed Diagnostics). The cultures were then incubated at 38°C for at least 72 h before searching for the parasite with a microscope (Bunbury *et al.,* 2005; Bunbury *et al.,* 2008).

Blood samples were collected from birds between October 2004 and September 2008. The capture procedure and blood collection methods have been previously described elsewhere (Bunbury *et al.,* 2007; Bunbury, 2006).

For reproductive data, I relied on the Mauritian Wildlife Foundation databases for IAA inbreeding data from 05/06/2000 to 20/08/2009.

5.2.3 DNA extraction, library preparation and sequencing

This work has been done in collaboration with The Genome Analysis Centre (TGAC), Norwich. Details on the tissue storage, DNA extraction, Quality Control (QC) measures, library preparation, sequence quality metric, RAD-seq protocol, reference assembly, processing and mapping of sequence reads, variant call analysis (including Z-chromosome and immune gene identification), population genomic statistics, and the genome wide association study (GWAS) are detailed in the Appendix 1 and 2.

5.2.4 Data analysis

To calculate the relationship between the genome-wide heterozygosity and fitness-related traits, the heterozygosity of immune and non-immune genes were calculated separately across individuals and regressed against the data on fitness-related traits. Heterozygosity was calculated after excluding the Z-chromosome, because this would introduce a sex bias, with females being the heterogametic sex (WZ). The data on reproductive traits were normalised by dividing the number of nests, eggs, hatchings and fledglings by the duration of pairing. A two-sample t-test was used to examine the difference in genome heterozygosity between a group of birds that were found negative to infection across their last three bimonthly screenings and a group of birds that were found positive in at least one of the last three screenings. A non-parametric test (Kruskal–Wallis test) was used to examine the difference in the genome-wide heterozygosity between fledged birds (140 birds) and birds that died before fledgling (35 birds). The General Linear Model (GLM) was performed to examine whether the variation in survival between fledged and unfledged squabs among families, could be explained by genome wide heterozygosity. The GLM was run where the fledged and unfledged squabs were nested within families which was used as a random factor in the model. All the statistical analyses were run using Minitab 17 and graphs were created using SigmaPlot v 13. GWAS was conducted using a limited number of available samples, as is often the case in studies on endangered species. It was conducted for IAA, the only subpopulation for which detailed reproductive data are available. There are reproductive data for 50 families. However, some females were excluded from the

analysis to avoid biasing. In detail, before conducting GWAS, eggs were counted during pairing time among adults. Therefore, there was a strong correlation between pair duration and number of eggs. When eggs/per day paired with mate was plotted a correlation was still observed due to the skewing of the data caused by pairings of short duration. Therefore, bias was avoided by minimizing pairing duration to at least 100 days. Hence, females with less than 100 days pairing duration were removed from the list. Furthermore, for a female that mated with more than one male, the total pair duration and total eggs were used to generate eggs/days for female with mate(s). This ensures that each female is represented only once during the GWAS.

5.3 Results

The association between genome-wide heterozygosity and fitness-related traits was examined using 45,841 SNPs. In addition to the infection status with *Trichomonas gallinae*, other life history traits including longevity, fledgling success, as well as various measures of reproductive success such as the number of nests, eggs laid and hatched young were examined. To examine whether there is a selection for body weight, the association between genome-wide heterozygosity and body weight was examined in both sexes. The association between genome-wide heterozygosity and the infection status with *Trichomonas gallinae* was examined in all subpopulations. However, for other life history traits including longevity, fledgling success, weight, as well as various measures of reproductive success such as the number of nests, eggs laid and hatched young were examined only in IAA because access to these data for mainland subpopulation was not possible due to nests in high trees being inaccessible. The SNP markers were divided into two groups; one group consisted of immune-related variants as adaptive genes that were within 10,000 base pairs of an *a priori* defined immune gene, and the second group consisted of SNPs that fell outside such regions (i.e. non-immune genes) as a reference group.

5.3.1 Estimated resistance to Trichomonosis across five subpopulations in non-immune genes

The individual genome-wide heterozygosity of the non-immune genes of 89 adult birds across all five subpopulations, including IAA, PW, CO, PL and BO, was regressed against the percentage of times that an individual was found to be infected with *T. gallinae* on

screening; this found that birds with a high genetic diversity are significantly less infected (Regression: $F_{1,87} = 11.15$, p = 0.001, R-Sq = 11.4%), (Appendix 9), (Figure. 5.2).

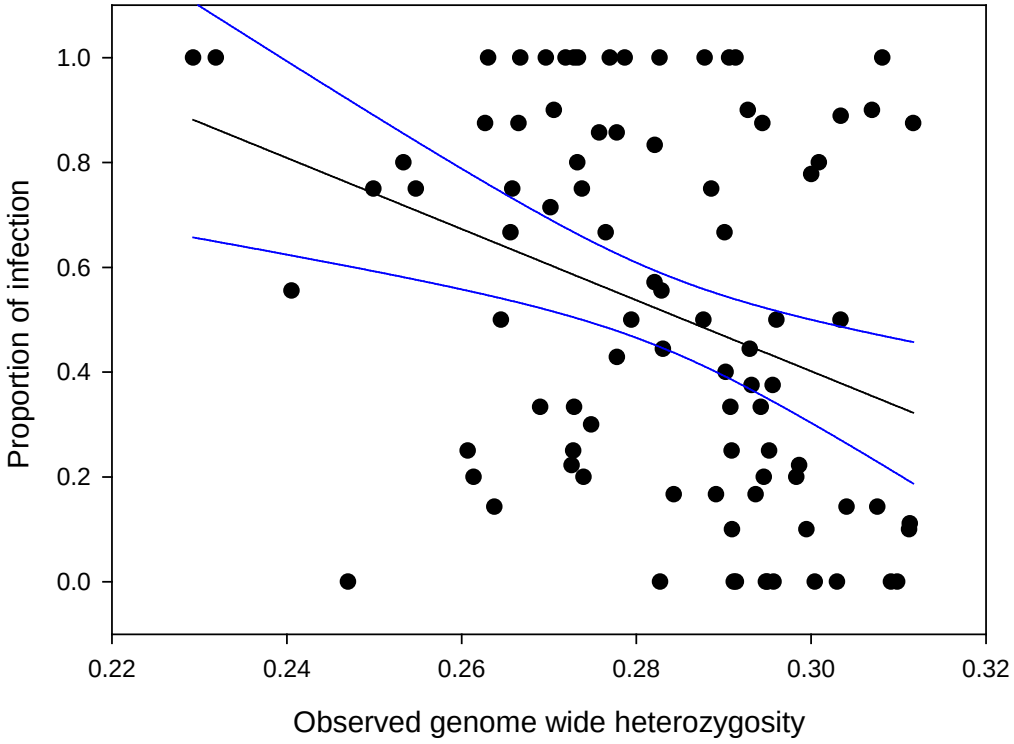

Figure 5.2 Linear regression of the observed heterozygosity of the non-immune genes of 89 adult birds from across all five subpopulations against the percentage of times that an individual is infected with *T. gallinae*. Shown are the regression line and the 95% confidence interval of the regression line (in blue).

5.3.2 Estimated resistance to Trichomonosis across five subpopulations in immune genes

The individual genome-wide heterozygosity of the immune genes of 89 adult birds across all five subpopulations, including IA, PW, CO, PL and BO, was regressed against the percentage of times that an individual was infected with *T. gallinae*. This found that birds with a high gene diversity in immune genes are significantly less infected (Regression: $F_{1,87} = 10.61$, p = 0.002, R-Sq = 10.9%), (Appendix 10), (Figure 5.3).

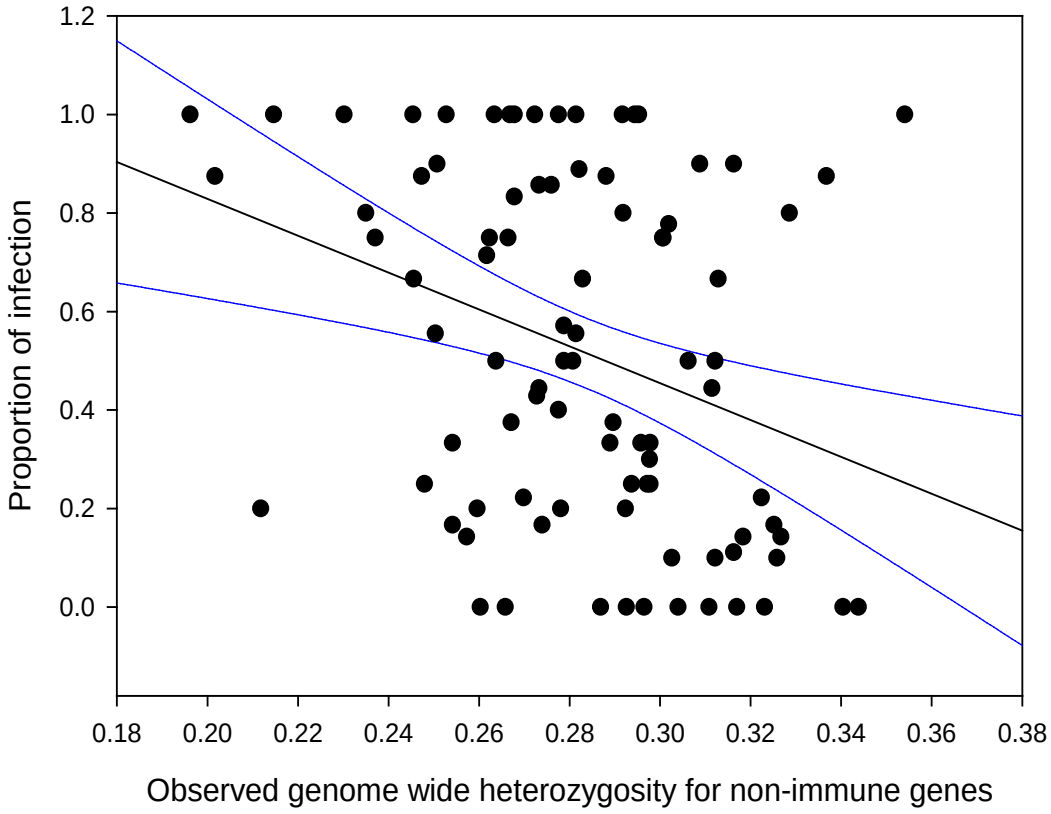

Figure 5.3 Linear regression of the observed heterozygosity of the immune genes of 89 adult birds from across all five subpopulations against the percentage of times that an individual tested positive for *T. gallinae*.

5.3.3 Estimated resistance to Trichomonosis for non-immune genes in the IAA subpopulation

The individual genome-wide heterozygosity of the non-immune genes of 47 birds from the IAA subpopulation was regressed against the percentage of times that an individual tested positive for *T. gallinae*; this found that birds with a high diversity in non-immune genes were significantly less infected (Regression: $F_{1,45} = 5.18$, $p = 0.028$, R-Sq = 10.3%), (Appendix 11), (Figure 5.4).

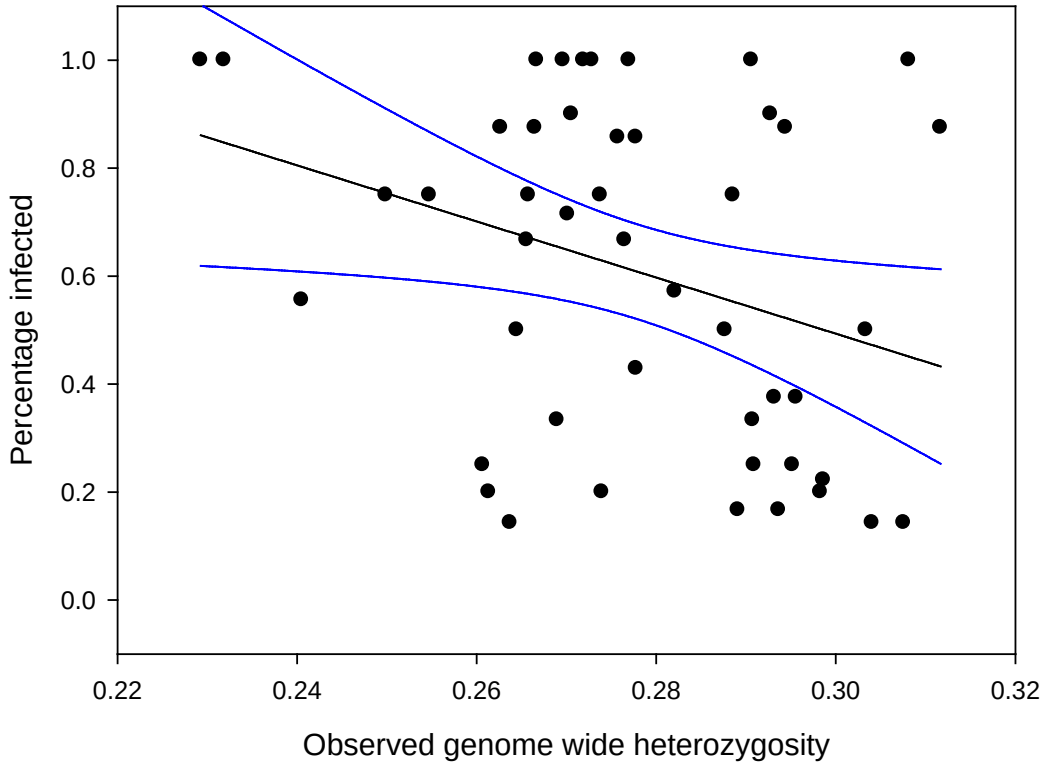

Figure 5.4 Linear regression of the observed heterozygosity of the non-immune genes of 47 adult birds in the IAA subpopulation against the percentage of times that an individual tested positive for *T. gallinae*.

5.3.4 Assessment of the relationship between chronic/clearance infection and genome-wide heterozygosity for non-immune genes

I then examined the difference in genome-wide heterozygosity between a chronically infected group of birds that was found positive to *T. gallinae* infection in at least one of their last three screenings and a group that was found to be negative to infection in the last three screenings. The test was run twice, for non-immune and immune genes.

The individual genome-wide heterozygosity of the non-immune genes of 89 birds across five subpopulations, including IAA, PW, CO, PL and BO, was tested to examine the difference between chronically infected birds and birds that recovered from infection. A two-sample t-test found a significant difference between the two groups ($t = 3.02$, d.f. = 60, $p = 0.004$), with the recovered group (N=29) having a higher genome-wide heterozygosity (Mean±SE = 0.2904 ± 0.0030) against Mean±SE = 0.2791 ± 0.0023) for chronically infected birds (N=60) (Figure 5.5).

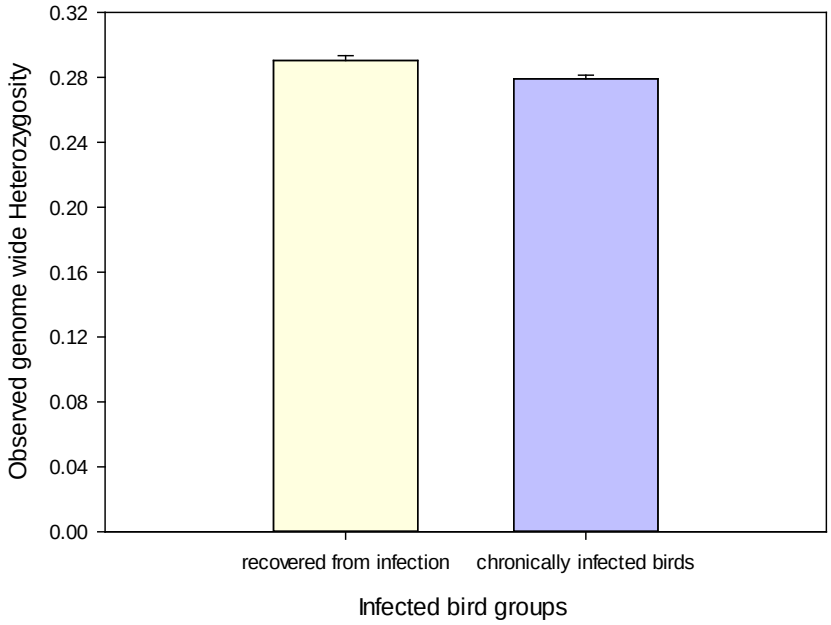

Figure 5.5 Bar chart showing the mean and standard error of the genome-wide heterozygosity of the non-immune genes of two groups of birds: birds that were found negative to infection in the last three screening times (Yellow bar) and birds that were found positive in at least one of the last three screening times (Blue bar).

5.3.5 Assessment of the relationship between chronic/clearance infection and genome-wide heterozygosity for immune genes

The individual genome-wide heterozygosity of the immune genes of 89 birds across five subpopulations, including IAA, PW, CO, PL and BO, was tested to examine the difference between chronically infected birds and birds that recovered from infection. A two-sample t-test showed a significant difference between the groups ($t = 2.75$, d.f. = 59, $p = 0.008$), with the recovered group having a higher genome-wide heterozygosity (Mean±SE = 0.2953 ± 0.0053) against Mean±SE = 0.2770 ± 0.0040) for chronically infected birds (Figure 5.6).

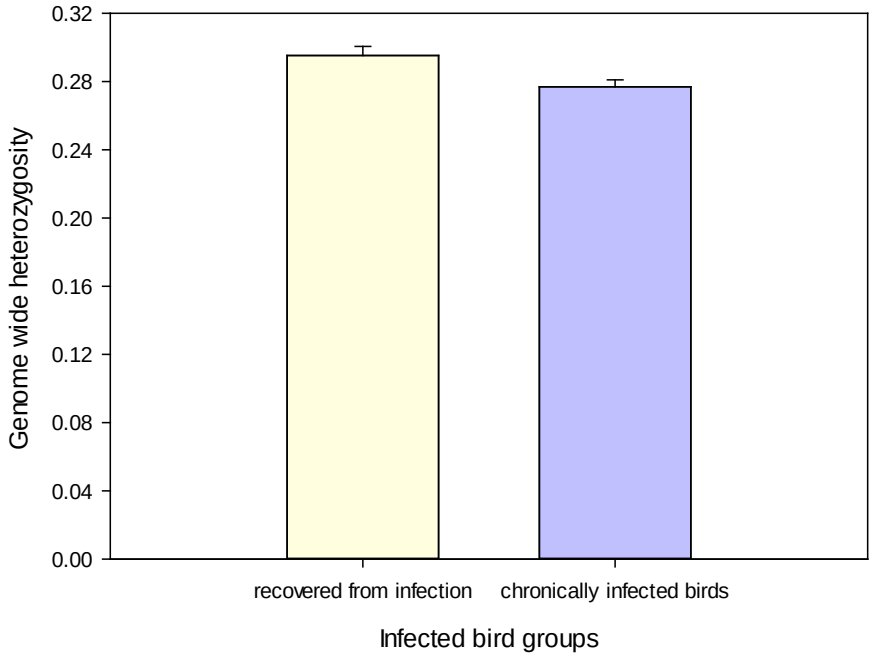

Figure 5.6 Bar chart showing the mean and standard error of the genome-wide heterozygosity of the immune genes of two groups of birds: birds that were found negative to infection in the last three screenings (Yellow bar) and birds that were found positive in at least one of the last three screenings (Blue bar).

5.3.6 Assessment of the relationship between individual fitness-related reproductive traits and genome-wide heterozygosity in the IAA subpopulation

Fitness-related traits, including the number of nests, number of laid eggs, number of fertile eggs, number of infertile eggs and number of eggs hatched as squabs, were regressed against the genome-wide heterozygosity for non-immune and immune genes. None of these traits showed a significant relationship with genome-wide heterozygosity, for either non-immune or immune genes (Tables 5.1 and 5.2).

Table 5.1 Analysis of Variance (ANOVA) of the linear regression of the non-immune genes of the IAA subpopulation against a number of fitness-related traits, including longevity, weight, number of nests, number of eggs laid , number of fertile eggs, number of infertile eggs, number of eggs hatched and number of fledged squabs.

Factor	R-Sq %	d.f.	F	P
Number of nests	0.0	1, 56	0.02	0.888
Number of eggs	0.0	1, 56	0.01	0.942
Number of infertile eggs	0.4	1, 56	0.22	0.641
Number of fertile eggs	0.5	1, 56	0.28	0.599
Number of hatched squabs	0.8	1, 56	0.45	0.506

Table 5.2 ANOVA of the linear regression of the immune genes of the IAA subpopulation against a number of fitness-related traits, including longevity, weight, number of nests, number of eggs laid , number of fertile eggs, number of infertile eggs, number of eggs hatched and number of fledged squabs.

Factor	R-Sq %	d.f.	F	P
Number of nests	0.6	1, 56	0.33	0.568
Number of eggs	0.8	1, 56	0.45	0.505
Number of infertile eggs	2.0	1, 56	1.15	0.289
Number of fertile eggs	0.0	1, 56	0.00	0.952
Number of hatched squabs	0.2	1, 56	0.14	0.709

5.3.7 Analysis of variation in life-history traits using a Genome-Wide Association Study (GWAS)

Above, the relationship between individual fitness-related reproductive traits and genome-wide heterozygosity was not found to be significant. This may indicate that the variation of these traits is significantly affected by a small number of genes with major effect, rather than being polygenic. In order to identify potential genes related to variation in this traits, a genome-wide association study (GWAS) was performed using the PLINK programme to identify the genomic region that can explain the variation in these traits among individuales pink pigeons from IAA. The GWAS failed to identify genomic regions associated to increased resistance or susceptibility to *T. gallinae*. However, it identified a genomic region associated with variation in egg-laying traits among 24 female pink pigeons from IAA. Only one marker scaffold188:6065267 (later renamed as Chromosome 813:6065267 for processing with the qqman (an R package for visualizing GWAS results manhattan plots) was statistically significant after Bonferroni adjustment with a probability of p=0.05 (Figure 5.7). The sequence (200 bp) around this statistically significant associated marker was blasted against the rock pigeon (*Columba livia*) genome reference and mapped to a position approximately 0.4 Mb from the progesterone receptor gene. However SNP markers in closer proximity to this progesterone receptor gene were found to not be significantly associated after multiple testing adjustments.

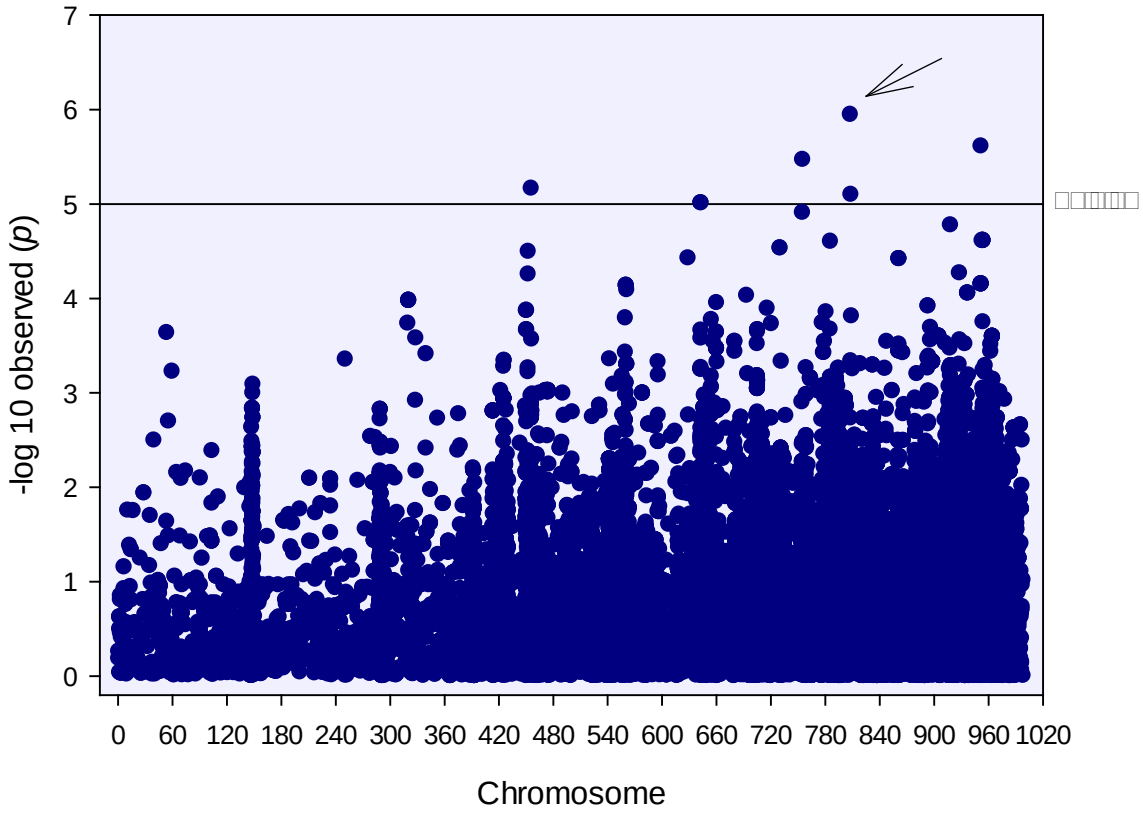

Figure 5.7 Manhattan plot showing the significance of association with the eggs-laid/day for SNPs in 24 females pink pigeon. Arrow indicates to the marker scaffold 188:6065267. This marker is found approximately 0.4 Mb away from the progesterone receptor gene (PRG).

5.3.8 Assessment of the relationship between body weight and genome-wide heterozygosity

Regressed fitness-related traits against genome-wide heterozygosity for non-immune and immune genes showed that number of birds with a high genome-wide heterozygosity is significantly higher among birds with higher body weight values (Regression: $F_{1,36} = 8.60$, p = 0.006, R-Sq = 19.3%; $F_{1,36} = 16.02$, p = 0.000, R-Sq = 30.8%, respectively), (Figure 5.8 and 5.9).

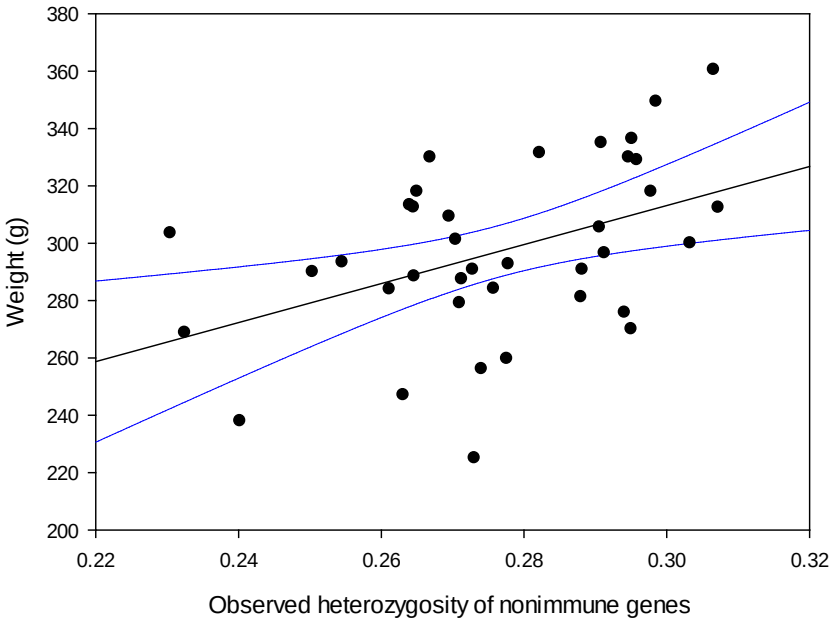

Figure 5.8 Linear regression of the observed genome-wide heterozygosity of the non-immune genes of 38 adult birds in the IAA subpopulation against their weight (g).

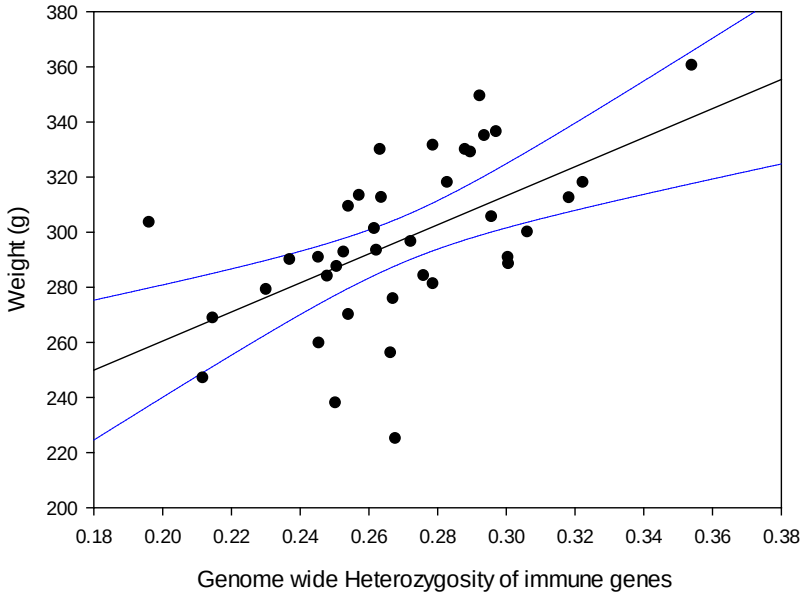

Figure 5.9 Linear regression of the observed genome-wide heterozygosity of the immune genes of 38 adult birds in the IAA subpopulation against their weight (g).

5.3.9 Assessment of the relationship between survivorship and genome-wide heterozygosity

The regressed longevity observation data of 60 birds from IAA against genome-wide heterozygosity for non-immune genes and immune genes showed that the number of birds with a high genome-wide heterozygosity was significantly higher only for non-immune genes among birds with a higher longevity (Regression: $F_{1,58} = 4.23$, $p = 0.044$, R-Sq = 6.8%; $F_{1,58} = 1.92$, $p = 0.171$, R-Sq = 3.2%, respectively) (Figure 5.10).

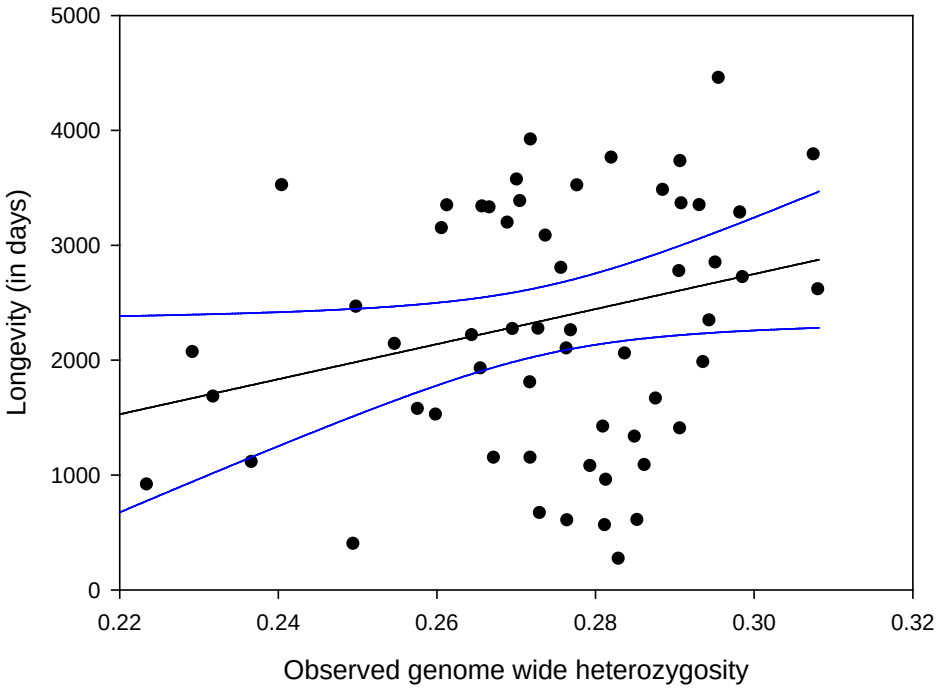

Figure 5.10 Linear regression of the observed heterozygosity of the non-immune genes of 60 adult birds from the IAA subpopulation against their longevity (days).

5.3.10 Assessment of the relationship between fledging and genome-wide heterozygosity

The genome-wide heterozygosity was examined in two groups: fledged birds (19 birds) and birds that died before fledgling (28 birds) in the IAA subpopulation. The GLM was run with genome-wide heterozygosity, with the fledged and non-fledged nested within families and families as factors. Families differ significantly in heterozygosity, but the rate of survival (dead or alive) did not explain the variation in heterozygosity. In other words, dead offspring are no more homozygous than those that fledged when analysed within families, although significant variation in fledging success is explained by families (Table 5.3 and Figure 5.11).

Table 5.3 GLM for calculating difference in genome-wide heterozygosity between fledgling and non-fledgling birds within families. The fledgling and non-fledgling young are nested within families and families as random factors.

Factor	Df	MS	F	p
Survival (Family)	8	0.0000403	0.32	0.951
Family	7	0.0010175	8.17	<0.001
Error	31	0.0001245		
Total	46			

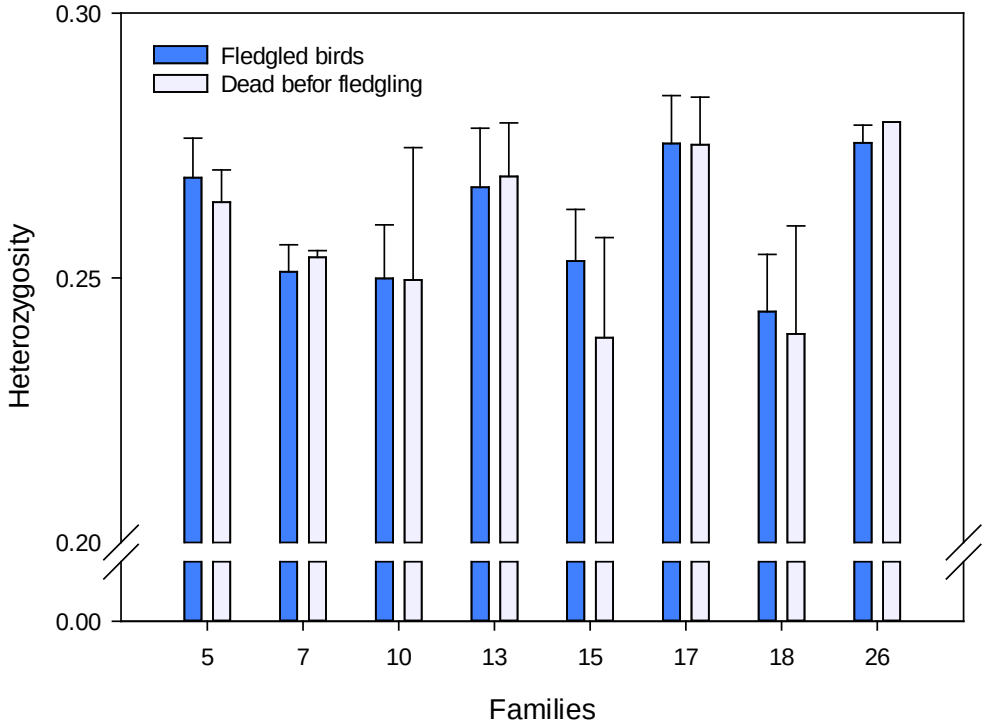

Figure 5.11 Bar chart showing the mean and standard error of genome-wide heterozygosity of two groups of squabs in the IAA subpopulation: Surviving squabs groups and squabs that died before fledgling.

However, when the difference between individual genome-wide heterozygosity was examined between fledged birds (140 birds) and birds that died before fledgling (35 birds) across five subpopulations, including IAA, PW, CO, PL and BO for both non-immune and immune genes, the difference was significant between two groups for non-immune genes (Kruskal–Wallis test: H = 10.10, d.f. = 1; p = 0.001), (fledged birds: Q1, Median and Q3 = 0.265, 0.279 and 0 291; and birds that died before fledging: 0. 0.2520, 0.2690 and 0.2770, respectively), and was found also significant between groups for immune genes (Kruskal–Wallis test: H = 6.44, d.f. = 1; p = 0.011), (Q1, Median and Q3 = 0.254, 0.278 and 0.298; 0.243, 0.257 and 0.284, respectively), (Figure 5.12 a-b).

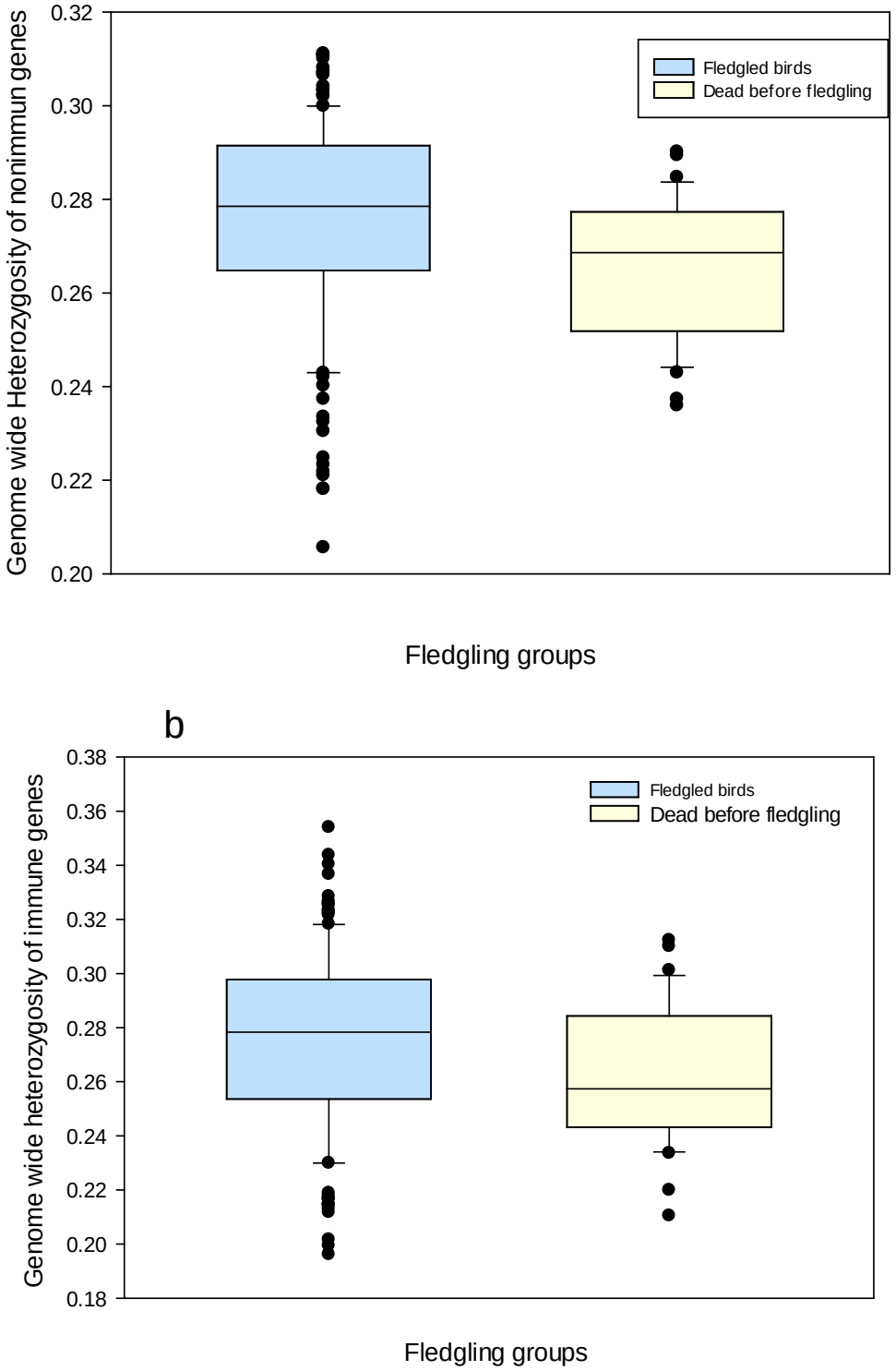

Figure 5.12 a-b Box plot showing the median and the first and third quartiles of the heterozygosity of fledged birds (Blue) and birds that died before fledging (Yellow). a) for non-immune genes, b) for immune genes.

5.3.11 Differences in genome-wide heterozygosity between male and females

The difference in genome-wide heterozygosity was examined between sexes in 61 females and 63 males across five subpopulations for both non-immune (44357 SNPs) and immune genes (1484 SNPs). The difference was not significant between females and males in non-immune genes (Q1, Median and Q3 = 0.26209, 0.27758 and 0.29182; 0.27102, 0.28220 and 0.29423, respectively) (Kruskal–Wallis test: H = 1.58, d.f. = 1; p = 0.209). However, a significant difference was revealed between females and males in immune genes (Q1, Median and Q3 = 0.24641, 0.26776 and 0.29808; 0.26708, 0.28808 and 0.30055, respectively) (Kruskal–Wallis test: H = 5.32, d.f. = 1; p = 0.021), (Figure 5.13).

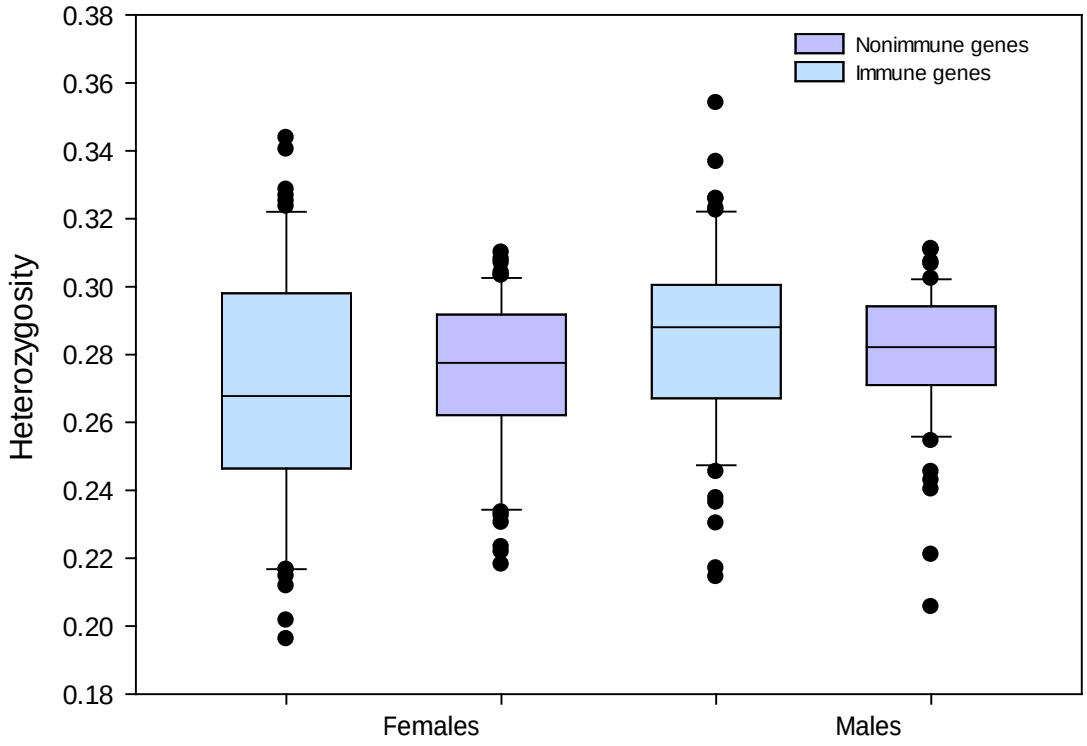

Figure 5.13 Box plot showing the difference in genome-wide heterozygosity between sexes, 61 females and 63 males, across five subpopulations for immune and non-immune genes.

5.3.12 Relationships between sex, body weight and genome wide heterozygosity

In this study, heterozygosity for non-immune and immune genes was found to be higher in birds with a higher weight (section 5.3.8). In addition, heterozygosity differed

significantly between males and females (section 5.3.11). In this analysis, I examined whether variation in bodyweight of 38 adult pink pigeons (16 females and 22 males) of the IAA subpopulation was explained by differences between the sexes and the level of heterozygosity at non-immune and immune genes. Interestingly, there was significant differences in bodyweight depending on the level of heterozygosity, but judging by the significant 'Sex' x 'Heterozygosity' interaction observed in the non-immune genes, this effect differed between the sexes (Tables 5.4 and 5.5). When bodyweight regressed against heterozygosity for males and females separately (Figure 5.14 a-b), the body weight and genome wide heterozygosity of immune genes and non-immune genes of males and females differed significantly between the sexes. Whereas males show a strong and highly significant positive relationship between the level of heterozygosity at immune genes ($F_{1,20}$=14.44, p=0.001 and R-Sq=39%) and non-immune genes ($F_{1,20}$=17.85, p<0.001 and R-Sq=44.5%). No significant relationship existed for females (immune genes: $F_{1,14}$=0.03, p=0.874 and R-Sq =0.0% and non-immune genes: $F_{1,14}$=0.97, p=0.341 and R-Sq=0.0%). These analyses indicates that with heterozygosity explains 44.5% and 39.0% of the variation in bodyweight in males when their weight was regressed against genome wide heterozygosity of non-immune genes and immune genes, respectively. In contrast, 0.0% of the variation in bodyweight of females was explained by heterozygosity both in the analysis of the immune and non-immune genes (Figure 5.14 a-b). This analysis suggests that if natural selection acts on increased bodyweight in the pink pigeon, it would help to maintain genome wide heterozygosity in males, whereas such selection has no effect on the gene diversity of females.

Table 5.4 General Linear Model (GLM) for difference between sexes in body weight with heterozygosity of non-immune genes as a covariate, crossed with sex as fixed factor.

Factor	d.f.	MS	F	P
Heterozygosity non-immune genes	1	3483.1	5.49	0.025
Sex	1	4021.9	6.34	0.017
Sex x Heterozygosity non-immune genes	1	4348.1	6.85	0.013
Error	34	634.7		
Total	37			

Table 5.5 General Linear Model (GLM) for difference between sexes in body weight with heterozygosity of immune genes as a covariate, crossed with sex as fixed factor.

Factor	d.f.	MS	F	P
Heterozygosity immune genes	1	5977.4	9.37	0.004
Sex	1	1023.1	1.60	0.214
Sex x Heterozygosity immune genes	1	1179.4	1.85	0.183
Error	34	637.9		
Total	37			

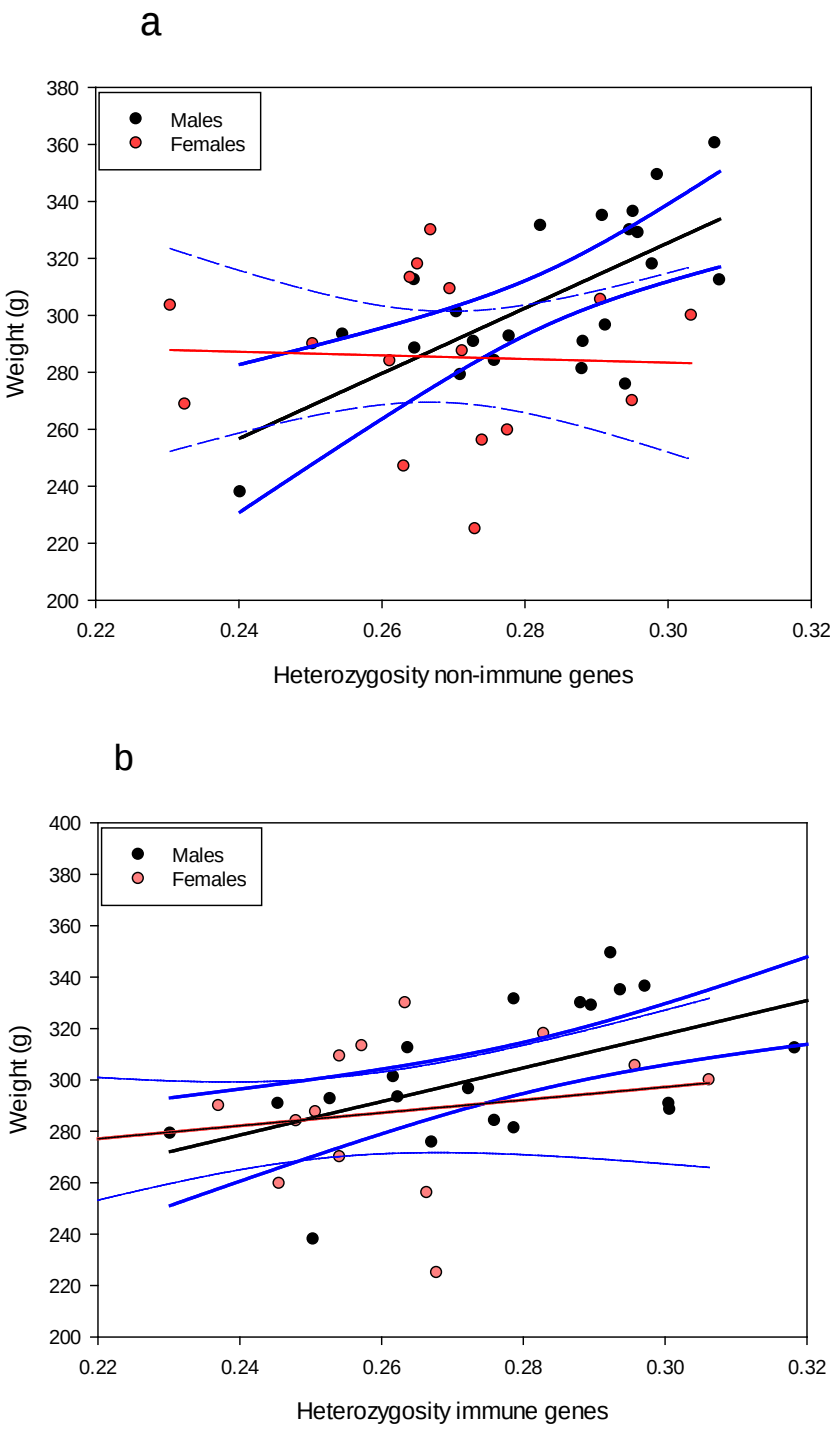

Figure 5.14 a-b Relationship between body weight and genome wide heterozygosity of a) non-immune genes, and b) immune genes of males and females. Males showed a strong and highly significant positive relationship between the level of gene diversity at immune genes ($F_{1,20}$=14.44, p=0.001) and non-immune genes ($F_{1,20}$=17.85, p<0.001), with heterozygosity explaining 44.5% and 39.0% of the variation in bodyweight, respectively. In contrast, no such relationship exists for females (immune genes: $F_{1,14}$=0.03, p=0.874 and R-Sq =0.0% and non-immune genes: $F_{1,14}$=0.97, p=0.341 and R-Sq =0.0%).

5.4 Discussion

This study analysed the genome wide heterozygosity of the Mauritian Pink Pigeon (*Nesoenas mayeri*) (Prévost, 1843) using restriction-site associated DNA sequencing (RAD-seq). A total of 175 birds were genotyped for 45,841 single nucleotide polymorphisms (SNPs), and the association between genome-wide heterozygosity and fitness-related traits was examined. The examined traits included the infection status with *Trichomonas gallinae*, longevity, fledgling success, as well as various measures of reproductive success such as the number of nests, eggs laid and hatched young. The SNPs were divided into two groups; one group consisted of immune related variants that were within 100,000 base pairs of an *a priori* defined immune gene, and the second group consisted of SNPs that fell outside such regions (i.e. non-immune genes). In addition, in order to avoid confounding effects caused by the Z-chromosome, SNPs on this chromosome were omitted from the analyses.

5.4.1 Association between genome-wide heterozygosity and susceptibility to parasitic infection

Variation in fitness-related traits between individuals can be explained by the variation in the genome-wide heterozygosity, as is observed in several other studies (e. g. Ruiz-López *et al.,* 2012). The first objective of this study sought to examine the relationship between infections with *T. gallinae* and genome-wide heterozygosity in 89 adult birds across five subpopulations. A significant negative relationship between the proportion of infections and the individual genome-wide heterozygosity was found, and this relationship was significant in both immune and non-immune genes. This result was furthermore supported by examining the genome-wide heterozygosity between chronically infected group of birds that were found positive to *T. gallinae* infection in at least one of the last three screenings and the group that was found negative to infection in the last three screenings (resistant birds). Interestingly, the genome-wide heterozygosity was highest in the resistant group of birds that cleared the infection, which indicates that the prevalence of infectious disease in the free-living pink pigeon population is linked to genetic factors. With small effective population sizes for pink pigeons (*Ne* < 10 birds) (Chapter 4), it is unlikely for natural selection to act to change allelic frequencies. However, changing allele frequencies can occur due to genetic drift

in small populations. The assessment of loss genetic variation in Chapter 4 showed a significant genetic drift in pink pigeons which indicate that genetic drift "overpowers selection" (Lande and Barrowclough, 1987). Although other forms of balancing selection cannot be excluded, linear regression analysis in Sections 5.3.1 and 5.3.2 showed a significant negative relationship between genome-wide genetic variation and the proportion of infection with *T. gallinae* in which birds with a high genetic diversity are significantly less infected, which is most consistent with over-dominant effects (i.e. heterozygote superiority). Arguably, this result cannot be explained by dominance effects, unless relatively homozygous individuals that express recessive mutations homozygous state are weak and less able to resist an infection. This study is amongst few that have proved the link between parasitic infection and host genetic variations in the free-living population using genomic data generated using genomic approaches (e.g. Hoffman *et al.,* 2014).

5.4.2 Relationship between reproductive traits and genome-wide heterozygosity in the IAA subpopulation

The second objective of this chapter was to examine whether gene diversity explains variation in reproductive traits, including the number of nests, number of laid eggs, number of fertile eggs, number of infertile eggs and number of eggs hatched as squabs. This analysis focused on the IAA subpopulation because it is the only subpopulation with detailed reproductive data. Fitness-related reproductive traits in the IAA subpopulation were studied for 12 months in 2004 by Bunbury (2006), who found a reduction in egg fertility and hatchability and the number of fledglings over this period. The current study used these data to perform a regression analysis, and these reproductive traits were regressed against genome-wide heterozygosity of both non-immune and immune genes. However, none of these traits showed a significant relationship with genome-wide heterozygosity for either group of genes. This suggests that inbreeding depression of reproductive traits is caused by deleterious effects at a small number of genes with large effect (e. g. Kaeuffer *et al.,* 2008). Genetic drift may have fixed recessive deleterious alleles at a small number of these loci, and the variation at these loci could explain the differences in reproductive fitness (Ashwell *et al.,* 2004). In order to examine this hypothesis in further detail, I applied a genome wide association study (GWAS) with

the aim to identify the particular genomic regions responsible for loss in fitness in reproductive success.

5.4.3 Analysis of variation in life-history traits using a Genome-Wide Association Study (GWAS)

In theory, the variation in fitness-related traits between individuals within population can be related general effect of genome wide heterozygosity, in which case the variation of these traits is partially explained by variation between individuals in their genome wide heterozygosity (Allendorf *et al.,* 2012). In the other hand, the variation in fitness-related traits can be related to local effects in which case variation in the alleles of one or few genes can explain the variation in fitness-related trait among individuals in the population (Allendorf *et al.,* 2012).

The GWAS failed to identify genomic regions associated to increased resistance or susceptibility to *T. gallinae*. This is because the infection rate across adult birds was normally distributed; hence, there were insufficient numbers of individuals testing constantly negative or positive. We showed in sections 5.3.1 and 5.3.2 that there was a significant negative relationship between genome wide genetic variation and proportion of infection with *T. gallinae* in which birds with a high genetic diversity are significantly less infected. This result is consistent with the considerable difference in severity within the population of pink pigeons who developed *T. galinae* infection, with some individuals testing consistently negative or positive across ten screenings, or having recovered/infected after one or more screenings, which can be explained by polygenic effects. In addition, environmental factors, pathogen genes and potential different *T. gallinae* strains may interact to cause this severity. Hence, it was challenge to perform GWAS for infection with *T. galinae*." In this thesis, I relied on a population-based approach to conduct GWAS. However, a case-control (family-based) approach can improve a GWAS to be more effective through the use of Family-pedigree data to detect fitness-related traits, but these data were not available for this thesis

The association between genome wide heterozygosity and number of eggs laid was not significant (Section 5.3.6), which suggests that inbreeding depression on this trait has a local effect. Using a genome-wide association study (GWAS), the significant sequence of 200 bp long related to variation in the egg-laying was mapped to a position of

approximately 0.24 Mb and 0.4 Mb from the progesterone receptor gene (PRG). This gene has been found to play an important role in ovulation in mammals and birds that grow oocytes which are captured inside follicles and prevent ovulation (Zhu et al., 2015; Conneely, 2010). Although detected SNP was associated with fecundity (egg laying) (Section 5.3.7). This SNP is however unlikely to be the causal SNP, given that nearby SNPs do not show a significant association with this trait. Future GWAS on this and other life history traits will require more additional samples.

5.4.4 Assessment of the relationship between body weight and genome-wide heterozygosity in the IAA subpopulation

A reduction in heterozygosity can negatively affect body condition, such as weight and height (e. g. Fareed and Afzal, 2014). In the case of the pink pigeon, the weight data of 38 birds in the IAA subpopulation were regressed against the genome-wide heterozygosity for non-immune and immune genes and found that genome-wide heterozygosity at both groups of genes was higher in birds with a higher weight. This is additional evidence that the pink pigeon population is suffering from inbreeding depression and that the rapid loss of heterozygosity due to the small effective population size and strong population fragmentation (see Chapter 4) pose a serious threat to the survival of this species. A reduction in body condition and body weight, especially during early development, can affect the growth, fecundity and immunocompetence of future generations (Lindström, 1999). This implies that inbreeding depression could have transgenerational effects, which further emphasises the need for immediate conservation interventions.

5.4.5 Assessment of the relationship between survivorship and genome-wide heterozygosity

The low hatchability and low fledgling numbers in the pink pigeon population were due to high mortality of squabs (Bunbury, 2006), which might be explained by inbreeding depression negatively impacting the survival rate. To test this hypothesis, the genome-wide heterozygosity was examined in relation to the longevity of adult birds and fledgling as indicators of squabs' survival. First, the longevity observation data of 60 birds were regressed against the genome-wide heterozygosity in both non-immune and immune genes. This result showed that the variance in longevity between birds is

significantly related to the level of genome-wide heterozygosity and that longevity is higher in birds that possessed higher heterozygosity. Most studies on the relationship between the level of heterozygosity and longevity have been conducted on domestic or captive populations. For example, it was found that longevity in a captive wolf population (*Canis lupus*) (Laikre and Ryman, 1991) and Landrace sows (Casellas *et al.,* 2008) was negatively affected by breeding. Second, I examined whether the genome-wide heterozygosity differed between fledged birds and squabs that died before fledging, analysing 18 families of full sibs in the IAA subpopulation. Heterozygosity differed significantly between families, but not between the survival categories. Nevertheless, a significant difference was detected between the successfully fledging and squabs that failed to fledge when it was analysed across the total population. This analysis was based on more birds, resulting in a stronger statistical power. This is additional evidence that a reduction in squab survival in the pink pigeon population is due to reduction in genome wide heterozygosity and inbreeding depression.

5.4.6 Examined difference in genome-wide heterozygosity between male and females

The genome wide heterozygosity was compared between 61 adult females and 63 adult males across five subpopulations which revealed that males showed a significantly higher heterozygosity at both immune and non-immune genes. This was surprising given that the analyses presented in Chapters 2 and 3 showed that both the survival and proportion of infection with *T. gallinae* did not differ significantly between adult males and females. Given that I aimed to remove SNPs present on the Z-chromosome and tried to use only autosomal SNPs, it is unlikely that the analysis is biased by heterogametic variation. This would decrease the heterozygosity of the heterogametic sex (i.e. the female birds), and as such, it could explain our results. Another possible explanation for this might be that that homozygous individuals may be more likely to contract an infection as squab, but can develop rapid resistance to infection in later life as an adult (Gay *et al.,* 2006). Further analysis is needed to answer the question about– what can explain the difference in heterozygosity between males and females?

5.4.7 Examine the relationships between sex, body weight and genome wide heterozygosity

I revealed a significant relationship between bodyweight of adult pigeons and their level of genome wide heterozygosity, and this pattern was observed both in the immune genes and non-immune genes. Males showed a strong and highly significant positive relationship between the level of gene diversity at both the immune genes and non-immune genes, and genetic variation at these genes explained between 39.0% and 44.5% of the variation in this important life history trait. Perhaps even more remarkably, no such relationship exists for females, with gene diversity explaining absolutely no variation in their bodyweight.

This observation also answers the addressed question about the genome wide heterozygosity that differed significantly between the 61 adult females and 63 adult males of the five subpopulations. I found that males showed a significantly higher heterozygosity at both immune and non-immune genes, which was completely unexpected given that the analysed birds came from the same current samples and subpopulations. Because this subpopulation is panmictic and samples were taken randomly, no differences in gene diversity between the sexes are expected unless the pressure of natural selection acting on heterozygosity differs significantly between the sexes. Furthermore, analyses presented in Chapters 2 and 3 showed that both the survival and proportion of infection with *T. gallinae* did not differ significantly between adult males and females, which suggests that pressure of natural selection acting on disease resistance and survival does not differ between the sexes. The findings can explain the difference in heterozygosity between males and females (see section 5.4.7). I propose that given the strong positive correlation between heterozygosity and body weight strictly in males, selection for body weight supports to maintain levels of genome wide heterozygosity in males but not in females. As a consequence of natural selection acting on body weight and the extremely strong positive correlation between male body weight and genome wide heterozygosity, males suffer less from the effects of genetic drift and inbreeding than females. Therefore, males maintain a higher level of gene diversity than females, even though they are in the same population and subjected to the same level of inbreeding. Furthermore, this may explain the male biased sex ratio, and possibly also the reduction in lifetime fecundity in females in this endangered population (Concannon, 2014). It might be that this finding is the first example of sex specific differences in the rate of inbreeding.

5.5 Implications for the conservation genetics of the pink pigeon population

One of the current aims of the conservation management of the pink pigeon population is to increase the number in the population to 600 individuals. Increasing the population size can secure the population from the risk of extinction. However, a number of issues have been found to affect the growth of the population, including infectious disease and reductions in fecundity and survival (Bunbury, 2006). In Chapter 4 of this thesis, it was found that a reduction in heterozygosity is due to a genetic drift which was accelerated by inbreeding. The current chapter provides evidence that infection with a parasitic disease caused by *T. gallinae* and a reduction in fitness related to reproduction and survival are linked to genetic variation. The analyses presented here clearly demonstrate that inbreeding depression is a significant threat to the pink pigeon population, and I therefore recommend the translocation of birds from one subpopulation to another which would convert genetic differentiation that exists between subpopulation into genome wide heterozygosity. Given that genome wide heterozygosity explains significant variation several fitness-related traits, conservation efforts should be aimed at increasing such gene diversity. In addition, the release of birds from different captive pink pigeon populations still present in zoos around the world could help to genetically supplement the wild pink pigeon populations. Genetic supplementation can be particularly efficient when prior genetic screening can identify genetic variants that are associated to increased fitness. For example, this study identified SNPs that are significantly associated with increased reproductive success nearby the progesterone receptor gene (PGR), and the reintroduction of birds with these genetic variants may significantly benefit the fitness in the wild Pink Pigeon population.

Findings in this chapter provide the following insights for future work on conservation management. First, for infectious disease, it is possible for a large population size to improve genetic resistance to parasitic diseases due to natural selection. In the case of pink pigeons, the population size is too small, which makes it difficult for species to select against infection. However, the results through this chapter showed a variety in the genome-wide heterozygosity, in which birds that have a higher heterozygosity represent birds that might gain gene/genes necessary for infection resistance. These birds and their progeny have been identified and pairing management is possible, especially in the case of establishing new subpopulations. Host-parasite coevolution is

recommended to understand the co-evolutionary adaption race between pink pigeons and *T. gallinae*. Currently, genomic data are available for both species, where colleagues, (Alrefaie, et al, unpublished work) have completed sequencing of the whole genome of *T. gallinae*. In doing so, insight into virulent strains can be realised and control strategies can be developed to stop or minimise infection.

Second, for reproduction success and survival, I recommend a further investigation to identify the gene(s) affecting these traits using quantitative trait loci (QTL) analyses or genome-wide association studies. Huge sequencing data sets of pink pigeons are provided by this study, which can be used to carry out such an investigation. In doing so, the fitness-related traits can be calculated for each individual in the entire wild population. The findings from these suggested analyses can improve the conservation management of pink pigeons with regard to increased genetic variability, increased resistance to diseases and minimised inbreeding depression.

5.6 Conclusion

The main aim of this chapter was to determine the association between genome-wide heterozygosity and fitness-related traits, including parasitic disease resistance and reproduction success and survival in the endangered pink pigeon population. The most significant result of this chapter is that genome-wide heterozygosity was highest in birds that were most resistant to *T. gallinae* infections. Secondly, differences in survival rate, including in longevity and fledgling success was significantly explained by variation in the genome-wide heterozygosity between birds. Intriguingly, reproductive success traits, including the number of nests, eggs and hatched young, appears to be affected by single or few genes, and we identified one genomic region nearby the progesterone receptor gene (PGR) that explain significant variation in reproductive success. Taken together, these findings implicate genetic factors as a major contributing factor to the inbreeding depression and the decline of the Pink Pigeon in the wild, and they call for urgent conservation genetic management interventions to save this species from extinction.

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Chapter 6
General Discussion

6.1 Introduction

Any large reduction in a wild population's size is a major conservation problem, and the primary cause of extinction. This is evident in the fact that extinction rates have been found to be higher in isolated and small populations than in large populations (Loehle and Eschenbach, 2012). There are two factors that can drive wild populations toward extinction: deterministic and stochastic factors (Caughley, 1994). Deterministic factors include negative human intervention, such as introducing invasive species (Blackburn *et al*., 2004) and habitat destruction (Manne *et al*., 1999). Stochasticity includes environmental, demographic and genetic factors, which can interact to increase the risk of extinction in small populations by reducing population size and increasing inbreeding (Frankham *et al*., 2010). Over generations, inbreeding leads to a loss of genetic variation, by decreasing heterozygosity and increasing homozygosity (recessive lethal alleles), which can accelerate genetic drift (loss of the genetic variation that allows adaptation to environmental conditions) (Allendorf *et al.,* 2012). Therefore, a small population size is more prone to fitness decline, including reductions in survival, reproductive success, individual health and resistance to infectious disease (Charlesworth and Charlesworth, 1999; Crnokrak and Roff, 1999).

The Mauritian pink pigeon (*Nesoenas mayeri*) is an endangered, recovered bottleneck species (Jones *et al*., 1992), growing from an estimated 10-20 birds in 1975 (Jones, 1987) to approximately 400 birds (currently) (Mauritius Wildlife Foundation, 2012). Through a captive breeding and release programme six further pink pigeon subpopulations have been established on Mauritius from the remnant population in Pigeon Wood, increasing the number of birds to around 400 over recent years (Mauritian Wildlife Foundation, 2012). These subpopulations include Bel Ombre, Plaine Lievre, Combo, Lower Black River Gorges and Pétrin, located in the Black River Gorges National Park (Mauritian Wildlife Foundation, 2012), which is the only suitable area of native forest remaining on the island (Jones, 1987). Another subpopulation was established on the Ile aux Aigrettes, a small islet off the eastern coast of Mauritius, from which the introduced mammalian predators had been eradicated and native lowland forest restored (Jones, 1987; Jones *et al*., 1992). The Lower Black River Gorges and Pétrin subpopulations were established

in 2007 and 2012, respectively (Mauritian Wildlife Foundation, 2012), and are not included in this thesis.

The primary aim of the conservation programme for the pink pigeon is to increase it's population size to a secure number to minimize the risk of it's extinction. The current aim is to reach a minimum number of 600 free living birds (Mauritian Wildlife Foundation, 2012). Previous studies of the pink pigeon have paid particular attention to those factors potentially limiting the growth of the population, including habitat destruction, predation, competition, lack of food sources, diseases, sex ratio bias and probable inbreeding depression (Swinnerton et al., 2004; Swinnerton *et al.,* 2005a; Swinnerton *et al.,* 2005b; Bunbury, 2006; Bunbury *et al.,* 2007; Bunbury *et al.,* 2008; Edmunds *et al.,* 2008). Together, these factors highlight the need to examine the causes behind the vulnerability of pink pigeons to these factors, and to further investigate the genetic structure of the pink pigeon population. This thesis focused on the following conservation related issues : a) sex ratio, to identify factors causing a deviation from parity; b) infection with *Trichomonas gallinae,* to determine the prevalence of infection in each individual over a two year period , and to examine which individuals, sexes and subpopulations are less likely to be infected; c) assess the genome-wide genetic variation, genetic drift, effective population size and degree of inbreeding; and d) the associations between genome-wide heterozygosity and the susceptibility to infectious disease and reduction in reproductive fitness.

6.2 Results

6.2.1 Sex ratio

The sex ratio in the adult pink pigeon population has been reported as male-biased in two previous studies (Bunbury, 2006; Concannon, 2014). However, neither study tested the deviation of the sex ratio from parity statistically. In addition, it was not clear what factors caused the biased ratio in the pink pigeon population, which raises the question of whether the persistent male bias in the adult pink pigeon population occurred as a result of a sex ratio skewed toward males at the primary or secondary levels, or because of the variations in longevity or mortality rates. In chapter 2, sex ratio was examined in the total population and each subpopulation, from 1987 to 2008, to determine whether the sex ratio was skewed significantly from parity in the pink pigeon population, and to identify the cause for the biased sex ratio in the adult birds.

This study found that the adult sex ratio has been biased towards males since 1995. Similar sex biases in other species have been explained by the differences in sex-specific mortality (Székely *et al.,* 2014) or survival rates (Maklakov and Lummaa, 2013). However, the present study found no evidence that either the adult mortality rate or longevity were sex-biased, when analysed across all of the subpopulations. In order to identify which subpopulations contributed to the biased sex ratio, the sex ratio was examined in each subpopulation over a 22 year period between 1987 and 2008. Two of the subpopulations (Pigeon Wood and Plaine Lievre) have been significantly biased towards males in different years, but no significant difference was found between the sexes in the annual mortality rate of those adults examined. Thus, it is likely that the biased sex ratio is driven by male bias at the primary or secondary levels.

The fledgling sex ratio was calculated, and was found to be biased toward males in several years in different subpopulations, especially in the founder Pigeon Wood subpopulation, which showed a male-biased in fledging rate in each of three years. To generate more evidence, the relationships between the numbers of fledgling males and females and the sex ratios in the adults were examined, and it was found that the number of fledgling males is strongly associated with the male-bias in adults. This finding suggests that the male-biased sex ratio in adult pink pigeons may be related to the deviation in the fledgling sex ratio, and could be due to a sex adjustment in the primary stage, or mortality in the embryos or squabs. In order to investigate hatchling sex ratio and to examine whether the hatchling sex ratio reflects the adult sex ratio, the sex ratio of 322 squabs in the Ile aux Aigrettes subpopulation, between 2004 and 2008, was examined. The sex ratio of the squabs was determined to be unbiased, suggesting a male-bias in fledgling. This also suggests a link between the sex ratios at the secondary and adult levels. The current finding of a male-biased fledging ratio can further increase the pink pigeon's extinction risk, and conservation management to reduce sex ratio bias is suggested.

6.2.2 Infection with *T. gallinae*

One of the most significant parasitic diseases that has been shown to affect the pink pigeon is Trichomonosis caused by the introduced protozoan parasite *Trichomonas gallinae* (Bunbury, 2006; Bunbury *et al.,* 2008). It is considered to be the main cause of death in nestlings (Bunbury, 2006). One aim of chapter 3 was to examine the variations

in the infection rates between the sexes and subpopulations, and to investigate the spatiotemporal incidence of infection. Another aim was to identify the birds that showed low probabilities of being infected, based on the average infection rates of each sex and subpopulation. The results showed that 86% of 418 screened birds tested positive for infection during at least one of the 10 bimonthly screenings. This may indicate a lack of immunity in the pink pigeon; however, a small number of individuals were found to be significantly less infected than by chance alone, and most of these birds belong to the Bel Ombre subpopulation. Interestingly, this subpopulation showed a high variation in the immune genes, compared to the non-immune genes, which may indicate some natural selection against infection (chapter 4). The percentage of screenings in which birds tested positive did not differ significantly between the males and females in this study, which does not support the prior studies reporting that the susceptibility to parasitic infection can be higher in males than in females, due to the effects of testosterone on immunocompetence (McCurdy *et al.,* 1998; Sheridan *et al.,* 2000). However, a study of the genome-wide heterozygosity among 61 females and 63 males revealed significant differences in 167 immune genes, with males having a higher amount of genome-wide heterozygosity (chapter 5).

The proportion of birds with the *T. gallinae* infection varies between subpopulations, and this variation was found to be statistically significant. When spatiotemporal variation was examined, the proportion of infection differed significantly between the subpopulations, and across time periods. This indicates that the *T. gallinae* infection rates in the five subpopulations are out of synchrony. This suggests that *T. gallinae* is either entirely more stochastic, and that temporal waves of infections percolate through the subpopulations, or that prevalence of *T. gallinae* is driven by social interaction.

In order to explore whether there is potential (immuno) genetic variation present in the pink pigeon population, all 418 individuals were analysed to determine the probability of each individual being less infected than by chance, based on subpopulation and sex infection average. A few of the birds (16; 3.8%) had a probability of being significantly less infected than expected by chance using $\alpha=0.05$, and two birds at Bel Ombre using a critical value $\alpha=0.01$.

Interesting additional findings were that the highest rates of *T. gallinae* infection occurred at Bel Ombre although this subpopulation also showed the highest longevity.

These findings are supported by results in chapter 4, which showed significant differences in the genome-wide heterozygosity between the immune and nonimmune genes, suggesting natural selection in Bel Ombre.

6.2.3 Genome wide genetic variation

Populations that are small in size are prone to losing genetic diversity through random genetic drift and inbreeding. In chapter 4, the important genetic parameters were calculated, including the observed heterozygosity (Ho), expected heterozygosity (He), potential genetic drift, inbreeding, population size and F statistics. The assessment of these parameters in the endangered pink pigeon population is of significant importance for the conservation management of this species.

In this thesis, RAD-seq techniques were used to generate 45841 SNPs that were used to genotype 175 pink pigeons in order to address these important research questions. The average observed heterozygosity (Ho) and expected heterozygosity (He) over all of the loci were similar across the subpopulations, suggesting that the subpopulations are approximating random mating, with no evidence of high inbreeding. However, rapid genetic drift was found from 2005 through to 2008 in three subpopulations (Ile aux Aigrettes, Combo and Bel Ombre). One potential contributing factor to the loss of genetic variation is a reduction in effective population size (Frankham *et al.,* 2010). The effective population sizes (Ne) were found to be reduced in this study, when calculated using the heterozygosity between two generations of parents and their offspring. A reduction in effective population size may increase inbreeding, which was found to be high in these offspring, leading to genetic drift in this subpopulation. Genetic drift may lead to a loss of the genetic variation required for adaptation to environmental conditions (Hedrick, 2001). In order to assess the adaptive genetic variation in pink pigeons, 167 immune genes were identified in 175 sequenced birds to genotype 1484 SNPs across the genome. The observed and expected heterozygosity and F_{IS} were calculated and compared with the non-immune genes. The aim was to determine whether these parameters differ between the immune and non-immune genes. The difference was significant in one subpopulation (Bel Ombre), with the F_{IS} being significantly lower in the immune genes than in the non-immune genes, indicating that the level of homozygous alleles is lower in the immune genes. This suggests that there

may be selection against infectious disease. Populations can develop immune resistance to infectious diseases after long-term exposure to pathogens (Staley and Bonneaud, 2015). However, this is conditional, based on the existence of those genes in some individuals in the population (Kilpatrick, 2006; Bonneaud *et al.,* 2011). In the case of the Bel Ombre subpopulation, which showed higher genetic variation in the immune genes, it has been found to contain more birds that were found to be less infected than other subpopulations. This suggests that the Bel Ombre subpopulation may have developed some resistance to *T. gallinae* infection; however, selection in this subpopulation may be small, and genetic drift may continue to affect these birds.

Additional effects of genetic drift can be deduced from an analysis of the genetic distance (F_{ST}) between the subpopulations. Genetic drift may expand the genetic divergence between subpopulations, especially between Ile aux Aigrettes and the other subpopulations, even with a positive significant relationship between the pairwise F_{ST} and geographical distances between the subpopulations. However, it is unlikely that the genetic divergence was caused by local adaptation, because the small population size may prevent selection. One possible explanation for this finding is that the genetic distance was likely driven by genetic drift.

6.2.4 Life history traits and resistance to infection

Inbreeding depression has been considered to be a major factor in increasing the extinction risk of a small population (Frankham *et al*., 2010).). It is a decline in the population fitness with regard to survival, reproductive success and individual health (Charlesworth and Charlesworth, 1999; Crnokrak and Roff, 1999), a decrease in the resistance to environmental stress (Reed *et al*., 2002) and an increase in the susceptibility to infection and disease (Trinkel *et al.,* 2011).

Previous studies have reported reductions in reproductive success, increased squabs mortality and continued infections with parasitic diseases in the pink pigeon population (Swinnerton *et al.,* 2004; Swinnerton *et al.,* 2005b; Bunbury, 2006; Bunbury *et al.,* 2008). Chapter 5 addressed the question of whether these conservation problems were related to genetic factors in the pink pigeon; therefore, the association between the genome-wide heterozygosity and variation in these traits was analysed using 45,841 single nucleotide polymorphisms (SNPs). A negative association was found between the

genome-wide heterozygosity and infection with *T. gallinae*, which was supported by the significant difference in the genome-wide heterozygosity that was examined between a chronically infected group and a group without infection. These findings can confirm that the prevalence of *T. gallinae* in the pink pigeon is associated with genetic variation in this species. Several researchers have performed studies to assess the relationship between the prevalence of pathogens and the host's genome-wide genetic variation in a natural wild population, using a huge number of genomic resources (e. g. Hoffman *et al.,* 2014) but our study is uniquely complete in the number of individuals investigated .

In assessing the relationship between survivorship and genome-wide genetic variation in the pink pigeon, longevity showed a strong positive relationship with the level of genome-wide heterozygosity. Studies on the relationships between longevity and heterozygosity in wild species are rare; however, this finding is consistent with previous studies conducted on domestic or captive populations, such as in a captive wolf population (*Canis lupus*) (Laikre and Ryman, 1991) and Landrace sows (*Sus scrofa*) (Casellas *et al.,* 2008). In chapter 3, longevity did not differ significantly between infected and uninfected birds, which means that the variation in longevity may be caused by a variation in the level of heterozygosity, and was not subject to infection with *T. gallinae*. To confirm this, an additional analysis was performed to examine whether there was a significant difference in the genome-wide heterozygosity between 140 fledged birds and 35 birds that died before fledgling, across five subpopulations, for both non-immune and immune genes. Surprisingly, the genome-wide heterozygosity was significantly higher in fledged birds group for the non-immune and immune genes, which provides additional evidence that a reduction in the fledgling in the pink pigeon population is related to a reduction in heterozygosity.

Low reproductive success in the pink pigeon subpopulation at Ile aux Aigrettes has been found, related to a reduction in fertility, hatchability and fledgling success (Bunbury, 2006). In chapter 5, the association between the variation in the life history traits and genome-wide heterozygosity was examined. The reproductive success traits, including the number of nests built, eggs and hatched young, did not show any significant relationship with the level of genome-wide heterozygosity, which may indicate that those traits are subject to local effects, with few recessive lethal alleles affecting the reproductive traits of the pink pigeon. In order to identify the genomic region that

explains the variation in one of these traits, the genome-wide association study (GWAS) method was performed for the egg-laying traits in 24 females. A genomic region approximately 0.4 Mb away from the progesterone receptor gene (PRG), a gene that has been identified previously as a gene related to ovulation in birds and mammals (Conneely, 2010; Zhu *et al.,* 2015), was identified. These findings suggest that a reduction in the number of eggs, as well as other reproductive traits in the pink pigeon, may be caused by a few deleterious alleles and genetic drift. This finding is encouraging to use quantitative methods, such as the quantitative trait locus (QTL) or GWAS, to identify the detrimental alleles affecting fitness in the pink pigeon.

The findings of this thesis show that the problems limiting the growth of the pink pigeon population, including infection with *T. gallinae*, a reduction in fledglings and probably a skewed sex ratio, are related to a reduction in genetic variation, suggesting that the pink pigeon is caught in a vortex that could drive this species to extinction. This emphasizes the urgent need for conservation management to avoid extinction.

6.3 Implications and recommendations for pink pigeon conservation genetics and management

Increasing population size is key to the successful conservation management of threatened species. For long-term conservation management, this can help to minimize inbreeding and genetic drift. The population size can be increased by expanding captive-bred birds to different locations, and selecting unrelated individuals for the additional captive populations. Even if the pedigree data are not available for the pink pigeon subpopulations (except the Ile aux Aigrettes), the genomic sources are informative data that can be used to identify the kinship of the required number of individuals. For short-term conservation management, one option is to translocate birds between subpopulations. The meta-population consists of subpopulations that are increasingly genetically differentiated, and translocation can enhance the gene flow between the subpopulations, increasing the heterozygosity necessary for natural selection.

With the genomic data from this study, it is possible to use advanced strategies for effective conservation management. For example, with the advent of genome sequencing in the pink pigeon, it may be possible to use assisted reproductive technologies, such as cryopreservation and artificial insemination, to overcome the

conservation problems related to reproductive success (Howard *et al.,* 2015). Artificial insemination can be used to increase fertility, and to obtain high fitness progeny from specifically mated pairs. Identifying pairs for any desirable fitness traits can be achieved by designing QTL or GWAS analyses that can provide accurate genotyping for one or more desirable fitness traits. Experiments in long-term conservation strategies require two groups of individuals, and their offspring from the second and third generations, carrying conflicting traits like high and low fertility. This research could help to decrease inbreeding depression but would not be practical or desirable in endangered species like the pink pigeon.

A short-term strategy for monitoring the inbreeding level in a population is to evaluate the parents' genetic similarity, and examine their relationships with different fitness-related traits. This analysis can shed light on the effects of genetic similarity on reproductive fitness.

For sex ratio conservation management, more females from captivate populations in Mauritius, Jersey or zoos around the world should be introduced into subpopulations to equalise the sex ratios. However, long-term conservation management requires increasing genetic variation to improve survival of squabs. It also requires improved predator control that may cause the male bias before fledging in these two subpopulations. Sex ratio management requires comprehensive data on reproductive success that should be recorded for each pair, including fertility, the number of eggs hatched, mortality before fledging and the number of young fledged. Collect blood or tissues samples from successful and failed nests including dead embryos and squabs can improve monitoring sex ratio in pink pigeon.

This study found a relationship between the level of genome-wide heterozygosity and infection with *T. gallinae.* In chapter three, we found that infection was a significant driver across subpopulations, which may indicate a particularly virulent strain of this parasite. Preliminary phylogenetic analyses did not find genetic differences between the samples of *T. gallinae* collected from the pink pigeons and Madagascar turtle-doves (*Streptopelia picturata*) in Mauritius (da Silva *et al*., 2007). However, this analysis was performed with few samples and primitive genetic markers. Currently, genomic data are available for *T. gallinae,* sequenced by Alrefaie et al. (unpublished work), which can enhance the ability to assess the local adaptation of *T. gallinae*. It can also provide

genomic data for studying the host-parasite coevolution between the pink pigeon and *T. gallinae,* which is recommended for understanding the co-evolutionary adaption race. Studying the evolutionary history of threatened species is important for understanding changes in gene frequency , causes of the loss of genetic diversity and reduction in population size over time (Wandeler *et al.,* 2007). In the pink pigeon, we have accessed museum specimens from the 1860s to investigate changes the level of genetic variation before population became bottlenecked.

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Appendix 1:

Details of DNA extraction, library preparation and Sequencing for chapter 4 and 5

1. Tissue storage

1.1 Muscle and carcass

Muscle tissue and whole carcass was received from the Durrell Wildlife Conservation Trust on dry ice and subsequently stored at -80 °C.

1.2 Blood

Approximately 5-100 ul of blood was stored in 1 ml of absolute ethanol or DNAzol (Life Technologies, Carlsbad, CA) in screw cap Eppendorf tubes. Samples where then stored at room temperature or 4 °C to reduce the evaporation rate.

2. DNA extraction

2.1 Muscle tissue

Approximately 5 g of frozen leg muscle was ground using a sterilized pestle and mortar in liquid nitrogen. Ground tissue was added to 4 x 50ml of pre-warmed (55 °C) extraction buffer (0.1M NaCl, 25 mM Tris-HCL pH 7.5, 100 mM EDTA, 1 % SDS and 0.5 ug ml $^{-1}$ proteinase K). Extractions were then incubated at 55 °C for 2.5 hours with gentle mixing before being placed on ice for 20 min. The samples were spun down at 3220 x *g* for 20 minutes at 4 °C. The supernatant was harvested and incubated at 80 °C for 15 minutes to denature the proteinase K, before being cooled to 37 deg C. RNase I (10U ul $^{-1}$) was added to each sample tube (to a final concentration of 1.25 U ml $^{-1}$) and incubated at 37 °C for 20 minutes before being placed on ice for 10 min. An equal volume of Chloroform: Isoamyl Alcohol 25:24:1 was added to each tube and mixed by gently inverting. Samples were then centrifuged at 3220 x *g* for 30 minutes at 4 °C. The aqueous phase was harvested and sodium acetate at pH 5.2 was added to a final concentration of 0.3 M. Ice cold isopropanol was then added (x 0.7 vol) before leaving to precipitate on ice, overnight. Samples were then spun down at 3200 x *g* for 30 min at 4 °C the supernatant was discarded and the pellet was washed in 10 ml of ice cold 80% ethanol. The pellet was again spun down at 3200 x *g* for 30 min at 4 °C and the wash procedure was

repeated twice more before removing all the supernatant and leaving to air dry for 20 minutes. Each pellet was finally resuspended in 300 µl TE plus RNAseI (10 mM Tris HCl, 0.1mM EDTA and 30 U ml $^{-1}$ RNAseI).

2.2 Blood tissue

A small amount (5-10 ul) of blood clot was removed from ethanol storage. To this sample 100 ul of TE buffer (10 mM Tris HCl pH 8, 1 mM EDTA) was added ad the sample vortexed for 2 min, then 100 ul of Detergent buffer (6.7% SDS and 7.1% Triton-X-100) was added and the sample was vortexed for 2 min. DNA was then purified using the using the Agencourt® GenFind™ V2 Blood & Serum Genomic DNA Isolation Kit in accordance with the manufacturers instructions, with the following amendments: Lysis was carried out at 55 °C for 1 hr; elution took place at RT for 1 hr or overnight at 4 °C.

3. DNA quality control (QC)

3.1 DNA quantification

DNA sample QC was performed using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) to measure DNA concentration.

3.2 Gel Electrophoresis

Electrophoresis was performed using the E-Gel electrophoresis system (Life Technologies, Carlsbad, CA). A precast 0.8% E-Gel cartridge was loaded with 10 ng of DNA sample. Gel-running time was ≈25 min at 60 V and 500 mA. The gel then was viewed and photographed using UVIdoc HD2 (UVitec, Cambridge, UK).

4. RAD-Seq library preparation

Genomic DNA (0.5–1 µg; from individual samples) was digested for 2 hr at 37°C in a 50 µL reaction with 20 units (U) of high fidelity HF-**Sbf**I (New England Biolabs). Samples were heat-inactivated for 20 min at 80°C. 1.2 µL of 100 nM P1 Adapter, a modified Illumina adaptor

P1 adaptor (post digest)

5’-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxTGC*A-3'

5’-Phos-xxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-3'

* = Phosphorothioate Bond

5’-Phos = 5' phosphate group

x = staggered barcode $d(x)_{4-8}$

Oligonucleotides were ordered from IDT

was added to the sample along with 0.72 µL of 100 mM rATP (Promega), 1 µL 10× **NEB** buffer 2, 1.2 µL (1000 U) T4 DNA Ligase (high concentration, NEB) and 5 µL H_2O and incubated at 22 °C for 30 min. Samples were then heat-inactivated for 20 min at 65°C. Samples were then pooled by row using 30 ul of each well and sodium acetate at pH 5.2 was added to a final concentration of 0.3 M. Ice cold isopropanol was then added (x 0.7 vol) before leaving to precipitate at -20 °C for 2 hr. Samples were then spun down at 18,000 x *g* for 30 min at 4 °C the supernatant was discarded and the pellet was washed in 1 ml of ice cold 80% ethanol. The pellet was again spun down at 18,000 x *g* for 30 min at 4 °C and the wash procedure was repeated twice more before removing all the supernatant and leaving to air dry for 20 minutes. Each pellet was finally resuspended in 130 µl TLE (10 mM Tris HCl, 0.1mM EDTA). Resuspended samples were then sheared using a Covaris S2 acoustic sonicator (Covaris Inc.) to an average size of 500 bp and purified by binding to an equal volume of AMPure XP beads (Beckman Coulter Inc.), washed twice in 80 % ethanol and eluted in 32 µl of EB (10 mM Tris–HCl, pH8). The NEBNext End Repair Kit (NEB) was used to polish the ends of the DNA. A-tailing was performed by adding 1µl of Taq DNA polymerase to each sample and incubating at 72°C for 20 mins. Following another AMPure XP bead (Beckman Coulter Inc.) purification, 2 µL of 10 µM P2 Adapter, a modified Illumina adapter

P2 adaptor (post a-tailing)

5'-
CAAGCAGAAGACGGCATACGAGATxxxxxxxCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCG
ATC*T-3'

5'-Phos-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGxxxxxxxATCAGAACA*A-3'

* = Phosphorothioate Bond

5’-Phos = 5' phosphate group

x = barcode

Oligonucleotides were ordered from IDT

was added along with 10 µL 5 x Quick ligation buffer and 2 µL T4 DNA Ligase (NEB) and incubated at 25 °C for 20 min. Samples were again purified using AMPure XP beads and eluted in 25 µL EB. 10 µL of this product was used in a PCR amplification with 25 µL Phusion Master Mix (NEB), 1.5 µL of 10 µM Illumina amplification primer mix

P1-forward primer: 5’-AATGATACGGCGACCACCGA-3’

P2-reverse primer: 5’-CAAGCAGAAGACGGCATACGA-3’

Oligonucleotides were ordered from IDT

and 12 µL H_2O. PCR reactions were performed on a Veriti thermal cycler with the following conditions: 95°C −3 minutes; 16 cycles of 98°C −20 seconds, 65°C −30 seconds, 72°C −30 seconds; 72°C - 3 minutes. Samples were again purified and eluted in 25 µL.

5. Whole genome sequencing library preparation

5.1 Amplification-free Illumina libraries

Genomic DNA (3 µg derived from leg muscle) was sheared to 500 bp using a Covaris S2 acoustic sonicator (Covaris Inc.) shearing parameters: Mode = Frequency Sweeping, Cycles = 1, Duty cycle = 5%, intensity = 5, cycles per burst = 200, time = 35 seconds. The fragmented was then purified by binding to an equal volume of AMPure XP beads (Beckman Coulter Inc.), washed twice in 80 % ethanol and eluted in 30 µl of EB (10 mM Tris–HCl, pH8). Size selection for 650 bp was performed on the Pippin Blue 1.5% cassette according to the manufacturers’ instructions. Fragmented ends were polished by adding 40 ul of TruSeq end repair mix to 60 ul of sample and incubating at 30°C for 30 min. The sample was once again purified with an equal volume of AMPure XP beads (Beckman Coulter Inc.), twice washed in 80 % ethanol and eluted in 17.5 µl of EB. A-tailing mix (12.5 ul). was added and the reaction was incubated at 37 °C for 30 min. Illumina Truseq adaptor 2 (1 ul) was then added alongside 2.5 µl of EB and 2.5 µl Truseq ligation mix followed by a 10 min incubation at 30 °C. Truseq stop ligation buffer (5 ul) was added

and the reaction purified using an equal volume of AMPure XP beads (Beckman Coulter Inc.), washed twice in 80 % ethanol and eluted in 20 µl of EB (10 mM Tris–HCl, pH8).

5.2 DISCOVAR library preparation (DISCOVAR, www.broad-nstitute.org/softwar/blog/?page_id=375)

Genomic DNA (500 ng) (prepared from leg muscle tissue), in a volume of 50 µl, was sheared to a size of ~400 bp using a Covaris S2 acoustic sonicator (Covaris Inc.) using Illumina's TruSeq PCR-free protocol shearing parameters: Duty cycle = 10%, intensity = 5, cycles per burst = 200, time = 45 seconds. Fragmented DNA was then cleaned up with 0.6x AMPure XP beads (Beckman Coulter Inc.) and eluted in 40 µl Tris-HCl pH8.0, following manufacturer's recommendations (Beckman Coulter Inc.). DNA fragments were then further cleaned up with 3.0x AMPure XP beads (Beckman Coulter Inc.), following manufacturer's recommendations (Beckman Coulter Inc.), but DNA was not eluted from the beads. Then using the KAPA Library Preparation Kit reagents (KAPA Biosystems, Catalog # KK8241), DNA fragments bound to the AMPure XP beads were subjected to end repair, A-tailing and Illumina 'PCR-free' TruSeq adapter ligation (Illumina, Catalog FC-121-3001) following manufacturer's recommendations (KAPA Biosystems). A second 0.7x AMPure XP bead clean up was performed following adapter ligation to remove adapter dimers and library fragments below ~150 bp in size. No library PCR amplification enrichment was performed. Sequence ready Illumina PCR-free library was then eluted off the AMPure XP beads following manufacturer's recommendations (Beckman Coulter).

5.3 Long mate-pair library preparation

Genomic DNA (2, 5, 8 ug) (prepared from leg muscle tissue), in a volume of 208 µl, was tagmented to a sizes of 4, 8, 12 Kb by adding 80 ul Illumina Mate Pair Tagment Buffer and 12 ul Illumina Mate Pair Tagmentation Enzyme followed by a 30 min incubation at 55°C. Neutralize Tagmentation Buffer (100ul) was then added and the sample incubated at RT for 5 min. DNA was then cleaned up with 500 ul Agencourt AMPure XP beads (Beckman Coulter Inc.) and eluted in 30 µl EB (Tris-HCl, pH 8.0), following manufacturer's recommendations. To the sample 132 ul of NFW, 20 ul 10 x strand displacement buffer, 8 ul dNTPs and 10 ul Strand displacement polymerase was added and incubated at RT for 30 min. DNA fragments were then further cleaned up with 100ul Agencourt AMPure

XP beads (Beckman Coulter Inc.) and eluted in 30 µl EB (Tris-HCl, pH 8.0), following manufacturer's recommendations (Beckman Coulter). Size selection was performed on the Pippin Blue. To the 30µl purified strand displacement DNA 10µl loading solution was added before following the manufacturers' instructions for loading and running the Blue Pippin using lane 1 for the S1 marker and lanes 2 to 5 for samples. Each sample was then topped up to 263 ul with NFW before the addition of 30 ul of 10 x Circularisation buffer and 7 ul of Circularisation Ligase followed by an incubation at 30 °C overnight. To each sample 9 ul exonuclease I was added before incubating at 37 °C for 30 min followed by 70 °C for 30 min. Stop Ligation Buffer (12 ul) was then added before shearing on the using a Covaris S2 acoustic sonicator (Covaris Inc.) shearing parameters: Cycles = 2, Duty cycle = 10%, intensity = 4, cycles per burst = 200, time = 37 seconds. For each sample 20ul of M280 Dynabeads (Life Technologies) were washed twice with 50 ul Binding Buffer before being resuspended in 300 ul Bead Binding buffer. Each sheared sample (300 ul) was then added to a 300 ul of prepared beads followed by incubation at 20°C for 15 minutes, resuspending the beads every 2 minutes. Beads were harvested to the side of the tube using a magnetic rack and the supernatant was discarded. Four times the beads were washed in bead wash buffer (200 ul) followed by harvesting to the side of the tube on a magnetic rack and removing the supernatant. Twice the beads were washed in resuspension buffer (200 ul) followed by harvesting to the side of the tube on a magnetic rack and removing the supernatant. End repair performed by adding 40 ul of End Repair Mix and 60 ul of nuclease free water and incubating at 30 °C for 30 minutes. Four times the beads were washed in bead wash buffer (200 ul) followed by harvesting to the side of the tube on a magnetic rack and removing the supernatant. Twice the beads were washed in resuspension buffer (200 ul) followed by harvesting to the side of the tube on a magnetic rack and removing the supernatant. A-tailing was performed by adding 12.5 ul of A-tailing Mix and 17.5 ul of nuclease free water and incubating at 37 °C for 30 minutes. Adaptor ligation was performed by adding 2.5 ul Ligation Mix ,1 ul DNA adapter index and 4 ul of nuclease free water before incubating at 30 °C for 10 minutes. This reaction was stopped by the addition of 5 ul Stop Ligation Buffer. Four times the beads were washed in bead wash buffer (200 ul) followed by harvesting to the side of the tube on a magnetic rack and removing the supernatant. Twice the beads were washed in resuspension buffer (200 ul) followed by harvesting to the side of the

tube on a magnetic rack and removing the supernatant. PCR was then performed by the addition of 25 ul PCR Master Mix, 5 ul PCR Primer Cocktail and 20 ul nuclease free water. PCR reactions were performed on a Veriti thermal cycler with the following conditions: 98°C –30 seconds; 16 cycles of 98°C –10 seconds, 65°C –30 seconds, 72°C –30 seconds; 72°C - 5 minutes. Amplified libraries were purified using AMPure XP beads at a ratio of 15:10 (sample to bead) and washed and eluted (20 ul Resuspension buffer) following the manufacturer's recommendations (Beckman Coulter Inc.).

6. Library validation

All purified libraries were QC checked with the Bioanalyzer DNA HS assay (Agilent Technologies, Inc, CA) and further quantified by both Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) before pooling (see figure 1).

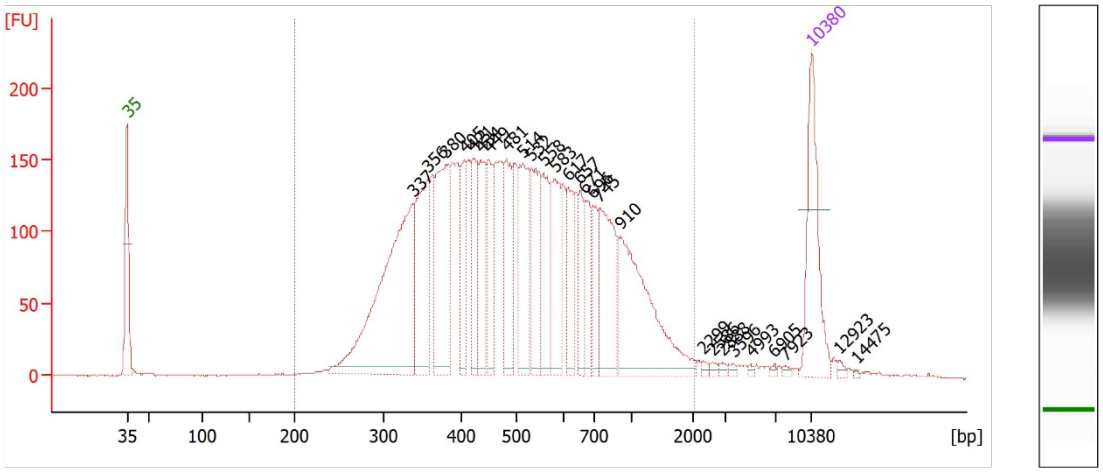

Figure 1. An example of a RAD-seq Library trace and simulated gel view produced using the Bioanalyzer.

Pooled libraries were further quantified by qPCR using a KAPA Library Quantification Kit - Illumina/ABI Prism (Kapa Biosystems, Inc. Wilmington, MA) on a StepOnePlus™ Real-Time PCR System (Life Technologies. Carlsbad, CA).

7. Sequencing Metrics

7.1 WGS – Amplification-free, DISCOVAR and Long Mate Pair

Amplification-free, DISCOVAR and Long Mate Pair libraries were sequenced using paired-end sequencing (2 x 250 bp for DISCOVAR and 2 x 150 bp for Long Mate Pair libraries) with a 1% PhiX spike, on an Illumina HiSeq 2500 in rapid run mode.

7.2 RAD-Seq

RAD-seq libraries were sequenced using paired-end sequencing. To tackle the low complexity sequence problem associated with restriction site mediated sequencing, modifications were made to the standard sequencing method: Read 1: 14 x dark cycles, 136 x light cycles, Rehyb Read1 sequencing primer, 14 x light cycles; Index read: 8 x light cycles; Read 2: 59 x light cycles. 2% PhiX spike, on an Illumina HiSeq 2500 in rapid run mode.

8. Long-mate Pair read QC

Long mate pair libraries were assessed and filtered for the presence of the junction adaptor in one or both reads using NextClip (Leggett *et al.,* 2013) (see figure 2).

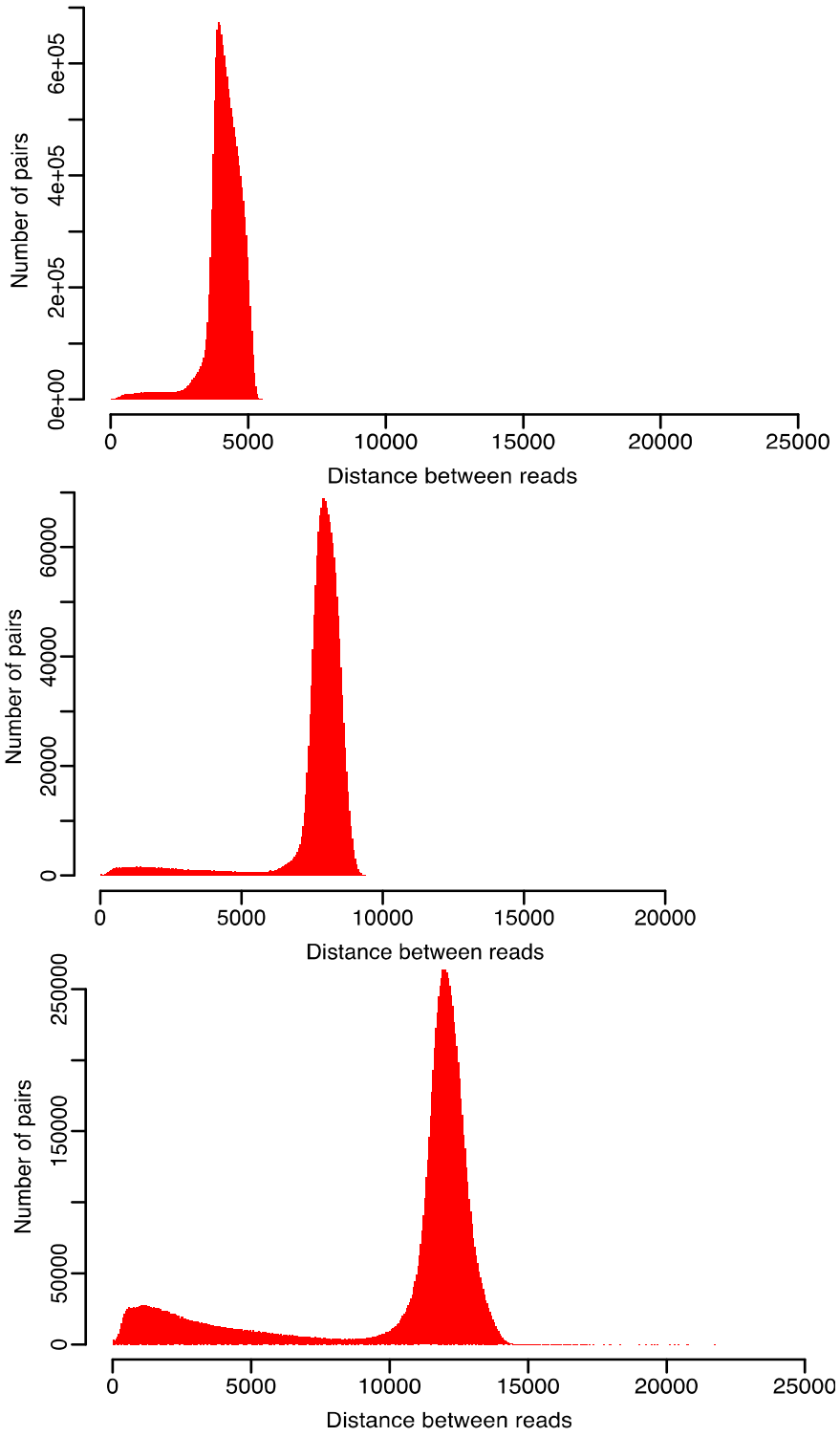

Figure 2. Insert size histograms of 4, 8 12 kb (top to bottom) mate pair libraries

9. Reference Assembly

The Pink pigeon reference was assembled using DISCOVAR (Weisenfeld, et al. 2015) and scaffolded with long mate pair libraries using SOAPdenovo (Li *et al.,* 2010). Analysis of

the draft pink pigeon genome assembly using Abyss-fac revealed the N50 to be 8 Mbp. Assessment using CEGMA (Parra, et al 2009) shows the assembly to be 60% complete (90% partial), using a set of 248 highly conserved protein family genes.

10. RAD-seq read processing

10.1 Processing sequencing reads

Base call (BCL) to FASTQ conversion was performed using CASAVA (using base mask y136,y14,y8,y59) then each second read was joined to the start of the corresponding first read to regain the full 150 bp read 1.

10.2 Demultiplexing

Demultiplexed using was carried out using RADplex (Leggett, 2013) with a mismatch of 0.

10.3 Sample read concatenation

Where, sample reads were generated on more than one run and even using more than one library, reads were concatenated together using the UNIX 'cat' command.

10.4 Read filtering and trimming

Reads were analysed and quality checked using FastQC (Andrews, 2010)

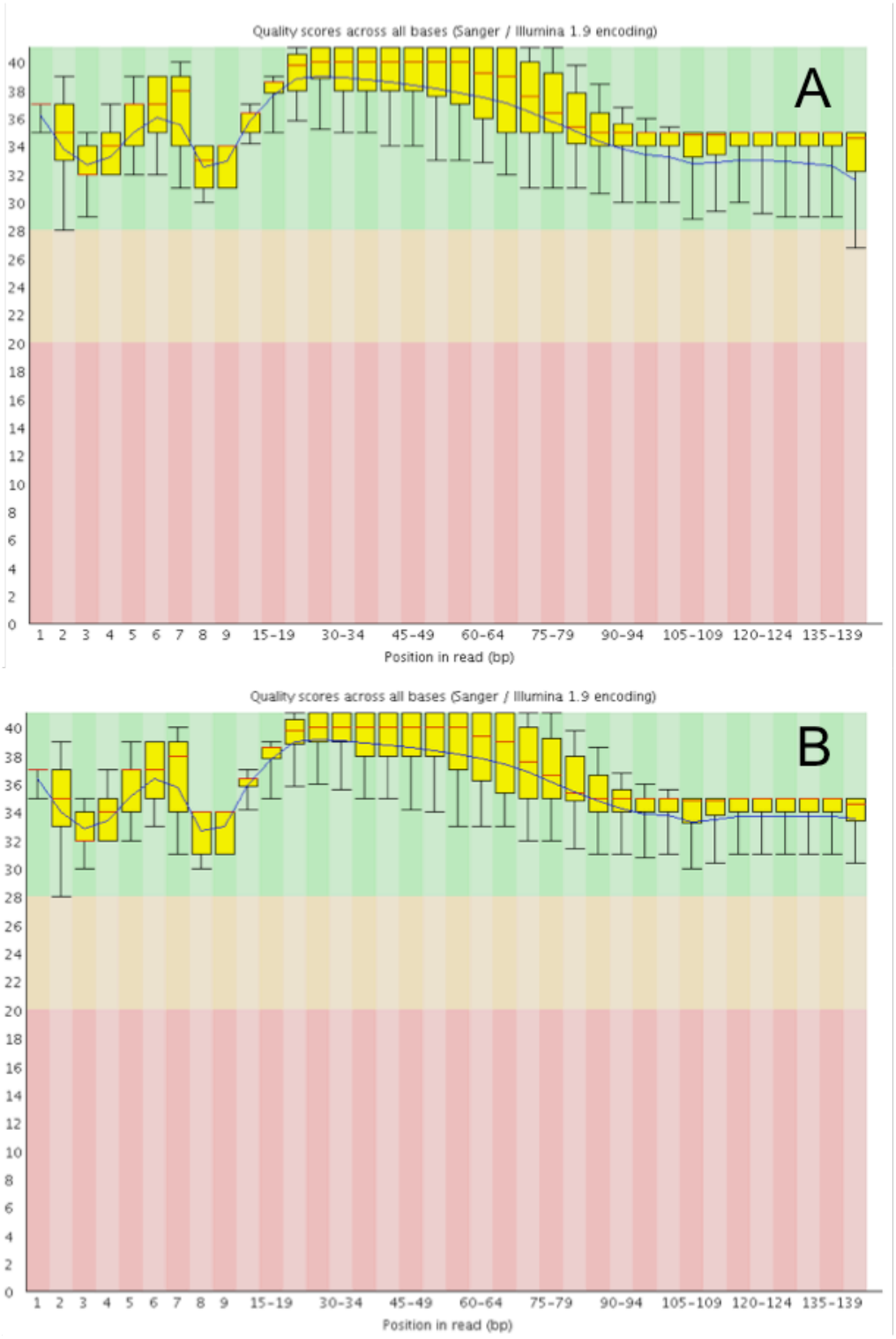

Figure 3. An overview of the range of quality values across all bases at each position generated by FastQC for reads before (A) and after filtering (B).

Reads were required to include the last 6 bp of the SbfI cut-site (TGCAGG) at the start of each read using a bespoke python script. Reads were then trimmed for RAD-seq

adapters and required to have an average quality score of 25, a ‘sKew’ percentage less-than 0, the maximum N-calls in a read to be 1 and the minimum remaining sequence length to be 25, using the "fastq-mcf" program from the ea-utils package (Aronesty. 2011). An example of read quality values before and after trimming and filtering is shown in figure 3.

10.5 Mapping

Reads were mapped to the pink pigeon reference sequence using the Burrows-Wheeler Aligner (BWA-mem) tool (Li, et al. 2013). Alignments were then quality (41) filtered (to ensure unique mapping) and sorted before removing duplicates and indexing with using SAMtools (Li, et al. 2009).

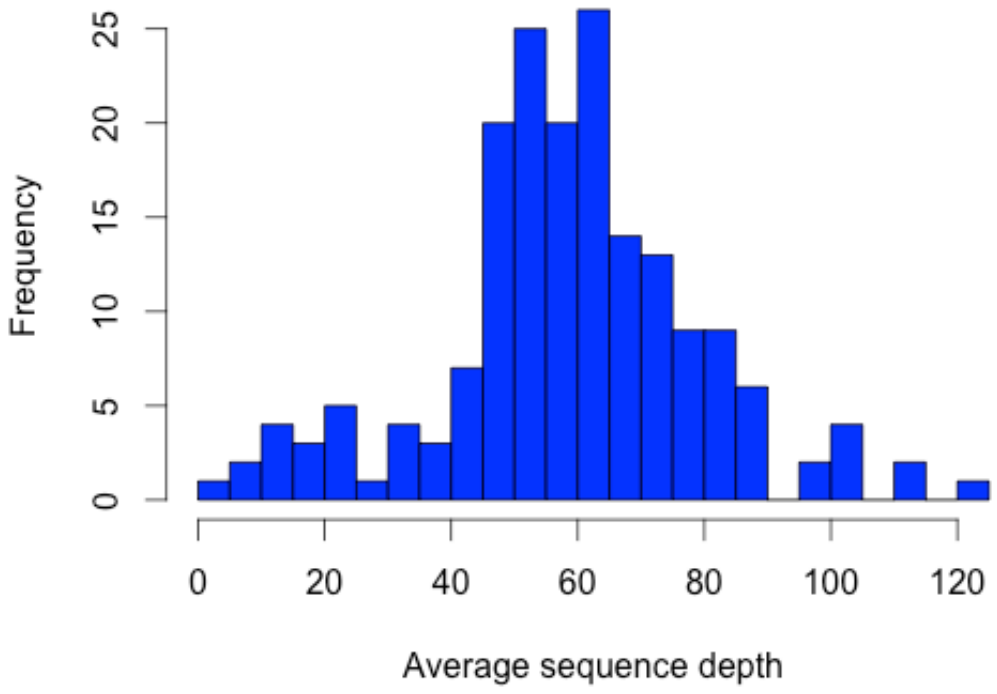

Figure 4. A histogram of sample sequence depth calculated using SAMtools.

Sequence depth was calculated using SAMtools and additional sequencing effort was performed until the depth of an average sample was > 50 (58.8 mean average) see figure 4.

10.6 Variant calling

SAMtools mpileup followed by piping to BCFtools (Li, 2011) was used to perform the variant calling.

samtools mpileup -uDf

- -u Compute genotype likelihoods and output them in the uncompressed binary call format
- -D Output per-sample read depth
- -f The faidx-indexed reference file in the FASTA format

bcftools view –bvcg

- -b Output in the BCF format. The default is VCF.
- -v Output variant sites only
- -c Call variants using Bayesian inference
- -g Call per-sample genotypes at variant sites

10.7 Sexing the pink pigeon population using FASTQs

Reads that were unmapped to the male (ZZ) pink pigeon reference from five females were compared against the unmapped reads from five males. Reads that were unique to female birds were blasted against the Chicken (*Gallus gallus*) W chromosome. This identified 21 sequence motif markers that were then used to perform sex evaluation (See figure 5).

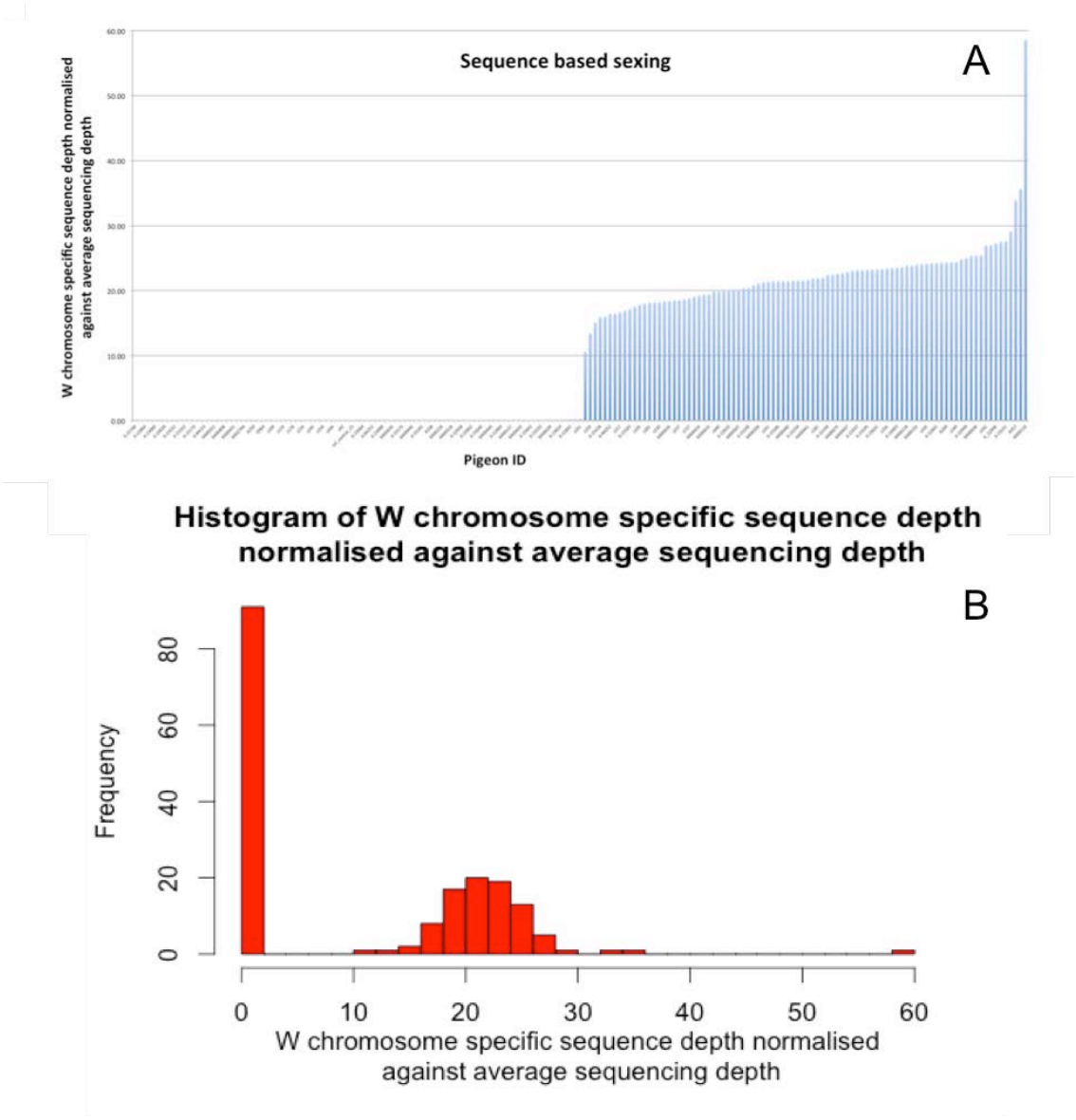

Figure 5. Sexing by sequencing, motif counts were normalised by sequencing depth. Males (0 - 0.22) and female (10 - 60) separate groups can be clearly identified when examining the bar graph (A) and the histogram (B).

Sex determination using sequence analysis identified seven birds to be the opposite sex to the observation data. Of these seven, one sample was removed from the dataset.

10.8 Variant call filtering

Variants were filtered using VCFtools (Danecek et al, 2011) so that only SNPS were present (indels were removed); a minimum depth of 10; a minimum allele count of 2; sites present in at least 96% of the samples.

Z chromosome identification

PLINK (Purcell, et al. 2007) was used to perform an association **--assoc** test using the sexing phenotypes to identify scaffolds and contigs relating to the sex chromosomes. The association tests were performed on both observation (based on behaviour) and sequence based sexing (see figure 6).

Sites were removed from the VCF file where scaffolds were identified as Z chromosome.

- C853946
- scaffold199
- scaffold1883
- scaffold9
- scaffold937
- scaffold729
- scaffold909
- scaffold1835
- scaffold476
- scaffold5
- scaffold480
- scaffold9
- scaffold21

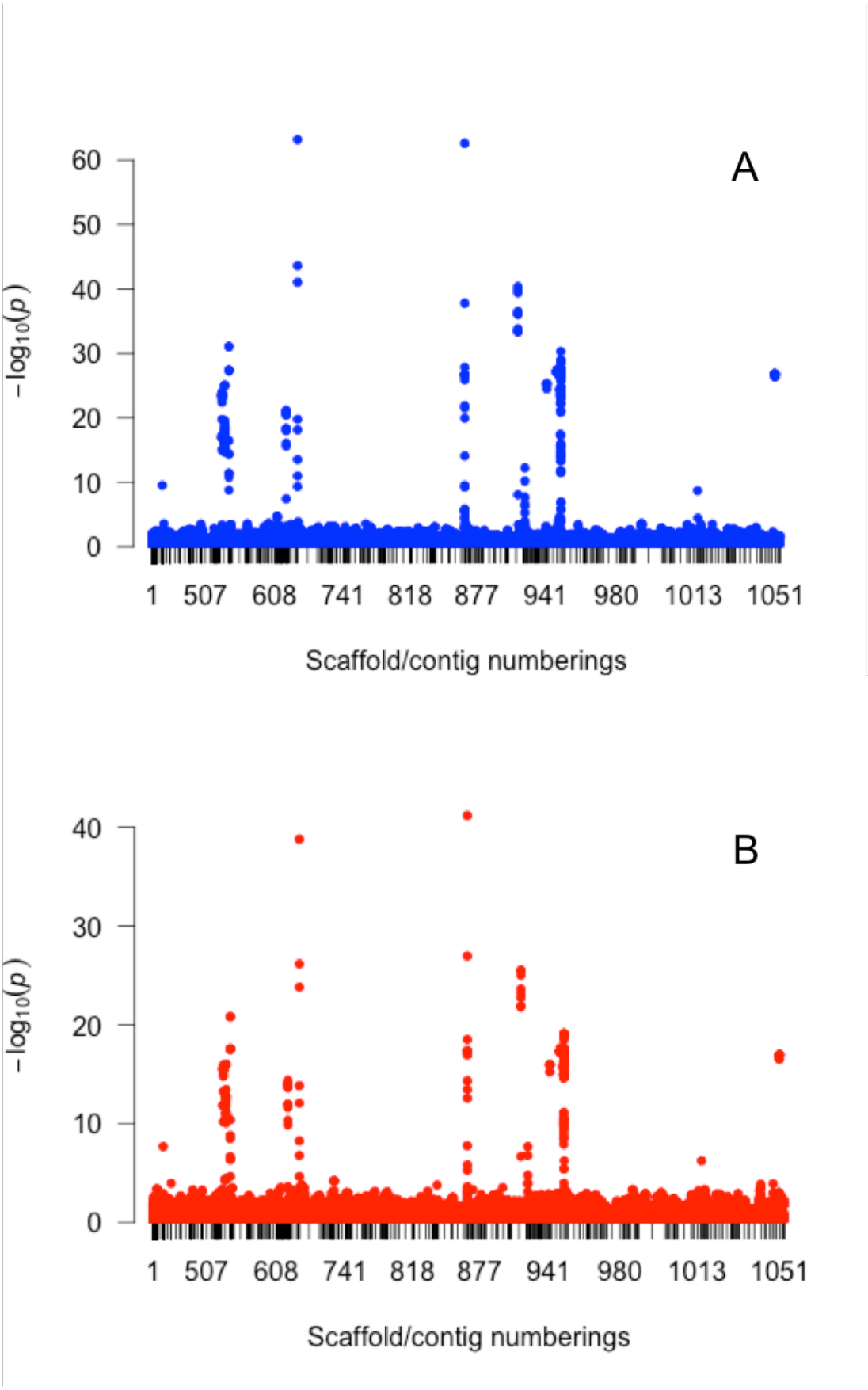

Figure 6. Manhatten plots revealing contigs that make up the sex chromosomes using observation (behavioural) data (A) and sequence based sexing (B).

10.9 Immune gene regions

Genes relating to the immune system and immunity (167) were collected from genes databases including GeneCards: The Human Gene Database (http://www.genecards.org/), GoPubMed (http://www.gopubmed.org/web/gopubmed/), GenomeNet (http://www.genome.jp/) using keywords (immune or immunity).These 369 genes were filtered to 167 by searching for these genes in the rock pigeon (Columba livia) using the National Center for Biotechnology Information databases (NCBI) (http://www.ncbi.nlm.nih.gov/) using keywords (gene name and Columba livia). (See the list in appendix 2).

A python script was written using the BioPython library to retrieve nucleotide identities from gene identities and subsequently the FASTA sequences. Locations for these genes were found by blasting the FASTA files to a local blast database of the Pink Pigeon reference. Python scripts were then used to identify marker sites that fell within 0.1 Mb of these regions.

11. Analysis

11.1 F-statistics and relatedness

Fst statistics between individuals of different populations was obtained using the method of Weir and Cockerham using VCFtools **--weir-fst-pop** and the inbreeding coefficient FIS of individuals were obtained using VCFtools **–het** while the relatedness was calculated using **–relatedness.**

11.2 Genome wide association

PLINK (.ped and .map) files were made using VCFtools –plink. Association tests were performed and adjusted for multiple testing using PLINK.

11.3 MDS plot of genome-wide identity-by-state

Identity-by-state (IBS) distances were obtained using PLINK --cluster –matrix plotting was performed using Rscript.

11.4 Heterozygosity

Heterozygosities were calculated from the PLINK (pedigree and map) files using a Bespoke python script

12. References

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Appendix 2 List of immune related genes that were used in this study.

Organism name	GeneID	Symbol	description
Columba livia	102095947	IK	IK cytokine, down-regulator of HLA II
Columba livia	102090447	IL-8	interleukin-8
Columba livia	102094329	IL4	interleukin 4
Columba livia	102087204	CD40	CD40 molecule, TNF receptor superfamily member 5
Columba livia	102086646	CD40LG	CD40 ligand
Columba livia	102095567	CD74	CD74 molecule, major histocompatibility complex, class II invariant chain
Columba livia	102092595	TICAM1	toll-like receptor adaptor molecule 1
Columba livia	102093907	TLR3	toll-like receptor 3
Columba livia	102096659	TLR4	toll-like receptor 4
Columba livia	102089513	TLR5	toll-like receptor 5
Columba livia	102093882	TLR7	toll-like receptor 7
Columba livia	102083634	ACKR2	atypical chemokine receptor 2
Columba livia	102092528	ACKR3	atypical chemokine receptor 3
Columba livia	102087275	ACKR4	atypical chemokine receptor 4
Columba livia	102083444	ADAM10	ADAM metallopeptidase domain 10
Columba livia	102098173	ADAM17	ADAM metallopeptidase domain 17
Columba livia	102094051	BLNK	B-cell linker
Columba livia	102098832	BRE	brain and reproductive organ-expressed (TNFRSF1A modulator)
Columba livia	102093308	CBLN2	cerebellin 2 precursor
Columba livia	102088383	CCL19	chemokine (C-C motif) ligand 19
Columba livia	102084533	CCL20	chemokine (C-C motif) ligand 20
Columba livia	102088203	CCL21	chemokine (C-C motif) ligand 21
Columba livia	102097362	CCR10	chemokine (C-C motif) receptor 10

Appendix 2 Continued

Organism name	GeneID	Symbol	description
Columba livia	102093527	CCR2	chemokine (C-C motif) receptor 2
Columba livia	102093340	CCR5	chemokine (C-C motif) receptor 5
Columba livia	102084598	CCR6	chemokine (C-C motif) receptor 6
Columba livia	102091339	CCR7	chemokine (C-C motif) receptor 7
Columba livia	102084932	CCR9	chemokine (C-C motif) receptor 9
Columba livia	102098737	CIAPIN1	cytokine induced apoptosis inhibitor 1
Columba livia	102091326	CLCF1	cardiotrophin-like cytokine factor 1
Columba livia	102095989	CMKLR1	chemokine-like receptor 1
Columba livia	102096373	CRLF1	cytokine receptor-like factor 1
Columba livia	102095018	CRLF2	cytokine receptor-like factor 2
Columba livia	102086336	CRLF3	cytokine receptor-like factor 3
Columba livia	102095493	CX3CR1	chemokine (C-X3-C motif) receptor 1
Columba livia	102092103	CXCL13	chemokine (C-X-C motif) ligand 13
Columba livia	102090944	CXCL14	chemokine (C-X-C motif) ligand 14
Columba livia	102086794	CXCR4	chemokine (C-X-C motif) receptor 4
Columba livia	102090272	CXCR5	chemokine (C-X-C motif) receptor 5
Columba livia	102092406	CYTL1	cytokine-like 1
Columba livia	102083486	DOCK1	dedicator of cytokinesis 1
Columba livia	102091102	DOCK10	dedicator of cytokinesis 10
Columba livia	102095013	DOCK11	dedicator of cytokinesis 11
Columba livia	102087155	DOCK2	dedicator of cytokinesis 2
Columba livia	102096641	DOCK3	dedicator of cytokinesis 3
Columba livia	102094475	DOCK4	dedicator of cytokinesis 4

Appendix 2 Continued

Organism name	GeneID	Symbol	description
Columba livia	102096339	DOCK5	dedicator of cytokinesis 5
Columba livia	102089424	DOCK7	dedicator of cytokinesis 7
Columba livia	102095213	DOCK8	dedicator of cytokinesis 8
Columba livia	102095141	DOCK9	dedicator of cytokinesis 9
Columba livia	102083777	LOC102083777	c-C motif chemokine 13-like
Columba livia	102083958	LOC102083958	c-C motif chemokine 13-like
Columba livia	102084092	LOC102084092	tumor necrosis factor receptor superfamily member 1A-like
Columba livia	102084147	LOC102084147	c-C motif chemokine 3-like
Columba livia	102084337	LOC102084337	c-C motif chemokine 3-like
Columba livia	102084454	LOC102084454	toll-like receptor 2 type-1-like
Columba livia	102084537	LOC102084537	c-C motif chemokine 3-like 1-like
Columba livia	102084767	LOC102084767	toll-like receptor 2 type-2-like
Columba livia	102084942	LOC102084942	class I histocompatibility antigen, F10 alpha chain-like
Columba livia	102085079	LOC102085079	autoimmune regulator-like
Columba livia	102085717	LOC102085717	dedicator of cytokinesis protein 2-like
Columba livia	102087718	LOC102087718	c-C motif chemokine 5-like
Columba livia	102087903	LOC102087903	mucin-17-like
Columba livia	102088992	LOC102088992	ephrin type-B receptor 3-like
Columba livia	102089774	LOC102089774	tumor necrosis factor ligand superfamily member 10-like
Columba livia	102090616	LOC102090616	toll-like receptor 2-like
Columba livia	102090750	LOC102090750	toll-like receptor 1-like
Columba livia	102090996	LOC102090996	stromal cell-derived factor 1-like
Columba livia	102091594	LOC102091594	class II histocompatibility antigen, B-L beta chain-like

Appendix 2 Continued

Organism name	GeneID	Symbol	description
Columba livia	102091663	LOC102091663	complement C1q tumor necrosis factor-related protein 1-like
Columba livia	102091843	LOC102091843	toll-like receptor 2 type-1-like
Columba livia	102093420	LOC102093420	class II histocompatibility antigen, B-L beta chain-like
Columba livia	102094010	LOC102094010	c-C chemokine receptor type 8-like
Columba livia	102094721	LOC102094721	chemokine-like receptor 1-like
Columba livia	102094827	LOC102094827	interleukin-8-like
Columba livia	102094906	LOC102094906	complement C1q tumor necrosis factor-related protein 1-like
Columba livia	102095127	LOC102095127	probable G-protein coupled receptor 141-like
Columba livia	102095140	LOC102095140	platelet factor 4-like
Columba livia	102095311	LOC102095311	c-C chemokine receptor type 4-like
Columba livia	102095323	LOC102095323	toll-like receptor 2-like
Columba livia	102095613	LOC102095613	uncharacterized LOC102095613
Columba livia	102095743	LOC102095743	c-C chemokine receptor type 4-like
Columba livia	102096229	LOC102096229	major histocompatibility complex class I-related gene protein-like
Columba livia	102096439	LOC102096439	suppressor of cytokine signaling 1-like
Columba livia	102096903	LOC102096903	toll-like receptor 1-like
Columba livia	102097081	LOC102097081	toll-like receptor 1-like
Columba livia	102097375	LOC102097375	c-C motif chemokine 3-like
Columba livia	102097596	LOC102097596	c-C motif chemokine 4-like
Columba livia	102097770	LOC102097770	c-C motif chemokine 5-like
Columba livia	102098016	LOC102098016	lymphotactin-like
Columba livia	102098631	LOC102098631	HLA class II histocompatibility antigen, DR alpha chain-like
Columba livia	102098807	LOC102098807	boLa class II histocompatibility antigen, DQB*0101 beta chain-like

Appendix 2 Continued

Organism name	GeneID	Symbol	description
Columba livia	102098998	LOC102098998	c-X-C chemokine receptor type 2-like
Columba livia	102088643	EDA	ectodysplasin A
Columba livia	102084601	EDA2R	ectodysplasin A2 receptor
Columba livia	102087618	EDAR	ectodysplasin A receptor
Columba livia	102085703	FADD	Fas (TNFRSF6)-associated via death domain
Columba livia	102093130	FAM19A1	family with sequence similarity 19 (chemokine (C-C motif)-like), member A1
Columba livia	102091352	FAM19A2	family with sequence similarity 19 (chemokine (C-C motif)-like), member A2
Columba livia	102095209	FAM19A4	family with sequence similarity 19 (chemokine (C-C motif)-like), member A4
Columba livia	102086701	FAM19A5	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5
Columba livia	102087399	FAS	Fas cell surface death receptor
Columba livia	102097854	FASLG	Fas ligand (TNF superfamily, member 6)
Columba livia	102094867	KIAA1324	KIAA1324 ortholog
Columba livia	102083869	KIAA1324L	KIAA1324-like ortholog
Columba livia	102092663	LITAF	lipopolysaccharide-induced TNF factor
Columba livia	102085992	NGFR	nerve growth factor receptor
Columba livia	102090786	PICALM	phosphatidylinositol binding clathrin assembly protein
Columba livia	102098416	PLSCR1	phospholipid scramblase 1
Columba livia	102087458	PLSCR5	phospholipid scramblase family, member 5
Columba livia	102091827	PSMG1	proteasome (prosome, macropain) assembly chaperone 1
Columba livia	102094995	PSMG2	proteasome (prosome, macropain) assembly chaperone 2
Columba livia	102090043	PSMG3	proteasome (prosome, macropain) assembly chaperone 3
Columba livia	102085021	RELL1	RELT-like 1
Columba livia	102093253	RELL2	RELT-like 2

Appendix 2 Continued

Organism name	GeneID	Symbol	description
Columba livia	102091604	RELT	RELT tumor necrosis factor receptor
Columba livia	102092369	SOCS1	suppressor of cytokine signaling 1
Columba livia	102097346	SOCS2	suppressor of cytokine signaling 2
Columba livia	102085285	SOCS3	suppressor of cytokine signaling 3
Columba livia	102086690	SOCS4	suppressor of cytokine signaling 4
Columba livia	102085447	SOCS5	suppressor of cytokine signaling 5
Columba livia	102093494	SOCS6	suppressor of cytokine signaling 6
Columba livia	102089506	SOCS7	suppressor of cytokine signaling 7
Columba livia	102095549	SPSB1	splA/ryanodine receptor domain and SOCS box containing 1
Columba livia	102096796	SPSB3	splA/ryanodine receptor domain and SOCS box containing 3
Columba livia	102097056	SPSB4	splA/ryanodine receptor domain and SOCS box containing 4
Columba livia	102087032	TNFAIP1	tumor necrosis factor, alpha-induced protein 1 (endothelial)
Columba livia	102084471	TNFAIP2	tumor necrosis factor, alpha-induced protein 2
Columba livia	102097352	TNFAIP3	tumor necrosis factor, alpha-induced protein 3
Columba livia	102085012	TNFAIP6	tumor necrosis factor, alpha-induced protein 6
Columba livia	102095396	TNFAIP8	tumor necrosis factor, alpha-induced protein 8
Columba livia	102091921	TNFAIP8L1	tumor necrosis factor, alpha-induced protein 8-like 1
Columba livia	102092671	TNFAIP8L3	tumor necrosis factor, alpha-induced protein 8-like 3
Columba livia	102094994	TNFRSF10A	tumor necrosis factor receptor superfamily, member 10a
Columba livia	102095161	TNFRSF11A	tumor necrosis factor receptor superfamily, member 11a, NFKB activator
Columba livia	102086049	TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b
Columba livia	102084582	TNFRSF13B	tumor necrosis factor receptor superfamily, member 13B
Columba livia	102086077	TNFRSF13C	tumor necrosis factor receptor superfamily, member 13C

Appendix 2 Continued

Organism name	GeneID	Symbol	description
Columba livia	102089901	TNFRSF14	tumor necrosis factor receptor superfamily, member 14
Columba livia	102086048	TNFRSF17	tumor necrosis factor receptor superfamily, member 17
Columba livia	102097904	TNFRSF18	tumor necrosis factor receptor superfamily, member 18
Columba livia	102097142	TNFRSF19	tumor necrosis factor receptor superfamily, member 19
Columba livia	102093466	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B
Columba livia	102092567	TNFRSF21	tumor necrosis factor receptor superfamily, member 21
Columba livia	102086629	TNFRSF25	tumor necrosis factor receptor superfamily, member 25
Columba livia	102085665	TNFRSF6B	tumor necrosis factor receptor superfamily, member 6b, decoy
Columba livia	102088093	TNFRSF8	tumor necrosis factor receptor superfamily, member 8
Columba livia	102088472	TNFRSF9	tumor necrosis factor receptor superfamily, member 9
Columba livia	102093335	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
Columba livia	102098042	TNFSF11	tumor necrosis factor (ligand) superfamily, member 11
Columba livia	102094183	TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b
Columba livia	102098274	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15
Columba livia	102098094	TNFSF8	tumor necrosis factor (ligand) superfamily, member 8
Columba livia	102089179	TRADD	TNFRSF1A-associated via death domain
Columba livia	102092613	TRAF1	TNF receptor-associated factor 1
Columba livia	102085500	TRAF3	TNF receptor-associated factor 3
Columba livia	102090831	TRAF4	TNF receptor-associated factor 4
Columba livia	102083770	TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase
Columba livia	102091345	VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor
Columba livia	102093823	XCR1	chemokine (C motif) receptor 1
Columba livia	102091818	C1QTNF1	C1q and tumor necrosis factor related protein 1

Appendix 2 Continued

Organism name	GeneID	Symbol	description
Columba livia	102093848	C1QTNF2	C1q and tumor necrosis factor related protein 2
Columba livia	102086599	C1QTNF3	C1q and tumor necrosis factor related protein 3
Columba livia	102084338	C1QTNF4	C1q and tumor necrosis factor related protein 4
Columba livia	102095994	C1QTNF5	C1q and tumor necrosis factor related protein 5
Columba livia	102090567	C1QTNF7	C1q and tumor necrosis factor related protein 7
Columba livia	102093791	C1QTNF9	C1q and tumor necrosis factor related protein 9

Appendix 3. Kruskal-Wallis test for assessment of difference between immune genes and non-immune genes in observed heterozygotes (Ho), expected heterozygosity (He) and (F_{is}) in the IAA subpopulation

	Position (100kb)	N	Median	Ave Rank	Z	H	p-v
Ho	Non-immune genes	44357	0.2754	22935.3	1.26	1.60	0.206
	immune genes	1484	0.2754	22494	-1.26		
	Overall	45841		22921	Overall		
He	Non-immune genes	44357	0.2873	22935.1	1.24	1.55	0.213
	immune genes	1484	0.2778	22500.6	-1.24		
	Overall	45841		22921			
F_{is}	Non-immune genes	44498	-0.02222	20862.5	-0.69	0.48	0.488
	immune genes	1343	-0.02178	21094.2	0.69		
	Overall	41739		20870			

Appendix 4. Kruskal-Wallis test for assessment of difference between immune genes and housekeeping genes in observed homozygosity (Ho), expected heterozygosity (He) and inbreeding (F_{is}) in the Pigeon Wood subpopulation

	Positions (100kb)	N	Median	Ave Rank	Z	p-v
Ho	Non-immune genes	44357	0.3	22893.8	-2.41	0.016
	immune genes	1484	0.3	23734	2.41	
	Overall	45841		22921		
He	Non-immune genes	44357	0.255	22896.2	-2.19	0.028
	immune genes	1484	0.32	23661.2	2.19	
	Overall	45841		22921		
F_{is}	Non-immune genes	44357	0.255	22896.2	-2.19	0.146
	immune genes	1484	0.32	23661.2	2.19	
	Overall	45841		22921		

Appendix 5. Kruskal-Wallis test for assessment of difference between immune genes and housekeeping genes in observed homozygosity (Ho), expected heterozygosity (He) and inbreeding (Fis) in the Bel Ombre subpopulation.

	Positions (100kb)	N	Median	Ave Rank	Z	
Ho	Non-immune genes	44357	0.25	22904.1	-1.49	0.136
	immune genes	1484	0.25	23425	1.49	
	Overall	45841		22921		
He	Non-immune genes	44357	0.2778	22905.6	-1.37	0.172
	immune genes	1484	0.2778	23382.5	1.37	
	Overall	45841		22921		
F_{is}	Non-immune genes	38326	-0.04348	19829.7	3.23	0.001
	immune genes	1265	-0.0614	18776.1	-3.23	
	Overall	39591		19796		

Appendix 6. Kruskal-Wallis test for assessment of difference between immune genes and housekeeping genes in observed homozygosity (Ho), expected heterozygosity (He) and inbreeding (F_{is}) in the Combo subpopulation.

	Positions (100kb)	N	Median	Ave Rank	Z	p-v
Ho	Non-immune genes	44357	0.2917	22905.8	-1.35	0.178
	immune genes	1484	0.2917	23376	1.35	
	Overall	45841		22921		
He	Non-immune genes	44357	0.2778	22909.7	-1	0.218
	immune genes	1484	0.3047	23258.4	1	
	Overall	45841		22921		
F_{is}	Non-immune genes	40634	-0.06667	21003.6	1.5	0.133
	immune genes	1340	-0.06667	20498.5	-1.5	
	Overall	41974		20987.5		

Appendix 7. Kruskal-Wallis test for assessment of difference between immune genes and housekeeping genes in observed homozygosity (Ho), expected heterozygosity (He) and inbreeding (F_{is}) in the Plaine Lievre subpopulation

	Positions (100kb)	N	Median	Ave Rank	Z	p-v
Ho	nonimmune genes	44357	0.25	22919.6	-0.13	0.900
	immune genes	1484	0.25	22963.6	0.13	
	Overall	45841		22921		
He	nonimmune genes	44357	0.2778	22907	-1.24	0.214
	immune genes	1484	0.2975	23340.6	1.24	
	Overall	45841		22921		
F_{IS}	nonimmune genes	37963	-0.04348	19598.3	-2.54	0.011
	immune genes	1286	-0.04348	20414.4	2.54	
	Overall	39249		19625		

Appendix 8. Kruskal-Wallis test for assessment of difference in observed heterozygosity (Ho) of the group of parents and offspring in the IAA population.

	Group	N	Median	Ave Rank	Z	p-v
Ho	Parents	44	0.2938	27.4	-3.27	0.001
	Offspring	20	0.3142	43.8	3.27	
	Overall	64		32.5		

Appendix 9. ANOVA of linear regression of relationship between the individual genome-wide heterozygosity of the nonimmune genes of 89 adult birds across all five subpopulations and the percentage of times that an individual is infected with *T. gallinae* (R-Sq = 11.4%).

Source	df	SS	MS	F	P
Regression	1	0.0031390	0.0031390	11.15	0.0.001
Residual Error	87	0.0245031	0.0002816		
Total	88	0.0276422			

Appendix 10. ANOVA of linear regression of the relationship between the individual genome-wide heterozygosity of the immune genes of 89 adult birds across all five subpopulations and the percentage of times that an individual is infected with *T. gallinae* (R-Sq = 10.9%).

Source	df	SS	MS	F	P
Regression	1	0.0093985	0.093985	10.61	0.0.002
Residual Error	87	0.0770430	0.0008856		
Total	88	0.0864414			

Appendix 11. ANOVA of linear regression of the relationship between the individual genome-wide heterozygosity of 47 IAA birds and the percentage of times that an individual is infected with *T. gallinae* (R-Sq = 10.3%).

Source	df	SS	MS	F	P
Regression	1	0.0016882	0.0016882	5.18	0.0.028
Residual Error	45	0.0146758	0.0003261		
Total	46	0.0163640			