Evaluating genetic diversity in the critically endangered orange-bellied parrot: informing species management

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DECLARATION

I declare that this thesis is the result of my own work and has not been submitted for another degree or qualification. All information derived from published or unpublished work of others has been acknowledged in the text, and a list of references is given at the end of this thesis.

Caitlin Morrison

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"The best of us are cursed with caring, with a bungling and undying determination to protect whatever looks like beauty, even if our vision is blurry."

- Jon Mooallem, Wild Ones

To all those who care, whether they want to or not: thank you, and keep going. This planet, and the myriad of astonishing beings with which we share it, are everything. They're worth understanding - and they're worth protecting.

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This thesis is presented as a series of chapters, some of which are versions of manuscripts that have been accepted for publication, or that have been submitted for publication. Published manuscripts and those currently under review are listed in the following section. Supplementary materials relating to each chapter follow in the appendices. A single reference list is provided at the end of this thesis.

Manuscripts included in this thesis:

Morrison CE, Johnson RN, Grueber CE / Hogg CJ. Genetic impacts of conservation management actions in a critically endangered parrot species. Conservation Genetics (submitted 10 November 2019).

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Authors' contributions

Caitlin Morrison, the candidate, performed sample selection, DNA extractions, processing of raw data, all subsequent data analysis and wrote the manuscript. Carolyn Hogg, Catherine Grueber and Rebecca Johnson assisted with project design, interpretation of results, and contributed to editing and finalising the manuscript.

I, as the primary supervisor, endorse that the level of contribution by myself, other co-authors and the candidate indicated above, is appropriate.

Catherine Grueber

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Caitlin Morrison, the candidate, performed sample selection, DNA extractions, DNA amplifications, design of primers, processing of Sanger sequencing results and all subsequent data analysis. Catherine Grueber provided training with DNA amplifications and gel extractions and assisted with primer design. Rosemary Gales provided information regarding OBP management and co-ordinated provision of samples. Catherine Grueber, Carolyn Hogg and Rebecca Johnson assisted in project development, project design, and interpretation of results. Caitlin Morrison, the candidate, wrote the manuscript and all authors assisted with editing and finalising the manuscript.

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ABBREVIATIONS

BFDV	*Beak and feather disease virus*
COI	Cytochrome oxidase I
CytB	Cytochrome B
DArTseq	Diversity Arrays Technology Pty. Ltd. sequencing method
F_{ST}	Fixation index
GTR	General Time Reversible
H_e	Expected heterozygosity
H_o	Observed heterozygosity
HA	Hemagglutination assay
HI	Hemagglutination inhibition assay
HKY	Hasegawa-Kishino-Yano
IUCN	International Union for the Conservation of Nature
IR	Internal relatedness
MAF	Minor allele frequency
OBP	Orange-bellied parrot
OBPRT	Orange-bellied Parrot Recovery Team
PBFD	Psittacine beak and feather disease
PCoA	Principal Co-ordinates Analysis
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
s.d.	Standard deviation
TLR	Toll-like receptor
VTRG	Veterinary Technical Reference Group
ZAA	Zoos and Aquarium Association Australasia

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ABSTRACT

As increasing numbers of species face decline and extinction, strategically designed conservation management programs are becoming more important than ever. The development of optimal species management plans often requires knowledge of species' genetic characteristics. As the cost of developing molecular genetics resources is decreasing, characterisation of genetic diversity in non-model organisms is becoming increasingly accessible, and study of genetics in endangered species possible. Information generated in such studies can be used to assess the current state of threatened species and populations, to review the impacts of past management actions, and to evaluate future directions for conservation of species.

This thesis examines patterns of genetic diversity in one of Australia's most endangered birds, the orange-bellied parrot (*Neophema chrysogaster*, OBP). The OBP is the subject of the longest running recovery program in Australia's history. However, the wild population size remains critically small, with fewer than 20 birds counted in the wild over several recent breeding seasons. A captive insurance population contains approximately 450 birds, small numbers of which are released to the wild each year. Among many threats to the species, infectious disease outbreaks are of particular concern in both the wild and captivity, with the OBP being challenged by several different pathogens including *Beak and feather disease virus* (BFDV) and *Pseudomonas aeruginosa*. Individual and population resilience in the face of disease outbreak can be compromised when genetic diversity is low.

Historically, conservation management of the OBP has involved multiple translocations of individuals between captive and wild populations. One of the most significant of these occurred in the austral summer of 2010/11, when approximately 40% of the wild population was transferred into captivity (21 of 52 individuals), to capture and preserve remaining wild genetic diversity. Subsequently, releases of captive birds to supplement the wild population have occurred annually since 2013. In this study I used molecular tools to assess contemporary genetic diversity in the OBP, to evaluate the genetic impacts of past management actions, and to provide

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recommendations for future genetic management of the species. These results contributed to the OBP's conservation management program.

As well as a critically small population size, previous preliminary studies have suggested that the OBP as a species has low genetic diversity. To explore genome-wide genetic diversity in the species I developed SNP markers using DArTseq, a method of reduced-representation sequencing (RRS). I typed 7,768 SNPs in 165 OBPs hatched between 2010 and 2016, from across both captive and wild populations. These data were used to assess the impacts of recent management actions on the OBP and profile the current situation of captive and wild populations.

I found that the population size reduction associated with removal of the 21 fledglings from the wild in 2010/2011, to bolster genetic diversity in the captive population, prompted a steep decline in heterozygosity in wild birds in the years immediately following. After the fledglings were incorporated into the breeding strategy in the captive population, heterozygosity of the captive population remained stable between 2013-2017. I confirmed that birds released from the captive population into the wild over this period successfully bred with both wild birds and other captive-release birds, and that this mixing is improving heterozygosity in the wild population after its decline. Captive and wild OBP populations have not been genetically distinguishable from each other through the period of my study, probably as a result of the high degree of exchange between the two populations, and low overall allelic diversity. Taken together, these findings demonstrated that current management of the OBP is preserving the remaining genome-wide diversity in the species. Past actions likely had a negative impact on diversity in the wild population, from which it is still recovering. Current actions (monitoring and translocations) are contributing to increase of genetic diversity in the wild population, which is returning to levels more similar to those found prior to the fledgling removal in 2010/11.

To learn more about pathogen threats to the OBP, I investigated whether a deficit in immunogenetic diversity may be playing a role in disease susceptibility. I discovered that diversity at Toll-like receptor (TLR) innate immunity genes is low in the OBP, with three out of the six TLR loci surveyed being monomorphic. A small amount of diversity was present at the other three loci. This diversity is comparable to low TLR

diversity found in other critically endangered birds and is consistent with a recent population bottleneck. No variation was detected at loci hypothesised to be involved in immune responses to *Beak and feather disease virus*, nor *P. aeruginosa*. It was concluded that sequence variation at TLR loci was therefore unlikely to be responsible for inter-individual variation observed in responses to these pathogens. Diversity at other immunogenetic regions, or other individual factors, are anticipated to be involved in differential OBP immune responses to these pathogens instead.

The RRS data generated in this thesis, alongside detailed reproductive and disease records, then enabled me to test for signs of inbreeding depression in OBPs via heterozygosity-fitness correlations. Inter-individual inbreeding depression is indicated when there is variation in fitness traits (between individuals) which is correlated with how genetically similar those individuals' parents were to each other. I assessed reproductive fitness in the form of number of fledglings produced and number of fledglings surviving to maturity. I also assessed disease response in the form of 1) individual test results for presence of BFDV, and 2) individual outcome following exposure to *P. aeruginosa*, during an outbreak in which many captive birds died. I found no strong indicators of inbreeding depression in the species in respect of either the reproductive traits or disease responses. I did find that parental age was a strong determinant of the number of fledglings produced by a pair of birds.

The results of these fitness analyses did not provide any indication that inter-individual inbreeding depression (variation in inbreeding within the OBP population) has been having a significant effect on BFDV infection status or individual response to *P. aeruginosa* exposure, nor on fertility rates measured in captivity at offspring fledging or beyond. It is important to collect data on earlier reproductive stages, and from the wild population, to determine the generality of these findings across contexts and across the life history of the species. It is also important to note that the possibility remains that low genetic diversity species-wide, as a result of historical inbreeding, has led to altered disease susceptibilities or fertility in the OBP as a species, *relative to other species*. This study did not address that question.

This project has revealed that, overall, the OBP has a low level of genetic diversity (similar to other endangered species). Low levels of genetic diversity can threaten

population viability, through an increased likelihood of compromised individual fitness due to inbreeding depression, and reduced overall population adaptive potential.

There has been some interest in determining the feasibility of increasing genetic diversity in the species by outbreeding (hybridising) OBP individuals with a closely related species, if a suitable candidate can be identified. I undertook a preliminary phylogenetic study of the genus *Neophema*, to which the OBP belongs, to investigate which other parrot is the most closely related to the OBP. I concluded from this work that there is evidence to support the current division of the genus into two subgenera, but that use of two mitochondrial fragments is insufficient for building a robust phylogeny of this genus. Confident identification of where the OBP sits phylogenetically relative to its relatives will require further investigation using more sequencing data, likely more complete mitogenome sequencing combined with data from select nuclear genes.

The work presented in this thesis has contributed significantly to our understanding of the contemporary genetics of OBP populations, revealing positive and negative aspects of past and current management of the OBP, and informing future conservation actions. The study has provided recommendations for management of the species: notably, that continued release of captive birds into the wild population is likely to be beneficial, from a population genetics perspective. Relevant to conservation management more generally, the study also found that when genetic diversity in a wild population has been compromised by removal of individuals as founders for a captive population, some of this diversity can be successfully reintroduced into the wild through release of captive individuals at a later date. Results from this work have been presented annually to the OBP Recovery Team to ensure timely integration of findings into management strategies. This thesis presents an example of the value and potential of undertaking genetic studies in critically endangered non-model species for which little genetic work has been previously published.

CHAPTER 1: INTRODUCTION

We are living in an era of unprecedented destruction of the natural world. The current rate of species extinction has led to the widespread understanding that the Earth is on the brink of a "sixth mass extinction" (Barnosky et al. 2011, McCallum 2015), with human impacts being the major cause (Pimm et al. 2014, Ceballos et al. 2015). The vast majority of species on the planet are under-studied and undescribed, and the majority of those described are of unknown conservation status (IUCN 2019). However, of the estimated 6% of described species evaluated by the International Union for the Conservation of Nature (IUCN), over 25% are threatened, including 12% of all bird species, 25% of all mammal species, and 32% of amphibians (IUCN 2019). In Australia, approximately 2% of birds and 11% of mammals have gone extinct since European settlement of the continent in 1788 (Garnett et al. 2011a, Woinarski et al. 2019), and of all mammal extinctions that have been documented since 1500, 35% have been Australian species (Woinarski et al. 2015). In Australia today, 28% of mammals are listed as threatened, 11% of birds and 13% of amphibians (IUCN 2019).

Members of Psittaciformes (parrots and cockatoos; hereafter parrots) are the most threatened avian order worldwide, with almost one third of all parrot species listed as threatened (Olah et al. 2016). In Australia (including offshore islands), 28% of parrot species are threatened (Olah et al. 2018), representing 10% of Australia's threatened bird species overall (Garnett et al. 2011a). Two of the nine bird species that have gone extinct since European settlement of Australia are parrots (the paradise parrot, *Psephotus pulcherrimus* [Irestedt et al. 2019], and the Norfolk Island kaka, *Nestor productus* [Garnett et al. 2011a]): these are two of only five parrots to have become extinct in Oceania since the 16th century, and two of 16 globally (Olah et al. 2016, Olah et al. 2018). Half of all parrot species listed at the most severe level of endangerment prior to extinction in the wild – critically endangered – occur in Oceania (Olah et al. 2018). In Australia, three parrot species are currently listed at this level (IUCN 2019). One of these is one of Australia's most endangered birds, and "arguably the most threatened parrot species in the world" (page 4, Heinsohn et al. 2018): the orange-

bellied parrot (*Neophema chrysogaster*, OBP). The OBP, and its conservation program, is the major focus of this thesis.

As species are increasingly threatened, efforts to understand and reverse species declines are also escalating (Stinchcombe et al. 2002, Di Marco et al. 2017). Conservation programs can be effective: research shows that active efforts to protect birds in the two most urgent categories of conservation concern likely decreased the rate of avian extinction by two-thirds between 1994 and 2004 (Butchart et al. 2006, Pimm et al. 2006). However, there are also many conservation programs which are not successful. Conservation strategies are more likely to succeed when there is robust understanding and mitigation of the key threatening processes specific to a species in question: processes inclusive of, for example, predation, habitat destruction (or modification), disease, compromised food availability, or genetic impacts which affect long-term population viability. The lengthy conservation program supporting the OBP has almost certainly prevented this species' extinction within the last ten years (Orange-bellied Parrot Recovery Team 2016, Troy and Hehn 2019). However, despite an intensive program involving captive breeding and extensive wild monitoring and management, to date the OBP remains at critical risk of extinction (BirdLife International 2018). In this introduction, I will highlight some of the history of the OBP and its conservation management, and draw attention to the questions that remain concerning conservation of the species and its outlook for the future.

1.1 Phylogenetic context

The OBP is one of six small Australian grass parrots belonging to the genus *Neophema* (Forshaw 2010). The diet of these parrots consists primarily of seeds, but they also eat fruits and flowers, and feeding usually occurs on the ground or in low shrubs. Grass parrots forage in small flocks or pairs, can breed from the age of one, and they nest once a year, mostly in tree hollows (Higgins 1999).

No complete molecular phylogeny of the *Neophema* parrots has been published to date, but the genus has been separated into two subgenera based on habitat preference and morphology (Juniper and Parr 1998). The OBP (*N. chrysogaster*), blue-

winged parrot (*N. chrysostoma*), rock parrot (*N. petrophila*) and elegant parrot (*N. elegans*) are currently placed within the subgenus *Neonanodes*, whilst the scarlet-chested parrot (*N. splendida*) and turquoise parrot (*N. pulchella*) are in the subgenus *Neophema*. The closely related Bourke's parrot, *Neopsephotus bourkii*, was previously included in *Neophema*, but is now positioned in its own (monotypic) sister genus *Neopsephotus*. Several members of *Neophema* are kept as aviary birds due to their attractive plumage, but most are poorly studied in the wild.

1.2 Distribution and migration

The OBP is endemic to Australia and is seldom found far from coastal habitat (Brown and Wilson 1981). Historically, its range spanned four Australian states: Victoria, South Australia, Tasmania and New South Wales (Jarman 1965), but the mainland range has contracted in more recent years, and currently spans from south-eastern South Australia to southernmost Victoria (Orange-bellied Parrot Recovery Team 2006a). The OBP's breeding range is in Tasmania, in the south-west wilderness World Heritage area. Breeding once occurred at several sites along a 150-km stretch of coastline within this area (Brown and Wilson 1980), but today only one known breeding location remains, at Melaleuca in the far south-west of Tasmania (Orange-bellied Parrot Recovery Team 2016).

Each year all wild OBPs migrate between Melaleuca, where breeding occurs over the austral summer, to mainland Australia where they over-winter (Orange-bellied Parrot Recovery Team 2006a). Migration south to the breeding site occurs in September-October each year, and northwards migration occurs in March-April. The movement of birds in their mainland habitat is not well understood: it is suspected that these movements may be nomadic, but specific individuals have also been documented returning to reside at the same over-wintering sites over multiple years (Monks et al. 2019). The specifics of the migratory route are also poorly understood, although for at least part of the population the route likely includes King Island (Brown and Wilson 1981). OBPs are thought to be mostly monogamous, have an average lifespan of 2.2

years in the wild (Holdsworth 2006; maximum age reported, 11.7) and generally lay clutches of 4-6 eggs (Holdsworth 2006).

OBPs are one of only two obligate migratory parrots in the world (the other is the swift parrot, *Lathamus discolor*, which also migrates between Tasmania and the Australian mainland). The only other species in the genus *Neophema* that has been observed to undertake true migratory behaviours (though much remains to be known about most of these species) is the blue-winged parrot (Higgins 1999, Peter 2016), which is at least partially migratory.

1.3 OBP population history

1.3.1 Population size

As with many Australian species, there is little documentation regarding OBP population sizes prior to European colonisation of Australia. Anecdotal reports exist from the 1800s, some describing flocks containing thousands of individuals in South Australia, but groups of 20-30 were more commonly seen (Jarman 1965). The veracity of many early anecdotal reports has been questioned, given that several other *Neophema* are very similar in appearance to the OBP (including having orange bellies) and ranges overlap (Jarman 1965). By 1917, the decline of the OBP was being noted (Mathews 1917), and in a thorough summary of recorded sightings of the species published in 1965, Jarman refers to the OBP as "undoubtedly one of [Australia's] rarest birds" (page 166, Jarman 1965).

Concerted efforts to monitor the OBP were first undertaken in 1978 (Loyn and Chandler 1978, Carr and Kinhill Planners 1979), and further surveys in the late 1970s and early 1980s in Victoria, South Australia, and Tasmania estimated the number of OBPs to range between 70-100 individuals (Brown and Wilson 1980; 1981). In 1983, a Recovery Team was assembled to plan and manage conservation of the species. This group consisted of representatives from state governments, universities and non-governmental agencies (Smales et al. 2000, Orange-bellied Parrot Recovery Team 2006a). In 1984, the first Recovery Plan outlining conservation management options

for the species was developed (Brown and Wilson 1984). This was the first single-species Recovery Plan in Australian history.

1.3.2 Threats and reasons for decline

The factors driving the decline of the OBP in the wild are not well understood. Loss and degradation of habitat are frequently cited among the major causes (Orange-bellied Parrot Recovery Team 2006a). This is particularly the case along the mainland coast, where development and transition from saltmarsh (the preferred habitat of the species) to agricultural grazing land has occurred (Weston et al. 2012). Introduction of feral predators and competitors (e.g. cats, foxes, starlings, honeybees), as well as the presence of native predators and competitors (snakes, currawongs, tree martins, sugar gliders) on the mainland and around the breeding range has also likely had an impact (Orange-bellied Parrot Recovery Team 2016), as has presence of invasive plants (mostly invasive grasses, compromising foraging sites; Orange-bellied Parrot Recovery Team 2006a). Infectious disease outbreak is also a likely contributing factor to declines (Orange-bellied Parrot Recovery Team 2006a, Peters et al. 2014, Yang et al. 2019; see section 1.7, below). Genetic diversity in the species has been little explored to date, but given the small size of the OBP populations it is also a concern (see below, section 1.5). Aspects of the OBP's biology, including its habit of ground feeding and species behaviours including carrying out challenging biannual migrations, make it a particularly vulnerable parrot species.

The OBP is listed as critically endangered by the IUCN Red List (BirdLife International 2018, IUCN 2019), as well as Commonwealth legislation in Australia (Department of the Environment 2019), and it is listed as threatened or endangered under state legislation in all Australian states where it has occurred (Orange-bellied Parrot Recovery Team 2016, Department of the Environment 2019). Over the last four breeding seasons (2015/16 to 2018/19), the average number of birds in the wild prior to breeding has been approximately 20 (Troy and Hehn 2019).

1.3.3 Management: establishment of captive population

One of the early actions of the OBP Recovery Team was establishment of a captive breeding program (Smales et al. 2000). Captive breeding and reintroduction programs are increasingly part of species conservation actions (Seddon et al. 2007, Bowkett 2009), although in general, *in situ* conservation (conservation efforts aimed at preservation of species within their natural range) is preferable to *ex situ* (such as captive breeding) (Rahbek 1993, McGowan et al. 2017). Captive breeding/insurance populations can be difficult to establish, expensive to maintain, and raise issues around adaptation to captivity, among other challenges (Snyder et al. 1996, Frankham 2007). However, in cases where species have reached extremely low numbers in the wild (e.g. the echo parakeet, *Psittacula eques* [Raisin et al. 2012] and the Puerto Rican parrot, *Amazona vittata* [Earnhardt et al. 2014]), or where conservation in the wild is particularly difficult, establishment of captive breeding populations can be one of few options remaining (Bowkett 2009, McGowan et al. 2017). In some cases, captive insurance populations have been essential for preventing extinction, with species restoration occurring for 'extinct in the wild' species through release of individuals from captivity. This occurred for Przewalski's horse, *Equus ferus przewalskii* (Liu et al. 2014) and the Arabian oryx, *Oryx leucoryx* (Spalton et al. 1999), and a similar reintroduction process is currently underway for the Hawaiian crow/'Alalā, *Corvus hawaiiensis* (Liao 2019).

After a short period of trialling husbandry techniques with species closely related to the OBP (rock and blue-winged parrots), the first 10 founders of the OBP captive breeding population were collected from the wild and moved into captivity in Tasmania in 1986 (Smales et al. 2000). The captive program grew to contain approximately 100 birds by the mid-1990s (Smales et al. 2000, Orange-bellied Parrot Recovery Team 2006a), and in 1994 expanded to include mainland facilities (Orange-bellied Parrot Recovery Team 2006a). Additional founders were slowly incorporated into the population from the wild over these decades, with a total of 25 founders acquired from the wild and listed in the studbook by 2010 (Smales et al. 2000, Everaardt 2018b). Now, the captive population numbers approximately 440 adult birds (Hogg and Everaardt 2019).

1.4 Inbreeding and loss of genetic diversity

Although increasing numbers of individuals provides some insurance against extinction, other critical factors must also be considered in best-practice species conservation. One factor that is extremely important for the long-term outlook of species is preservation of genetic diversity (Allendorf et al. 2013). Loss of genetic diversity occurs stochastically when a species declines (e.g. Bouzat et al. 1998, Segelbacher et al. 2014); small populations are then at further risk of stochastic diversity loss via genetic drift. Overall, low genetic diversity can compromise species fitness through loss of adaptive potential and inbreeding depression. Loss of adaptive potential limits a species' ability to evolve in the face of changing environments in the future, and inbreeding depression compromises population size and growth through decreased individual fitness of inbred individuals (Frankham et al. 2002). Generations in captivity are also associated with adaptation to the captive environment, which can lead to individuals struggling to survive in the wild upon release (Chargé et al. 2014).

There are well-documented examples of inbreeding depression and loss of adaptive potential increasing the risk of extinction in both laboratory and wild settings (e.g. Bürger and Lynch 1995, Saccheri et al. 1998, Frankham et al. 1999, Frankham 2005, O'Grady et al. 2006, Wright et al. 2008). Increased expression of recessive deleterious alleles (resulting in recessive disease, one common cause of inbreeding depression) is thought to be responsible for high rates of chondrodystrophy (lethal dwarfism) in California condors, *Gymnogyps californianus* (Ralls et al. 2000), and emergence of lethal nestling blindness in a Scottish population of red-billed choughs, *Pyrrhocorax pyrrhocorax* (Trask et al. 2016). Inbreeding depression has also been identified as being responsible for low reproductive outputs in many bird species, including the kākāpō, *Strigops habroptilus* (White et al. 2015) and the South Island robin, *Petroica australis* (Heber et al. 2013). Examples of the negative outcomes arising from loss of adaptive potential in the wild are more difficult to identify, but are likely to exist (Evans and Sheldon 2008).

In captive management, various strategies can assist in maintenance of genetic diversity over time, to avoid inbreeding depression and loss of adaptive potential. The

predominant method is management of breeding by minimising mean kinship (Ballou et al. 2010), where pedigree records of all individuals in a population are used to prioritise individuals for breeding based on their calculated relatedness to the population as a whole.

For the OBP breeding program, birds in the captive population were not initially bred according to the mean kinship strategy; instead, birds were housed in group aviaries for breeding, which precluded control over founder contributions, or certainty over which birds had contributed at all (Smales et al. 2000). Relatedness between the initial founders, and parentage of all offspring generated early in the captive breeding population, are unknown factors (Everaardt 2018b), making successful later establishment of mean kinship strategies more difficult. Nevertheless, the population grew, and as time progressed management became more intensive, with single-pair aviaries established and minimising-mean-kinship strategies deployed to pair birds specifically for retention of genetic diversity (Smales et al. 2000).

1.5 Recent population management

1.5.1 Species management circa 2010

By 2010, pedigree analysis of the captive OBP population indicated that three founders (one male and two females) were dramatically over-represented in the population (Hockley and Hogg 2013). Fecundity in the population was by that point in serious decline, with high levels of egg infertility a particular concern (Orange-bellied Parrot Recovery Team 2016). Taken together, these observations suggested that inbreeding depression was occurring in this population.

In the wild population over the same period, efforts were focussed on supporting breeding, management of threats at the wild breeding site, habitat preservation, and studies of the species' behaviour. Modest reintroductions of captive birds at the main breeding site at Melaleuca also occurred over this time, with 38 captive birds released there from 1991/92 to 1993/94 (Orange-bellied Parrot Recovery Team 2006a). Despite these releases, and other efforts by the Recovery Team to minimise threats to the

species, the wild OBP population remained in decline throughout the 2000s. By the end of 2010, only 25 adult birds were detected at the wild breeding site. At this time, it was predicted to be highly likely that the species would become extinct in the wild in 3-5 years (Orange-bellied Parrot Recovery Team 2016).

1.5.2 OBP population genetic studies

Amid these concerns, in the late 2000s, genetic diversity in the OBP was assessed using 13 microsatellite markers (Coleman and Weeks 2012, Miller et al. 2013). The focus of those studies was diversity across the remaining wild OBP population and the two main captive populations that existed at the time, in Hobart at Taroona (Tasmania), and at Healesville Sanctuary (Victoria). Diversity in the species over time was also assessed, including changes in microsatellite diversity from 1991 to 2011.

These microsatellite data have not been formally published, but reports to the OBP Recovery Team at the time concluded that both wild and captive populations had lost genetic diversity since the 1990s, with the wild population having lost 25% of its diversity since 1992 (mostly through 1992-1995, Coleman and Weeks 2012). Small differences between wild and captive OBP populations were detected (F_{ST} values were small, but statistically significant), and all three populations were found to have private alleles (1-3 alleles in total across 13 loci), indicating some unique genetic diversity was present in each relative to the others (Coleman and Weeks 2012). The unique genetic diversity within both wild and captive populations was considered at risk of disappearance given the decline in reproductive output of the captive population at this time and the high probability of extinction predicted for the wild population (Orange-bellied Parrot Recovery Team 2016). Furthermore, the reported recent loss of genetic diversity was a concern for the viability of the OBP, as low genetic diversity is known to put species at greater risk of the emergence of negative effects associated with small population size and inbreeding (Frankham 2005, Allendorf et al. 2013).

1.5.3 Founder intake 2010/2011

The OBP Recovery Team was thus faced, in 2010, with a wild population at imminent risk of extinction, which likely contained genetic diversity not currently represented by birds in captivity, and a captive breeding population that was sizeable but highly inter-related, and suspected to be suffering from inbreeding depression. In response, the Recovery Team mobilised quickly and the decision was made to prioritise preservation of the remaining genetic diversity present in the wild, through transfer of 21 fledglings from the 2010/2011 breeding season into captivity (Hockley and Hogg 2013). This group represented approximately 75% of that year's wild offspring cohort (Troy and Hehn 2019). Of these, 18 birds survived to become additional founders to the captive population, and were rapidly incorporated into the reproductive strategy therein. At the most recent assessment, there were few captive OBPs which do not have the 2010/11 founders in their heritage (Everaardt 2018b). Decisions surrounding the number of birds harvested from the wild population at this time were based on information suggesting that 25 individuals ought to provide a reasonable genetic foundation for a captive breeding program (Frankham et al. 2002, Orange-bellied Parrot Recovery Team 2010).

The integration of the 2010/11 founders into the captive population appeared to improve the reproductive issues previously observed (Orange-bellied Parrot Recovery Team 2016). In the near-decade since, the captive population has grown from approximately 150 individuals to approximately 440, and today five institutions are involved in OBP breeding, overseen by the Zoo and Aquarium Association Australasia. As with many breeding programs, the captive OBP population is managed as a single meta-population, and birds are transferred between captive facilities when recommendations require this. Some issues with fertility in the captive population are still observed, with infertile eggs, low hatch rates and low fledgling survival rates still reported, albeit not to the extent observed in 2010 (Orange-bellied Parrot Recovery Team 2019).

Although the 2010 decision was made in part to preserve the genetic diversity of the OBP, no genetic follow-up to examine the outcomes of the action were undertaken.

Whether captive and wild populations remained genetically distinct, and what extent of genetic diversity was preserved in either over the subsequent years, remained unknown. Addressing these questions forms part of this thesis.

1.6 Infectious disease affecting the OBP

As well as the very small population size in the wild, there is another recent threat to both captive and wild populations of the OBP which deserves highlighting: infectious disease. Infectious disease increases the probability of extinction in endangered species (Smith et al. 2009, MacPhee and Greenwood 2013), and has compromised conservation efforts in many critically endangered avian species, including the echo parakeet, *P. eques* (Psittacine beak and feather disease, Kundu et al. 2012), the pink pigeon, *Nesoenas mayeri* (trichomonosis, Swinnerton et al. 2005, Bunbury et al. 2008), and several Hawaiian birds (avian malaria and avian pox, Warner 1968, van Riper III et al. 1986, van Riper III et al. 2002). Given the complexity of both detecting and deriving evidence for the population-scale impact of infectious disease, this threat can be difficult to quantify - in wild populations in particular.

1.6.1 Psittacine beak and feather disease

One of the most researched disease threats to both captive and wild OBP populations is Psittacine beak and feather disease (PBFD). PBFD is caused by a circular single-stranded DNA virus in the family Circoviridae (circovirus), known as *Beak and feather disease virus* (BFDV, Pass and Perry 1984, Ritchie 1995). BFDV infects parrots across the globe, though it is thought to have originated in Australia (Harkins et al. 2014). Infection with the virus can result in a spectrum of outcomes, ranging from rapid fatality to chronic subclinical disease, or even clearance of the virus with no clinical signs. One outcome of PBFD is immune system depression in otherwise healthy individuals, increasing susceptibility to secondary infections (Todd 2000). When susceptible species develop clinical disease, it can lead to beak and feather deformities and is often fatal in young birds or those with complicating conditions. All forms of

the disease have been observed in the OBP. The acute form results in rapid health decline (weight loss, depression, greenish diarrhoea and death - most common in juvenile birds), whilst the chronic form involves viral shedding in faeces and in feather material, beak and feather deformities, and often ultimately results in death. Temporary infection followed by clearing of the virus has also been recorded (Pass and Perry 1984, Ritchie 1995, Sarker et al. 2014b). There are no known effective treatment options available for this disease, and it is currently unclear exactly which intrinsic or extrinsic factors determine this variation in outcomes among OBP individuals, other than age.

There is an extensive history of recorded outbreaks of BFDV in both wild and captive OBP populations, including the death of seven of the ten birds that were the original founders of the captive population in 1986 (Smales et al. 2000). Though there was no evidence of BFDV infection or clinical disease in the wild from 2000-2006, outbreaks have been documented since (Peters et al. 2014) and a significant recent outbreak occurred in the wild population in 2015. At this time several wild offspring succumbed to the disease (though there was no evidence of adult mortality), leading to even lower juvenile recruitment than usual that year (Das et al. 2015, Troy et al. 2016). Outbreaks in the captive population are frequent, reflecting the difficulties in managing this disease owing to its environmental persistence, and the potential for subclinical carriers that shed virus into the environment. To attempt to combat this, birds are tested for presence of the virus by polymerase chain reaction tests (Shearer et al. 2009), and also sometimes HA (haemagglutination assay) or HI (hemagglutination inhibition) tests. HA tests are used to measure viral load and HI tests are used to detect and measure presence of BFDV-specific antibodies (Raidal et al. 1993). Birds that test positive for the presence of the virus are quarantined. All candidates for captive release are tested for BFDV presence repeatedly every 4-6 weeks over the months preceding their release (Williams and Baker 2015).

Presence of infectious disease makes management of translocations between populations of threatened species difficult and contentious. Most BFDV outbreaks in the OBP, however, are suspected to be spill-over events from neighbouring parrot species that are acting as reservoirs (e.g. long-billed corellas [*Cacatua tenuirostris*],

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Bourke's parrots [*Neopsephotus bourkii*]) (Peters et al. 2014, Raidal et al. 2015, Sarker et al. 2015). The presence of reservoir species and potential for cross-species transmission of disease has been identified as one of the major factors increasing risk of extinction due to disease in endangered species (de Castro and Bolker 2005).

Complicating efforts directed towards elimination of BFDV and PBFD from the OBP is the fact that there are other both biotic and abiotic reservoirs of the virus. There are examples of other Australian parrots which, in much larger populations, appear to have endemic BFDV infection, but where individuals only very rarely show clinical signs of PBFD (e.g. rainbow lorikeets *Trichoglossus haematodus* [Wildlife Health Australia 2014]), or they do so at a higher rate but it doesn't affect population viability (e.g. sulphur-crested cockatoos *Cacatua galerita* [Raidal and Peters 2018]). It seems to be the case that BFDV can be present in a species without compromising survival of that species, as long as clinical expression of PBFD and mortality in juveniles is low. Such populations are, however, a risk factor for spreading the virus to other species with which they come into contact (i.e. as reservoir species). Populations of several other wild parrot or cockatoo species that come into contact with the OBP are reservoir population candidates, as they have been found to retain disease in their populations at low or endemic levels (e.g. Sarker et al. 2014a, Sarker et al. 2015, Fogell et al. 2016).

Because BFDV is also very stable in the environment, and can persist in nesting material from year to year (Todd 2000), as well as being spread through faeces and on feathers, it is very difficult to eliminate exposure of the OBP to the virus when other birds are sharing sites or coming into contact. Nesting material provided in the OBP nesting boxes is removed each year so these boxes can be cleaned, and new material provided for subsequent years, to protect the OBP from pathogens remaining at these sites. However, as there are some birds that still use wild nests, the possible presence of BFDV in nesting material, exposing the vulnerable young, remains an issue.

1.6.2 Other infectious agents detected in the OBP

In addition to risks from BFDV infection, several other significant pathogen outbreaks have been recorded in the captive OBP population in recent years. These pathogens include *Pseudomonas aeruginosa*, a common agent of disease in captive birds. *P. aeruginosa* is a gram-negative bacterium which causes a variety of symptoms depending on its localisation in the body (Gerlach 1994). In birds, it frequently causes respiratory infections (Bailey et al. 2000), although it should be noted that this pathogen often acts as a secondary invader rather than a primary pathogen. Thus, host immune status, and co-morbidities, likely play a role in the manifestation of disease following exposure.

At the beginning of 2017, at least 16 birds died in the major OBP captive breeding facility due to infection with *P. aeruginosa* (O'Connor 2017, Yang et al. 2019). The source of this outbreak was eventually traced to the sprouted seed being fed to all birds in the facility. The seed was treated with an antimicrobial agent to prevent bacterial growth, but the strain of *Pseudomonas* involved in this outbreak had developed resistance to that agent (Whitson 2017), which occurs frequently in *P. aeruginosa* (Carmeli et al. 1999, Aloush et al. 2006). All birds were therefore likely exposed to the pathogen during this outbreak, however only some succumbed. Each of these was necropsied and cause of death was identified; in many cases cause of death was either pneumonia or septicaemia with splenitis, hepatitis or stomatitis from *P. aeruginosa* infection.

Other infectious agents identified in OBP captive populations include *Psittacid Adenovirus-2*, *Aspergillus* species, and members of the *Mycobacterium avium complex* (OBP VRTG 2019, Orange-bellied Parrot Recovery Team 2006a, Yang et al. 2019). Prevention or control of pathogen outbreaks offers wide-ranging challenges for species managers, and a paucity of detailed information about many pathogens and their interactions with birds or other components of the environment hampers biosecurity efforts. When outbreaks do occur there is a spectrum of scenarios that species managers must be prepared for. These range from limited outbreaks (such as the *P. aeruginosa* in the captive OBP population in 2017) to pathogens or diseases that

persist in populations chronically or are continuously reintroduced (such as BFDV in the OBPs). Outbreaks also range in the severity of their impact on a species and its conservation – depending on fatality levels, ages of birds affected, availability of reliable tests for pathogenic agents, and availability/effectiveness of treatments.

One area, in particular, where biosecurity concerns complicate management of endangered species is surrounding actions such as translocations of individuals – in the case of the OBP, between different captive facilities for breeding, or between captive facilities and the wild. Decision-making regarding transfer of individuals between sites, when there is suspected or confirmed disease present at one of the sites, involves careful establishment of complex risk-assessment frameworks. Risk-averse attitudes for prevention of disease spread in these scenarios sometimes conflict with management priorities, and difficult evaluations must be undertaken (for a more detailed discussion of these procedures and associated challenges, see Hartley and Sainsbury [2017]). This work is ongoing for teams responsible for management of the OBP.

The frequency and range of infectious disease outbreaks in the OBP has long concerned the Recovery Team, and there are questions concerning the species' apparently high susceptibility to disease in general. Lack of population genetic diversity has been shown to influence dynamics of disease in other species (e.g. Hawley et al. 2005, Whiteman et al. 2006, King and Lively 2012), and this has been proposed as a potential factor contributing to the prevalence of infectious disease in the OBP (Crates et al. 2017).

1.7 Recent OBP status

As part of the current Recovery Plan, the OBP Recovery Team undertakes intensive monitoring and management of the wild population and the breeding site (Orange-bellied Parrot Recovery Team 2016). However, in the years immediately following the 2010/11 removal of fledglings from the wild population, juvenile recruitment in that population declined (Troy and Hehn 2019). Wild population size over recent years has been critically small, with fewer than 20 wild birds arriving at Melaleuca for the

breeding season in 2016/17 and 2017/18. There has also been a large sex bias in birds returning from mainland migrations, with only 3-4 wild females returning to the breeding site over the last three seasons (2016/17 to 2018/19, Troy and Hehn 2019).

The main factor that appears to be keeping the current wild OBP population from extinction is the continued annual releases of captive birds, to supplement the population and correct sex ratios during the breeding season. An average of approximately 24 birds have been released to the wild population at Melaleuca each year since 2013 (Troy and Hehn 2019). Birds released to the wild are chosen based on age, general health, sex, and kinship values (Hockley and Hogg 2013). Female captive-release birds appear to successfully produce clutches, but the identities of their partners, and knowledge about released male birds' contributions to the wild population have been areas of uncertainty. Overall, current breeding rates in the wild population are lower than historical averages (Troy and Hehn 2019), and the cause of this is unknown. Low survival of released birds through the migratory season has also been a major impediment to wild population growth (Stojanovic et al. 2018, Troy and Hehn 2019).

There are many threatening processes affecting persistence of the OBP. Infectious disease threatens individual survival and decreases recruitment, whilst coastal development on the mainland and fire management of habitat near the breeding site threaten food availability. The requirements of migration to and from the mainland cause high mortality, and infertility and hatch failure affect population growth. The extent to which each of these may be interacting with, or a result of, underlying genetic dynamics in the species is not known, but investigating some of these possibilities is among the aims of this thesis.

1.8 Thesis overview

There are many uncertainties regarding the genetic status of both captive and wild OBP populations, and what actions will best manage these. In particular, questions remain around the genetic distinctiveness of captive and wild populations today, what impact the release of captive birds into the wild population may be having on wild

genetic diversity, and whether any further transfer of wild individuals into captivity is likely to be beneficial to the captive population. Meanwhile, inbreeding depression remains an unquantified concern (Orange-bellied Parrot Recovery Team 2019).

With the continuous improvement of genetic technologies providing greater scope for analysis of non-model populations, I had the opportunity in this thesis to use molecular genetics to explore principles concerning management of critically endangered species, including the impacts of founder removals and captive releases on genetic diversity.

As a species with a well-documented recent management history, the OBP provides a good candidate as a case study for analysis of the genetic impacts of translocation events: both translocation of individuals out of a wild population, and release of individuals back therein. Further, as a species with a documented history of declines in reproductive output, and encounters with disease agents, it is a promising species in which to search for interactions between genetics and these other factors.

In this study I was able to address concerns from the OBP Recovery Team, and evaluate the overall genetic status of the OBP, including impacts of translocations. I do so in four empirical chapters, as outlined below.

1.8.1 Genome-wide genetic diversity

In order to evaluate the current status of genetic diversity in both wild and captive OBP populations, I genotyped single-nucleotide polymorphism (SNP) markers in 165 OBPs from across the modern populations, as well as the founder individuals from 2010/11. I used DArTseq (Cruz et al. 2013), a form of reduced-representation sequencing (RRS; Davey et al. 2011). RRS is particularly suitable for studies of non-model organisms, and has been used to undertake genetic analyses of many other species of conservation interest (Garvin et al. 2010). SNP markers are now one of the most common marker types used in population genetics (Seeb et al. 2011), having several advantages over microsatellites, the markers previously used to assess OBP genetic diversity (Morin et al. 2004, Allendorf 2017).

In this component of my thesis, I measured levels of genome-wide diversity across captive and wild OBPs and assessed changes in diversity over time in each population. I investigated measures of population structure, and used SNP markers to conduct parentage analysis in the wild population. Through the parentage analysis, I evaluated whether captive release birds successfully contribute reproductively to the wild population after their release. I found low levels of overall genome-wide diversity in the species, but determined that captive release birds were successful contributors to the wild population. I also identified that current management actions appear to be successfully acting to preserve genetic diversity in the species over time.

1.8.2 Functional diversity

Given that infectious disease, specifically BFDV, is a known factor impacting conservation of the OBP, I then set out to investigate links between immunogenetic diversity and disease in this species. Such links between disease susceptibility/response and immunogenetic diversity have been identified in a range of other bird species (e.g. Bumstead 1998, Bonneaud et al. 2006, Boonyanuwat et al. 2006, Sepil et al. 2013). For my study in the OBP, I targeted a specific set of immune genes: the Toll-like receptors. Toll-like receptors are components of the innate immune system that are part of the first line of defence against all pathogens (Aderem and Ulevitch 2000). TLRs contain molecular pattern recognition regions that recognise a broad variety of components found in pathogens, beginning a chain of signalling that leads to initiation of host immune responses (Cormican et al. 2009). TLRs are generally well-conserved among species (Roach et al. 2005), with less complex genomic architecture than other immunogenetic regions such as the major histocompatibility complex (Velová et al. 2018). Because of their pattern recognition roles, association between specific Toll-like receptors and resistance to specific pathogens is more straightforward to evaluate (e.g. Misch and Hawn 2008, Skevaki et al. 2015). TLRs have been investigated in many bird species, including other critically endangered birds (e.g. Dalton et al. 2016), and, significantly, specific TLR receptors

have been previously linked with two of the pathogens of interest in the OBP, BFDV and *P. aeruginosa* (Zhang et al. 2005, McIsaac et al. 2012, Knafler et al. 2016).

I evaluated and compared levels of TLR diversity in the group of wild OBP founders from 2010/11, in captive OBPs from 2016, and in representatives of all other *Neophema* species. I assessed the predominant forms of selection acting at these loci, and looked for evidence of recent population dynamics. I then surveyed diversity at two TLR loci (implicated in disease response in other species) in a wider sample of OBPs that had been involved in recent disease outbreaks. I found TLR diversity and selection patterns in the OBP to be similar to measures in other critically endangered bird species, and did not identify any associations between TLR diversity and disease response.

1.8.3 Genetics and inbreeding depression

Following TLR characterisation, I investigated whether individual genome-wide diversity (in contrast to the functional diversity investigated at TLRs) plays a role in individual disease response or reproductive success in the OBP via inbreeding depression. I used heterozygosity-fitness correlations (HFCs; Hansson and Westerberg 2002, Grueber et al. 2008) to study this. Underlying the HFC methodology is the understanding that, in general, more inbred individuals are less heterozygous (Franklin 1977). Correlations between heterozygosity and fitness, therefore, indicate that low heterozygosity (caused by inbreeding) is having effects on fitness, which is inbreeding depression (Mitton 1993). The increasing availability of large numbers of SNP markers for non-model populations makes these forms of analysis more powerful than ever (Hoffman et al. 2014).

Many HFC studies have examined both reproductive fitness and disease response vs. genetic diversity in other endangered species, with a variety of findings (for reviews see Chapman et al. 2009, Fox and Reed 2010, Szulkin et al. 2010). In my study, however, I found no robust evidence that inbreeding depression is currently affecting fitness in the OBP, whether measured as disease response or reproductive output.

1.8.4 Phylogenetic analyses

Although I found no direct evidence of genetic diversity compromising individual fitness in the OBP, the low species levels of both functional and genome-wide diversity suggest a high risk of problems developing in the future. In many endangered species, fragmented populations contain slightly distinct genetic diversity, and translocation of individuals among populations is recommended to manage genetic diversity in a species overall (Frankham et al. 2017, Ralls et al. 2018). For the OBP, however, captive and wild populations are genetically similar, and no secondary population of the species exists from which to gain diversity.

The only remaining viable option for increase of genetic diversity in such cases is to consider outbreeding with a closely related species. There is increasing interest in this option as a conservation tool generally (e.g. Jackiw et al. 2015, Chan et al. 2019), but few examples exist where interspecies hybridisation has been put into practise for conservation (exceptions include hybridisation between American and Chinese chestnut, *Castanea dentata* x *C. mollissima* [Steiner et al. 2017], and between several coral species in the genus *Acropora* [Chan et al. 2018]). Examples of hybridisation for conservation purposes in animal populations primarily consist in outbreeding between distinct populations of the same species: e.g. South Island robin, *P. australis* (Heber et al. 2013), or mountain pygmy possum, *Burramys parvus* (Weeks et al. 2017); or between subspecies: e.g. Florida panther, *Puma concolor coryi* (Johnson et al. 2010), Norfolk Island boobook, *Ninox novaeseelandiae undulata* (Garnett et al. 2011b), and dusky seaside sparrow, *Ammodramus maritimus nigrescens* (Zink and Kale 1995, though this program was abandoned). Such actions are contentious, with debates existing around the legal status of "hybrid" individuals, the definition of a species, conservation value of hybrid individuals, and concerns for species health due to the risks of outbreeding depression (Rhymer and Simberloff 1996, Allendorf et al. 2001, Fitzpatrick et al. 2015). Results of outbreeding depend on the phylogenetic context of the species involved, in addition to other factors (in particular, chromosomal incompatibilities, Frankham et al. 2017), and can be unpredictable (Edmands 2002). Though controversial, in cases where species have declined to single small

populations, there are currently no alternatives for generation of genetic diversity on timescales likely to be relevant when facing extinction.

Hybridisation is known to occur among several of the *Neophema*, and hybrid individuals resulting from OBP/blue-winged parrot crosses have been previously documented in the wild (Hockley 2011, Orange-bellied Parrot Recovery Team 2016). To fully explore the viability of this option for the future management of the OBP, it is necessary to identify which species of *Neophema* is likely to be the most compatible with the OBP (i.e. its closest relative), and to assess whether these species likely have a recent common ancestor or an ancient one. As no molecular phylogenies of the complete genus *Neophema* had been previously published, I undertook a preliminary study to investigate this question. Results of this component of my study revealed that, although there is molecular evidence to support distribution of the six *Neophema* into their two current subgenera, placement of the OBP is not easily resolved, and the two mitochondrial sequences which I produced were insufficient to do so. Larger amounts of sequencing data will be required in the future to resolve this question with more certainty.

1.9 Closing remarks

Over the course of my candidature, I have helped reveal the past impacts of management actions on genetic diversity of the OBP, assessed the present status of wild and captive populations, evaluated the effectiveness of current management strategies, and determined which future actions are likely to be necessary or worth consideration. I have, over this time, shared all of my results with the OBP Recovery Team, and it is my hope that this thesis acts as a useful demonstration of the contributions that molecular genetics can make towards monitoring and management of threatened species into the future.

CHAPTER 2: Genetic impacts of conservation management actions in a critically endangered parrot species

2.1 BACKGROUND

Conservation management of the OBP has involved many actions which are likely to impact genetic diversity in the species over time, including establishment of a captive population, repeated intakes of founders into this population, and close management of captive breeding by minimising mean kinship. However, the genetic status of the species has not been assessed since prior to the last major intake of captive founders from the wild in 2010/11. The work presented in this chapter outlines the genetic impacts of recent species management actions, investigated using genome-wide SNP markers developed for the purpose. The chapter reports changes in population-wide heterozygosity subsequent to the last founder intake, compares genetic diversity from captive and wild populations, investigates parentage in the wild population, and evaluates the genetic impacts of releases of captive birds into the wild, which have been occurring since 2013.

This chapter comprises a manuscript that has been submitted for publication in Conservation Genetics:

Morrison CE, Johnson RN, Grueber CE / Hogg CJ. Genetic impacts of conservation management actions in a critically endangered parrot species. Conservation Genetics (submitted 10 November 2019).

The work has been formatted for consistency with this thesis. Supplementary material for this chapter is available in Appendix 2.

I led the research presented in this chapter. Carolyn J. Hogg, Catherine E. Grueber and Rebecca N. Johnson provided guidance in the project design and interpretation of results, and assisted in editing and finalising the manuscript.

2.2 MAIN ARTICLE

Genetic impacts of conservation management actions in a critically endangered parrot species

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ABSTRACT

Establishing populations of endangered species in captivity is becoming an increasingly common component of species recovery programs, for insurance against extinction and/or as source populations for reintroductions. It is important for the success of these efforts that captive populations are genetically representative of wild populations, and that genetic diversity is maintained over time. Our study presents SNP data from wild and captive populations of the critically endangered Australian orange-bellied parrot (*Neophema chrysogaster*). We examine the genetic effects of a decision implemented in 2010/2011, where three quarters of the juvenile cohort from that season (N = 21) were recruited from a wild population in decline to supplement an existing captive program. We report that heterozygosity among wild birds decreased in the years after this action. Following multiple releases of captive-reared birds back into the wild (occurring annually since 2013), captive and wild populations have attained similar overall levels of heterozygosity, and genetic differentiation between these populations is low. Parentage analyses confirm that captive-bred

released individuals have successfully paired with both wild and other captive release birds and produced offspring. Our study suggests that translocation of wild individuals into captivity, from declining wild populations, can have deleterious lasting impacts on genetic diversity levels in these populations. However, our data also confirm that in captivity, the addition of wild founders can improve population health, and founder diversity can be successfully preserved over time. The genetic diversity retained in captive populations can also be reintroduced to wild populations at a later date, provided that captive-release individuals are able to reproductively contribute to the recipient wild population.

Keywords: captive breeding, reintroduction, conservation genetics, reduced representation sequencing

INTRODUCTION

In the contemporary era, where unprecedented human impacts are resulting in increased habitat destruction, environmental instability and growing numbers of species threatened with extinction, conservation of wildlife is becoming more challenging yet more critical than ever. For species where protection *in situ* proves especially difficult (e.g. where threats are not well understood, or very difficult to manage) or where extremely low numbers of individuals remain, captive conservation breeding programs can provide a lifeline to perpetuation of a species (Rahbek 1993, Seddon et al. 2007). This has been the case with several high-profile conservation and captive breeding success stories, such as the black-footed ferret (*Mustela nigripes*), California condor (*Gymnogyps californianus*), and Mauritius kestrel (*Falco punctatus*), among others (Cohn 1993, Jones et al. 1994, Biggins et al. 2011). Captive conservation breeding populations are generally established and managed to 1) act as insurance populations so a species persists if numbers continue to decline in wild populations, and/or 2) provide a source of individuals for reintroduction/translocation programs, to boost wild population numbers or to establish populations at new sites (Ebenhard 1995).

In order to maintain wild-sourced genetic diversity over time, it is recommended that captive populations are managed using a mean kinship minimisation strategy (Ballou et al. 2010, Ivy and Lacy 2012). This strategy aims to equalise founder representation over time, maintain genetic diversity, and reduce inbreeding by prioritising (for breeding) those individuals with the smallest number of relatives (i.e. kin) already present in the captive population. However, many captive populations are started from modest numbers (<20 individuals) of founders, often sourced from small, fragmented wild populations, which is not ideal for capture or long-term preservation of representative genetic diversity (Ralls and Ballou 1986).

For many species with captive conservation breeding programs, further transfers of individuals between captive populations and the wild occur in order to manage population size and genetic diversity (Mills and Allendorf 1996). Such actions can be crucial to the success of these programs (Frankham 2008), but they also carry substantial risks, particularly in species with very few individuals remaining. Such risks include compromised wild populations due to introgression from captivity-adapted individuals, disruption of demographic stability in small wild populations due to removal of founders, substantial losses of individuals due to poor post-reintroduction survival, or transfer of infectious diseases between populations (Ewen et al. 2012). Unfortunately, decisions about these types of actions frequently have to be made without adequate information to predict their likely impacts on genetic diversity or on population demographics and ecology (Snyder et al. 1996, Fischer and Lindenmayer 2000).

Here, we utilise genomic data to conduct a retrospective assessment of management actions undertaken to secure the captive population of a critically endangered small Australian parrot, the orange-bellied parrot (*Neophema chrysogaster,* OBP hereafter), which is at high risk of extinction in the wild (Orange-bellied Parrot Recovery Team 2010). The OBP is one of only two obligate migratory parrots in the world, with all individuals nesting on the island of Tasmania, off the south coast of Australia, and migrating to coastal mainland Australia to over-winter. Wild populations of the OBP were observed to be in decline in the 1970s, when comprehensive surveys of historical range sites were undertaken (Brown and Wilson 1980). As a result, a Recovery Team

was established in 1983 and a Recovery Plan (the first single-species Recovery Plan in Australian history) was developed in 1984 (Brown and Wilson 1984).

Due to critically low numbers of birds in the wild, the OBP captive insurance population was established in 1986 with an intake of 10 wild founders (three of which survived to breed). Further founders were soon after incorporated and these birds bred yearly in flocking aviaries as the population grew to a stable size of around 100 individuals by the late 1990s (Orange-bellied Parrot Recovery Team 2006a). Further intakes of approximately 25 founders occurred sporadically between 1986 and 2009 (Smales et al. 2000, Everaardt 2018a). Despite active *in situ* conservation efforts, including release of 38 captive birds at the breeding site from 1991-1994, wild population numbers remained low (approximately 100 birds or fewer) over this period (Orange-bellied Parrot Recovery Team 2006a). Over time, management of the species became more intensive, and today breeding in the captive population is managed by mean kinship strategy and a species studbook is maintained.

Between 2000 and 2008, wild OBP population size was in decline by an average of 12% per year (Holdsworth et al. 2011, Orange-bellied Parrot Recovery Team 2016). By 2010 in the captive population, hatch rates were also declining and 96% of the living population was primarily descended from one male and two females (C. Hogg, *unpublished data*). A report commissioned by the OBP Recovery Team to investigate genetic diversity in both the wild and captive populations, using 13 microsatellite markers developed by Miller et al. (2013), suggested both captive and wild populations had been losing genetic diversity over the prior two decades (Coleman and Weeks 2012). This preliminary analysis also suggested that wild and captive populations were highly related, but did exhibit some genetic differentiation (in allele frequencies, with significant F_{ST} values), and the detection of private alleles indicated that captive and wild populations contained some genetic diversity unique to each (Coleman and Weeks 2012).

There were concerns that extinction of the species in the wild was imminent, and that this could entail an associated permanent loss of novel wild genetic diversity. Thus in 2010, the decision was made to undertake a further major intake of founders for the captive population, and 21 fledglings that hatched in the wild in 2010/11 were

transferred into captivity. This harvest ultimately represented 75% of the wild juvenile cohort for that year (and 40% of the total wild population; Troy and Hehn 2019). Of these birds, 18 are still genetically represented (through their descendants) in the current captive population (Everaardt 2018a).

In the period 2010-2017, the wild OBP population size at the beginning of each breeding season (prior to any captive release events) has averaged approximately 22 birds (Table 2.1, Troy and Hehn 2019). The population follows a consistent pattern of increase during the breeding season followed by only small numbers of birds completing migration to return for breeding in the following years (Holdsworth et al. 2011, Troy and Hehn 2019). The wild population has also shown a highly skewed sex ratio of migration-return birds, with male overrepresentation (Stojanovic et al. 2018, Troy and Hehn 2019). The captive population has numbered 300-400 individuals over this time, and releases of captive birds to supplement the wild population and correct the sex ratio imbalance have occurred annually during the breeding season since 2013/14 (Orange-bellied Parrot Recovery Team 2016).

Table 2.1: Total orange-bellied parrot (OBP, *Neophema chrysogaster*) population size in each year of the study. Captive and juvenile wild population census dates are at the end of the breeding season in March each year i.e. 2010/11 census is March 2011. Wild adult population census figures represent the number of animals returning at the start of the breeding season in November each year i.e. 2010/11 census is November 2010. Released birds were released at the sole remaining OBP breeding location.

Year	Captive population		Wild population		Captive cohort released
	Adult	Juvenile	Adult	Juvenile	-
2010/11	97	69 +21*	25	27^	0
2011/12	170	62	22	14	0
2012/13	174	106	19	24	0
2013/14	211	104	18	39	24
2014/15	236	107	35	29	27
2015/16	240	85	23	18	13
2016/17	250	109	17	20	23
2017/18	272	139	19	33	23

* 21 wild juveniles acquired; ^ total number of fledglings at the end of the breeding season

Here, we use SNP markers generated by reduced representation sequencing to undertake a comprehensive assessment of the genetic impacts of the founder intake in 2010/11. Specifically, we sought to address the following questions: 1) What impact did the 2010/11 harvest of wild individuals have on subsequent wild population diversity; 2) What impact did addition of those founder individuals have on diversity within the captive population; 3) Are wild and captive populations genetically distinct; 4) Are the captive-bred birds that were released in 2013-2016 successfully breeding with wild birds and, if so, 5) What impact is the release of captive birds having on genetic diversity in the wild population? The results of this analysis will be useful knowledge for current conservation management of the OBP and has implications for other conservation breeding programs, particularly those involving transfer of individuals between wild and captive insurance populations.

MATERIALS AND METHODS

All OBP samples used in this study were collected as part of ongoing conservation monitoring of the species and are accessioned in the Australian Museum collection (for details of samples, see Supplementary Table S1.1). Blood samples of up to 80 μL were collected via capillary tube following brachial wing venipuncture and stored dry on filter paper or FTA cards (for captive and wild samples collected 2013 onwards) or in Queen's lysis buffer (samples collected in 2010/11). Sampling was performed by professionals associated with institutions housing OBPs (at Taroona, Hobart, and other Zoo and Aquarium Association members), and individuals from the Tasmanian Government responsible for monitoring of wild OBPs.

DNA was extracted using one of two methods. The first method was using the Bioline Isolate II genomic DNA kit (Bioline, Eveleigh, NSW, Australia), following manufacturer's recommendations for extraction of genomic DNA from either blood or dried blood, as appropriate. The second method, used for blood samples stored on Whatman® FTA® classic or FTA® elute cards – which contain a proprietary chemical mixture for cell lysis and storage of DNA on dried cards (GE Healthcare, Illinois, USA) - was by chelex extraction based on a combination of protocols (GE Healthcare 2010,

Casquet et al. 2012). Briefly, a small section of sample card (approx. 0.5cm^2) was cut and surface scored with a clean scalpel blade. The section was washed twice with sterile water (incubated at room temperature for 10 minutes with occasional vortexing, then water removed). 150 µL of a 10% w/v suspension of chelex was added to each tube containing the washed paper, and samples were incubated overnight at 56°C with shaking at 350 rpm. After incubation, sample tubes were vortexed for 15 seconds then centrifuged for 3 minutes at 20,000 x g and the supernatant (eluate) was removed and pipetted into a clean tube.

For all extracts (N = 165 individuals), a 20 µL volume was submitted to Diversity Arrays Technologies PL (plus replicates, to a total of N = 188 samples) for amplification and sequencing using DArTseq (Cruz et al. 2013). DArTseq is a sequencing method similar to ddRAD. Enzymes used for DNA digestion were *PstI* and *SphI*. Raw data was processed using the "*de novo* assembly" pipeline in *Stacks* 2.2 (Rochette and Catchen 2017). Parameter testing following Mastretta-Yanes et al. (2015) was undertaken on a subset of samples (N = 20) to evaluate optimal settings for data processing. The aims of parameter testing were to maximise the number of polymorphic loci detected but avoid incorporating significant error rates or false positive loci. Briefly, testing occurred as follows: minimum read depth (m) was fixed at 3, as this value has been recommended to be suitable for data from most organisms (Paris et al. 2017). Number of mismatches allowed between stacks (putative loci) between individuals (n) and number of mismatches allowed between stacks/putative loci within individuals (M) were covaried, given values 1 – 6 inclusive. Error rates were found to be similar between all datasets and so the set of parameters yielding the greatest number of loci was chosen. These parameters were m = 3, n = 3 and M = 3. 100 samples with high coverage representing all sub-populations included in the study (based on hatch year and captive/wild location) were used to build the catalog (within *Stacks* module "cstacks"). The final set of filtering performed within *Stacks* included cutoffs of minimum allele frequency (MAF) = 0.01, maximum observed heterozygosity = 0.8, and inclusion of loci present in a minimum of 40% of samples (r = 0.4). When more than one SNP occurred at a locus, only one SNP was retained, selected at random using *Stacks*. All other *Stacks* settings were defaults.

Further data filtering was completed using a custom R script (Wright et al. 2019a), using the following thresholds for filtering: minimum read depth ≥ 2.5, MAF ≥ 0.03, observed heterozygosity ≤ 0.8, call rate ≥ 80%, reproducibility (calculated per locus, using technical replicates included by Diversity Arrays Technologies) ≥ 95%, and difference in coverage between reference and alternate alleles ≤ 80%.

Subsetting of data by year was based on a bird's location at the beginning of the breeding season (October) in that year. Birds are included in year group categories for all years where they were present/alive, with the exception of the 2010/11 founder group of birds (N = 16). These birds were excluded from all year groups subsequent to 2011 to prevent their signal obscuring patterns of change (seven of these birds remain in the population to date). As there were no samples available for our study of captive birds in 2010/11 or 2011/12, nor wild birds in 2011/12 or 2012/13, where comparisons between wild and captive populations were made for a given year, the 2010/11 wild founders were compared to the 2012/13 captive birds. Sampled birds which survived after release to the wild (N = 1) were included in the captive dataset prior to release, the release dataset in year of release, and the wild dataset subsequent to that, until death. To investigate population diversity, internal relatedness (IR, Amos et al. 2001) and observed heterozygosity (H_o) were calculated with GENHET for R v3.1 (Coulon 2010) using default settings. To evaluate population differentiation, principal co-ordinates analysis (PCoA) plots were produced using the R-package adegenet v2.1.1 (Jombart 2008), and F_{ST} values using R-package StAMPP v1.5.1 (Pembleton et al. 2013).

Parentage assignment was performed using the R-package SEQUOIA v1.1.1 (Huisman 2017). Recommendations for SEQUOIA include use of hundreds rather than thousands of SNPs for optimal assignment of parentage (Huisman 2019). We therefore further filtered our data to develop a set of highly informative markers for this type of analysis, with more stringent filtering on MAF (>0.05) and call rate (>0.98), as outlined in the SEQUOIA guidelines (Huisman 2019). Error rate was set as 0.03. Parentage assignment accuracy was confirmed by manually examining known sire-dam-offspring trios from pedigree studbook data.

RESULTS

After filtering, our final dataset contained 7,768 SNPs. No sex-linked loci were detected. Error rates derived from controls submitted in duplicate or triplicate, between and across plates, averaged 0.54% (range 0.15-1.15%). The more stringent filtering for parentage analysis in SEQUOIA generated 917 SNPs.

Pairwise F_{ST} values between the captive and wild populations in each given year were very low (near zero) indicative of limited genetic population differentiation (Table 2.2). However, all p-values were statistically significant ($\alpha = 0.05$) and confidence intervals did not span zero, indicating that population differentiation could nevertheless be detected with our SNP dataset. The greatest difference detected was between wild birds in 2010/11 and captive birds in 2012/13 (founders excluded) (Table 2.2). PCoA plots were consistent with F_{ST} findings (Figure 2.1). Observed and expected heterozygosities were similar to each other through the sampling period, particularly in the years with larger sample sizes (Table 2.3).

Table 2.2: Pairwise F_{ST} values for wild versus captive orange-bellied parrot (*Neophema chrysogaster*) populations in each year included in the present study. 95% confidence intervals were calculated from bootstrapping over 10,000 replicates. 2010/11 founders were excluded from all years subsequent to 2011 but otherwise year groups contain all other birds present in the listed population in that year.

	founders 2010/11 vs captive 2012/13	2013/14 wild vs captive	2014/15 wild vs captive	2015/16 wild vs captive	2016/17 wild vs captive
F_{ST} value	0.0738	0.0251	0.0140	0.0244	0.0194
95% CI range	0.0683 - 0.0794	0.0217- 0.0286	0.0131- 0.0151	0.0229- 0.0259	0.0184- 0.0205

Table 2.3: Observed heterozygosity (H_o) and expected heterozygosity (H_e) and their standard deviations (s.d.) calculated from 7768 SNPs, for wild, captive and released orange-bellied parrot (*Neophema chrysogaster*) cohorts by year. Note all individuals in a given year are represented in only one cohort: released, captive, or wild. Data for released birds is not included in captive nor wild figures.

Year	Population	N	H_o (s.d.)	H_e (s.d.)
2011/12	wild	16	0.296 (0.007)	0.281 (0.164)
2012/13	captive	3	0.273 (0.021)	0.228 (0.202)
2013/14	wild	4	0.287 (0.012)	0.254 (0.188)
	captive	33	0.287 (0.015)	0.291 (0.152)
2014/15	wild	26	0.277 (0.015)	0.282 (0.158)
	captive	46	0.290 (0.014)	0.294 (0.150)
	released	11	0.282 (0.015)	0.268 (0.173)
2015/16	wild	15	0.280 (0.012)	0.277 (0.164)
	captive	67	0.290 (0.014)	0.294 (0.148)
	released	4	0.292 (0.011)	0.239 (0.199)
2016/17	wild	28*	0.290 (0.014)	0.288 (0.158)
	captive	50	0.290 (0.014)	0.292 (0.152)
	released	17	0.289 (0.013)	0.288 (0.157)

*includes one bird released in the previous season which survived migration and returned to the breeding site

Our parentage analysis identified several instances of captive release birds successfully breeding post-release (Table 2.4). A total of 11 captive release birds were identified as sires or dams of nestlings hatched in the wild from 2014/15 to 2016/17. One brood was determined to be the result of a captive-born:captive-born pairing, whilst three other broods were identified as the result of captive-born:wild-born pairings. For remaining broods involving captive-born parentage only one parent was able to be identified.

Individual genetic diversity (measured as internal relatedness (IR), which has an inverse relationship with heterozygosity), was variable both within and between population groups in our study (Figure 2.2), although overall variation was low. The lowest median IR (corresponding to highest median heterozygosity) was evident in the 2010/11 founder group, relative to all other population groups assessed. The highest spread of IR values was detected in our 2012/13 captive sample (Figure 2.2a), which contained only three birds, two directly descended from 2010/11 founders and one a descendant of prior captive birds. This year also showed the highest median IR (lowest heterozygosity) value of our study. The second highest median IR was derived from the 2014/15 wild juvenile cohort (Figure 2.2b). Median IR was high in the captive population in 2012/13 but was stable at a lower value (i.e. higher heterozygosity) through the rest of the study period (Figure 2.2a). Wild median IR peaked in 2014/15, from a low in 2010/11 and declined 2014-2016 (Figure 2.2b) (i.e. heterozygosity initially dropped then increased). Median IR values of released cohorts were generally representative of/similar to captive birds in any given year (Figure 2.2a,c; Supplementary Figure S2.1).

Table 2.4: Parentage assignment results for orange-bellied parrots *(Neophema chrysogaster*) hatched in the wild population between 2013 and 2017, based on analysis of 917 SNPs in the R package SEQUOIA. Where parents were positively assigned their wild or captive origin is specified. No wild-origin females of breeding age were included in the dataset so no wild/wild parental pairs were identified.

Parental pair characteristics	Number of broods with parentage identified	Total number of individuals with parentage identified
Wild/captive	3	7
Captive/captive	1	2
Captive dam, sire unidentified	9	17
Captive sire, dam unidentified	2	3
Wild sire, dam unidentified	3	7
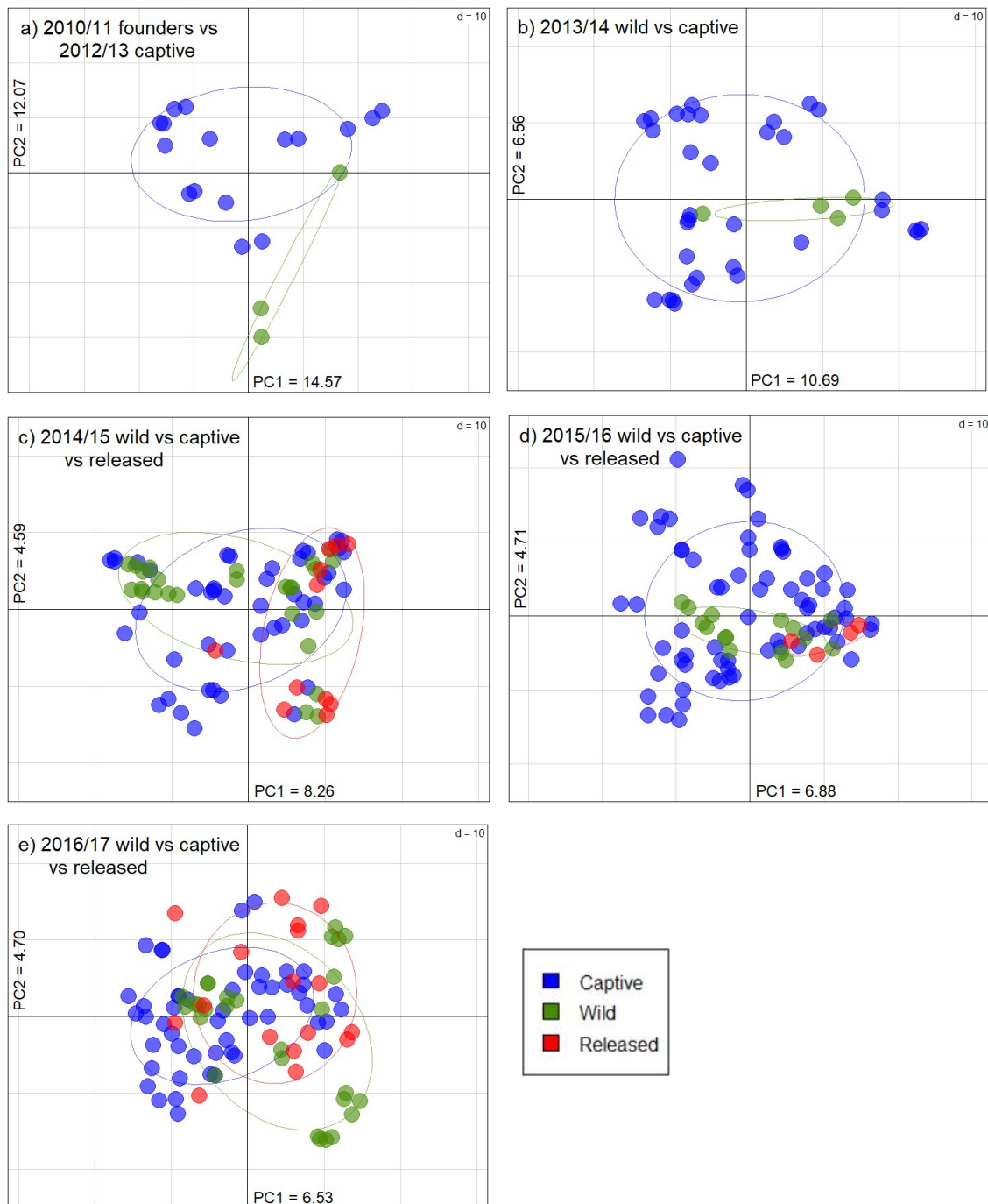
Figure 2.1: Principal co-ordinates spatial plots of SNP data for wild vs captive orange-bellied parrot (*Neophema chrysogaster*) populations from 2010-11 to 2016/17, based on principal components 1 and 2. Green dots represent wild birds, blue dots captive and orange dots release cohorts. Ellipses represent 95% confidence intervals. PC is an abbreviation of principal component.

a) Captive juvenile vs captive adult

b) Wild adults vs wild juveniles
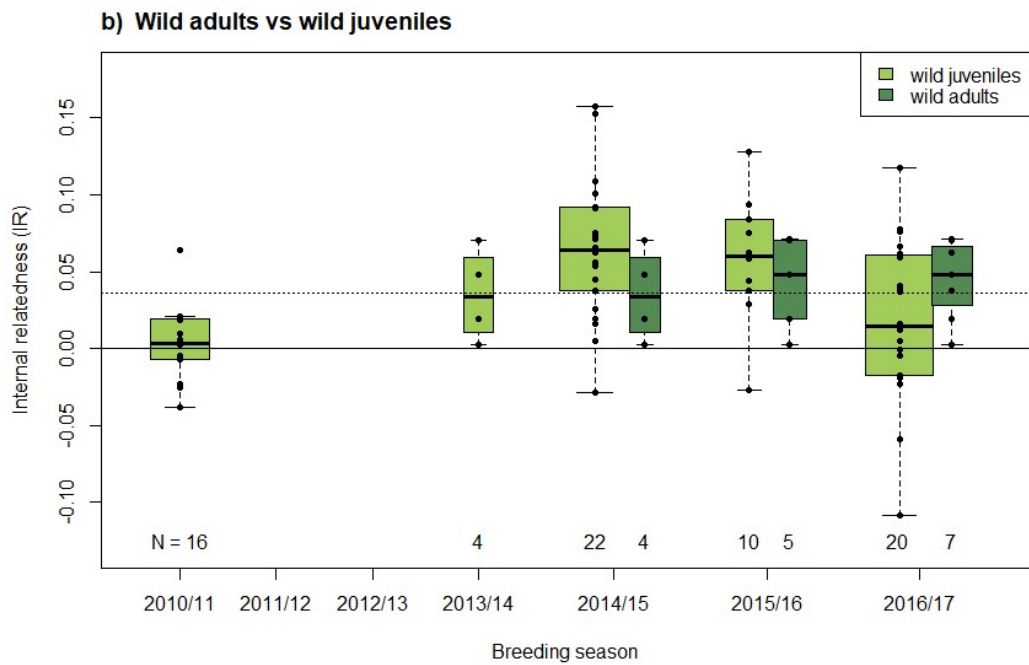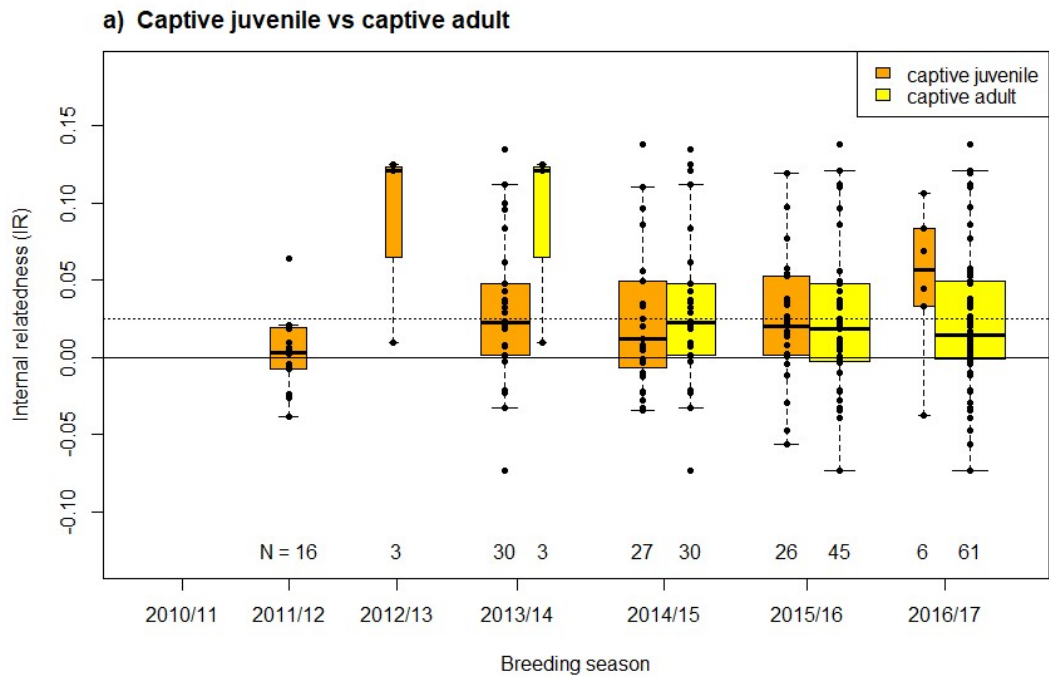
Figure 2.2: (continues on next page)

c) Wild vs released birds
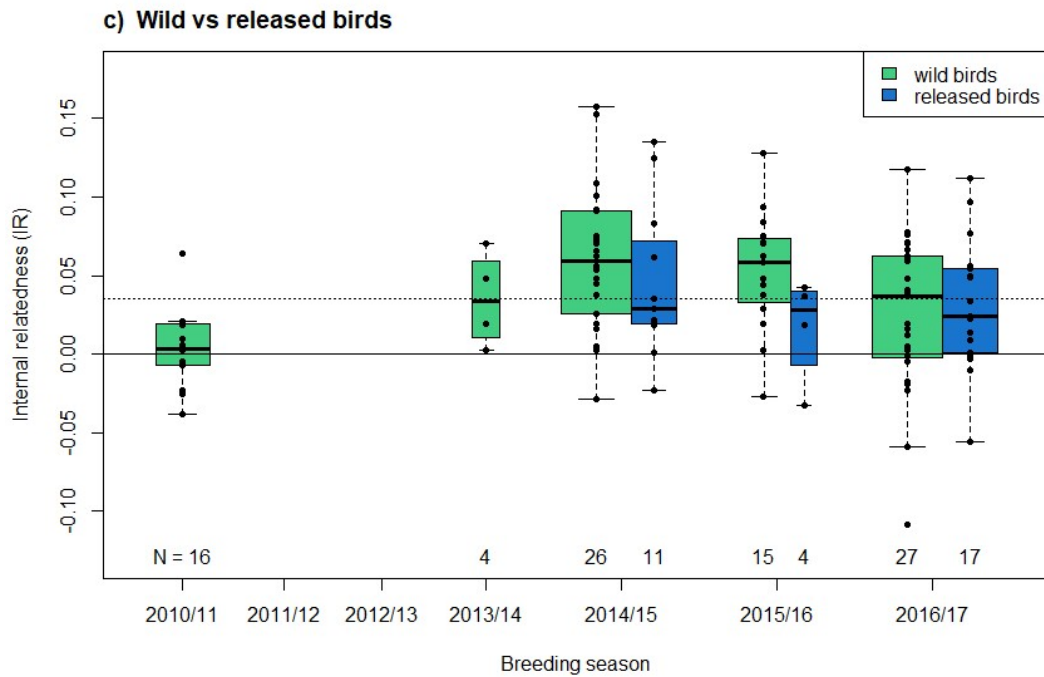
Figure 2.2: Internal relatedness (IR) over time in captive and wild populations and release cohorts of orange-bellied parrots *(Neophema chrysogaster)*, between breeding seasons spanning years 2010/11 to 2016/17. Width of boxes corresponds to sample size and dots represent individual IR values. "N =" refers to sample sizes. Boxes are bounded by quartile (Q)1 and Q3, with the median value in each box indicated by the bold bar. The whiskers represent +/- 1.5 times the interquartile range. Dotted horizontal lines represent means and are derived from data contained in each graph separately. Solid horizontal lines at x = 0 are provided as a reference point. a) Captive birds including released cohorts by year, separated into adults and juveniles; mean IR = 0.0249; b) Wild birds separated into juveniles and adults per year; mean IR = 0.0358; c) Wild birds versus released cohorts by year; mean IR= 0.0354. 2010/11 founder data was excluded from all years subsequent to 2011/12 and released birds are excluded from wild cohorts in the year of their release, but other year groups contain all other birds present in the listed population in a given year. There is overlap of individuals between years.

DISCUSSION

The action we explore in this paper – a large harvest of OBP individuals from a very small wild population – was undertaken in extreme circumstances. Extinction of the wild population was predicted to be imminent, and removal of wild individuals to captivity was at the time justified on the basis of improving the genetic health and diversity of the captive insurance population. Although this action had the intended effect of increasing captive population reproductive health, it had near-immediate negative impacts on genetic diversity within the wild population.

The removal of 21 fledglings from the wild OBP population in 2010/11 reduced wild population size and heterozygosity (Figure 2.2b, Tables 2.1 and 2.2). Preliminary data on microsatellite diversity in the species at that time suggested that the wild population was not only declining, but simultaneously losing genetic diversity prior to 2010/11 (as was the captive population). The halving of the juvenile cohort in 2010/11 likely accelerated this genetic decline, with there being fewer individuals available to contribute reproductively in 2012/13. Data presented here suggests that the large reduction in juvenile cohort size likely had a negative impact on genetic diversity. Heterozygosity (which has an inverse relationship with IR [homozygosity]) levels decreased from a study-wide high in the 2010/11 juveniles harvested for captivity to the second-lowest median value observed in our study in the 2014/15 wild juvenile cohort (Figure 2.2b). Were the pattern of increasing IR in individuals from the wild population to have continued, the situation would likely have become increasingly detrimental for the health of the wild population, with a higher and higher likelihood of that population experiencing inbreeding depression and loss of adaptive potential.

Levels of average heterozygosity (inversely proportional to IR) in the captive population were stable through the majority of our sampling period (2013-2016; Figure 2.2a, Table 2.3), indicating that the current breeding strategy (minimising mean kinship) is effectively preserving individual heterozygosity in the population. Consistency between observed and expected heterozygosities (Table 2.3) suggests that no deviation from Hardy-Weinberg equilibrium is occurring, and that no population structure was detected. This pattern also implies that the current breeding strategy is successfully equalising founder contributions. Lower heterozygosity detected in

2016/17 captive juveniles (Figure 2.2a) relative to juveniles from other years is difficult to interpret given the low sample size. The close clustering of IR values in general across the study indicates that there is relatively low variation between individuals, and between groups of individuals across years. This may be due to overlap of individuals between years, may be due to genetic homogenization of the population, or may simply be a sign that genetic changes over the period examined, whilst observable, have not been dramatic.

Because we lack captive samples suitable for direct comparison between captive birds and wild founder birds in 2010/11, our data do not allow us to assess what differences there were between heterozygosity within the two populations at that time. However, observations from the captive population indicate that this group was experiencing decreased fertility prior to the intake in 2010/11, which has subsequently improved (A. Everaardt, *unpublished data*). It is likely that the addition of novel genetic diversity into the captive population, through the introduction of wild birds collected in 2010/11, improved overall population health – but contributions from other factors to changes in fertility has not been definitively ruled out.

Recent releases of captive OBPs back into the wild at Melaleuca (the remaining breeding site) started from 2013/14 (Orange-bellied Parrot Recovery Team 2016). The parentage analysis conducted herein confirms that pairings between captive and wild birds occur post-release, that they produce viable offspring, and that they have done so over multiple years of releases (Table 2.4). We saw in each year where releases of captive OBPs occurred that median heterozygosity values of the release cohort were higher than those of the wild population, but that in successive years this difference reduced due to wild population heterozygosity levels increasing. We infer that this increase in wild heterozygosity from 2014/15 to 2016/17 was most likely driven by interbreeding of captive release and wild birds. The placement of juvenile wild cohort medians for each year in between the release and wild median IR values supports this interpretation (Figure 2.2b,c).

The wild juvenile cohort of 2016/17 had the highest median heterozygosity in our study other than that for the 2010/11 founders. That these two populations had very similar levels of heterozygosity appears to indicate that the decline in heterozygosity

in the wild OBP population since 2011 is being remedied through the release of captive birds back into the wild, and their effective reproductive contributions therein.

Genetic impacts of species management actions are the focus of this study; however, management actions may of course have unforeseen ecological impacts. A deficit in reproductive success in captive-reared individuals after their release has been observed in other species, for example the steelhead trout (*Oncorhynchus mykiss*, Araki et al. 2007). This deficit was observed in one study of the OBP (Stojanovic et al. 2018), however data for other years indicate that wild and captive release reproductive success figures are generally similar, albeit lower than historical figures (Troy and Hehn 2019). Poor condition in captive release birds has been observed, and captive-origin OBPs have been documented to depart for northward migration later than wild birds, with timing of captive-release adult migration more similar to timing of wild juveniles (Orange-bellied Parrot Recovery Team 2006a). Furthermore, the vast majority of released adult birds do not return to their breeding/release sites in years after their release (presumed dead), and additional captive individuals are currently released each year to maintain numbers in the wild population, which is costly and is not self-sustaining (Williams and Baker 2015, Stojanovic et al. 2018). Though the genetic situation of the wild population appears to be improving, its critically small size remains a serious concern. The captive population is still challenged by low rates of fertility and offspring survival (Orange-bellied Parrot Recovery Team 2019) but not to the same extent to which these were occurring in 2010.

We conclude from this study that founder intakes which remove a significant percentage of a small population in a short span of time can have strong negative impacts on genetic diversity in wild populations. A preferable option would be the integration of smaller numbers of wild individuals into a captive population over a longer period of time, rather than significant harvests in a single year, or simultaneous transfer of individuals from wild to captivity and from captivity to wild, to maintain population size, if this is an option. However, in the case where large founder harvests have been undertaken in the past, we show that release of captive individuals into wild populations can go some way towards mitigating the negative genetic impacts of those

harvests – though care must be taken to address other demographic impacts such actions may have on wild populations.

CHAPTER 3: Impact of low innate immune-gene diversity in the critically endangered orange-bellied parrot (*Neophema chrysogaster*)

3.1 BACKGROUND

Conservation of the OBP has been challenged by outbreaks of a diversity of pathogenic agents in both wild and captive populations over the course of many years. Questions exist surrounding the potential role genetics may or may not play in susceptibility of OBPs to infectious disease. Given the low genome-wide genetic diversity revealed in the OBP in the previous chapter, work presented in this chapter set out to measure diversity at functional loci – specifically, immunogenetic loci – in the species. This chapter investigates diversity at six innate immune gene (Toll-like receptor, TLR) loci in the OBP and in other parrots belonging to genera *Neophema* and *Neopsephotus*. Groups of OBPs that showed different responses to two disease agents, *Beak and feather disease virus* (BFDV) and *Pseudomonas aeruginosa*, were a particular focus. Better understanding of the role functional diversity may play in susceptibility to disease in the OBP could assist with determining the priority of species management actions into the future.

This chapter comprises a version of the following manuscript published in Emu – Austral Ornithology:

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The work has been formatted for consistency with this thesis. Supplementary material for this chapter is available in Appendix 3.

I led the research presented in this chapter. Catherine E. Grueber, Carolyn J. Hogg and Rebecca N. Johnson provided guidance in the study design, interpretation of results and drafting of the manuscript. Rosemary Gales provided information regarding captive OBP management and co-ordinated sampling. All co-authors assisted in editing and finalising the manuscript.

3.2 MAIN ARTICLE

Impact of low innate immune-gene diversity in the critically endangered orange-bellied parrot (*Neophema chrysogaster*)

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ABSTRACT

Outbreaks of disease in threatened species can increase the likelihood of extinction by reducing population size. Low immunogenetic diversity in such species can increase susceptibility to, and severity of, disease outbreaks. Our study assessed genetic diversity at innate immune genes the Toll-like receptors (TLRs) in a critically endangered Australian bird, the orange-bellied parrot (*Neophema chrysogaster*). Conservation management of this species has recently been challenged by several infectious disease outbreaks. We found low diversity at six TLR loci in both captive and wild orange-bellied parrot populations, similar to results seen in other threatened bird species. Three loci were found to be monomorphic across all samples, including birds which were recorded to have been involved in recent infectious disease outbreaks. Phylogenetic analysis of TLR sequences from *Neophema chrysogaster* as well as representatives of all other species within parrot genera *Neophema* and *Neopsephotus* revealed a small number of sites under pervasive and/or episodic

positive selection, and a number of sites under negative selection. This study is the first to investigate functional genetic diversity in the orange-bellied parrot.

INTRODUCTION

Disease outbreaks in small isolated populations of endangered species are a major threatening process that can accelerate entry into the extinction vortex (Gilpin and Soulé 1986, Caughley 1994). Previous studies have implicated disease outbreaks as contributing to near-extinction events in several species, including the Bellinger River snapping turtle (Zhang et al. 2018), land snails *Partula turgida* (Cunningham and Daszak 1998), and amphibians affected by chytrid fungus (Schloegel et al. 2006). Low genetic diversity can increase both individual and population susceptibility to infectious disease (e.g. O'Brien et al. 1985, Roelke et al. 1993, Whiteman et al. 2006), and has also been implicated in increased disease severity (e.g. Ilmonen et al. 2008, Elbers et al. 2018). Measurement and monitoring of genetic diversity, especially immunogenetic diversity, has become an increasingly common component of endangered species management and research (Schwartz et al. 2007).

The critically endangered orange-bellied parrot (*Neophema chrysogaster*, OBP) is one of six small Australian grass parakeets in the genus *Neophema*. This species is unusual in that it is one of only two obligate migratory parrot species in the world (Juniper and Parr 1998). The sole wild population nests over the austral summer at a single wild breeding site, Melaleuca (south-west Tasmania), and in autumn the birds migrate to over-winter along the south coast of mainland Australia. In the past three years (2015-2017), at the beginning of each breeding season the wild population has numbered fewer than 25 birds. Historical records since European colonisation of Australia are limited, but indicate that much larger population sizes were likely present in the relatively recent past (Jarman 1965).

More detailed monitoring has been undertaken since 1979, and the population size was estimated to be in the vicinity of 70 - 90 individuals for much of the 1980s (Brown and Wilson 1980). For unknown reasons, a slow decline began in the 2000s, until in 2010 there were fewer than 50 birds known in the wild (Pritchard 2012). A captive OBP insurance population was founded in 1986 for conservation purposes with 3 birds, followed by supplementation in 1987 (5 birds), 1988 (1), 1993 (5), 1996 (2), 1997 (3), 2001 (2), 2008 (2), 2010 (3) and 2011 (21) (ZAA 2018). In 2018, the captive population contained 344 individuals (Everaardt 2018a) and the wild population fewer than 20 individuals. Captive breeding is managed through a mean kinship minimisation strategy based on pedigree analysis to maintain genetic diversity (Ballou *et al.* 2010).

There are multiple significant threats to OBP populations (Orange-bellied Parrot Recovery Team 2016). Key infectious disease threats include Psittacine beak and feather disease (PBFD), caused by *Beak and feather disease virus* (BFDV, Peters et al. 2014, Raidal et al. 2015), aspergillosis, caused by *Aspergillus* spp. (Orange-bellied Parrot Recovery Team 2006a), tuberculosis, caused by members of the *Mycobacterium avium complex* (OBP VRTG 2019), adenoviral infection (*Psittacid adenovirus-2*, Yang et al. 2019), and infection with *Pseudomonas aeruginosa* (O'Connor 2017). In other species of *Neophema* or *Neopsephotus*, the only publications addressing disease susceptibility or outbreak frequency to date have been case studies (e.g. Jung et al. 2009, Shivaprasad and Phalen 2012). Broader disease research has been published on other endangered Psittacids (e.g. Raidal et al. 2015, Tollington et al. 2015, Vaz et al. 2017).

Given the critically small population size of the OBP, disease outbreaks pose a special risk to this species (Stojanovic et al. 2018). Two recent disease outbreaks have highlighted the severity of this risk. In 2014/15, an outbreak of BFDV at Melaleuca (Das et al. 2015), resulted in the deaths of several nestlings and led to lower juvenile recruitment that year than in any of the previous five years (Troy et al. 2016). The wild population in 2015/16 numbered only 23 individuals as a result. In a captive parallel, 16 of the highest-priority captive breeding pairs fledged no young due to the death of at

least one of the parents from *P. aeruginosa* infection, which led to an overall reduction in captive population size (Hogg and Everaardt 2019).

Toll-like receptors (TLRs), a family of innate immune genes, are part of the first line of defence against invading pathogens. Variation at TLR loci has been implicated in differential responses to diseases in many species (e.g. Heng et al. 2011, Skevaki et al. 2015, Ruiz-Rodriguez et al. 2017), including birds (Bateson et al. 2016, Knafler et al. 2016). There are 10 TLR genes currently known to exist in birds (Brownlie and Allan 2011): note that the naming conventions of TLR1 (previously TLR1LB), and TLR10 (previously TLR1LA), have recently changed (Velová et al. 2018). Of significance to the situation of the OBP, TLR3 has been tentatively associated with BFDV immunity/susceptibility in another species of parrot (red-crowned parakeet, *Cyanoramphus novaezelandiae*; Knafler et al. 2016), and TLR5 has been specifically linked to immune recognition of *P. aeruginosa* in many studies in humans and mice (e.g., Zhang et al. 2005, McIsaac et al. 2012), as well as other mammals (Hayashi et al. 2001), and reptiles (Voogdt et al. 2016).

No previous studies have investigated immunogenetic diversity in the OBP nor in any related *Neophema* species. Given the importance of disease processes in OBP conservation, this study aimed to: 1) quantify functional genetic diversity in OBPs by sequencing six TLR loci; 2) test whether diversity at TLR loci may have influenced OBP responses to two recent pathogen outbreaks: *beak and feather disease virus* and *P. aeruginosa*; and 3) use TLR sequences from the OBP and closely related species to learn more about how TLR genes have evolved in parrots.

METHODS

Sample collection and DNA extraction

All OBP samples used in this study were collected as part of ongoing conservation monitoring of the species and are accessioned in the Australian Museum collection (Supplementary Table S1.1). Sampling was via brachial wing venipuncture followed by

collection of up to 80 μL of blood by capillary tube and subsequent storage either dried on filter paper (captive and wild samples collected 2013 onwards) or in Queen's lysis buffer (samples collected 2010/2011). Sampling was performed by professionals associated with OBP housing institutions or those responsible for monitoring of wild OBPs.

The other species examined in this study were: blue-winged parrot (*N. chrysostoma*); rock parrot (*N. petrophila*); elegant parrot (*N. elegans*); turquoise parrot (*N. pulchella*); scarlet-chested parrot (*N. splendida*); and Bourke's parrot (*Neopsephotus bourkii*). Each of these parrots is native to Australia, with varying distributions covering much of the country. All are categorised on the IUCN Red List as of Least Concern. Samples of *Neophema*/*Neopsephotus* species were sourced from frozen tissue collections of Museum Victoria (Melbourne, VIC) and the Australian National Wildlife Collection (Canberra, ACT) (Supplementary Table S1.1). DNA from all samples was extracted using Bioline Isolate II genomic DNA kits (Bioline, Eveleigh, NSW, Australia), following manufacturer's guidelines.

TLR amplification and sequence analysis

DNA samples from 20 OBPs, as well as additional representative samples of *Neophema* (five further species, as above) and *Neopsephotus bourkii*, were used for TLR screening. OBP samples included 10 individuals hatched in the wild in 2010, later removed for supplementation of the captive population (hereafter referred to as "2011 founders"), and samples collected between 2013 and 2015 from 10 captive birds. Efforts were made not to include individuals that were directly descended from the 2011 founders, however, as there have been a number of translocation events between wild and captive OBP populations since the 1990s the two sets of 10 samples likely do not represent fully distinct groups.

Full methods describing the PCR amplification and direct sequencing of binding regions of TLR loci are provided in Supplementary Methods (Appendix 3). In short, we used a combination of primers developed for other bird species (Alcaide and Edwards 2011, Grueber and Jamieson 2013), and primers developed specifically for

Neophema/*Neopsephotus* in this study following previously published methods (Grueber and Jamieson 2013) (Supplementary Tables S3.2, S3.3). For diversity screening at each TLR locus, our alignment of 20 OBP sequences was imported into DnaSP v.5.10.01 (Librado and Rozas 2009). Sequence data was phased into haplotypes (default settings), and DnaSP was then used to calculate haplotype number and diversity, nucleotide diversity (π), and Tajima's D (as a test of neutrality, Tajima 1989).

Disease association analysis

We used additional wild and/or captive OBP samples to test whether TLR diversity is associated with response to BFDV and *P. aeruginosa* following recent outbreaks in the OBP. We focused on birds involved in either a *P. aeruginosa* outbreak (one captive breeding site in 2017), or in any of the several small and/or ongoing outbreaks of BFDV in both wild and captive populations. Individuals were considered for inclusion in this analysis if they were present in the relevant population at the time of the outbreak. Individuals were included as "affected" if they died (*P. aeruginosa*) or tested positive for presence of BFDV (via a PCR test). Individuals were included as "unaffected" if they survived *(P. aeruginosa*), or tested negative for presence of BFDV. Captive birds included individuals hatched between 2010 (including founders) and 2017, and wild samples included samples from birds collected in 2015 and 2016. Using our TLR amplification methods, we targeted TLR3 for investigation of BFDV immunity (N = 93 OBPs) and TLR5 for investigation of *P. aeruginosa* (N = 49 OBPs). These loci have been (to varying extents) associated with responses to these two pathogens in other species (McIsaac et al. 2012, Knafler et al. 2016).

Evolution of *Neophema* TLR sequences

We conducted phylogenetic sequence-evolution analysis using our polymorphic OBP TLR alignments, as well as a multispecies alignment that included up to seven sequences (six *Neophema* spp. (including OBP), plus *Neopsephotus bourkii*) for each TLR gene. Evidence of selection on TLR codons was tested using HyPhy (Kosakovsky

Pond et al. 2005) on the Datamonkey web server (Weaver et al. 2018) as well as CodeML within PAML 4.0 (Yang 2007). Episodic selection may affect residues within immune genes such as avian TLRs (e.g., Grueber et al. 2014, Raven et al. 2017), so we used a range of standard pervasive as well as episodic (i.e. branch-site) methods to examine positive and negative selection. Full selection model specifications are provided in the Supplementary Methods (Appendix 3). MEGA-X (Kumar et al. 2018) was used to build neighbour-joining phylogenetic trees (Supplementary Figure S3.1). Further details of phylogenetic methods are outlined in Appendix 3.

RESULTS

TLR diversity of the OBP

A total of 260 sequences were produced in this study (Table S1.1). This included 20 OBP sequences for diversity screening of 6 TLR loci (N = 10, 2011 founders; N = 10 modern captive birds). TLR3, TLR5 and TLR7 fragments were monomorphic, whilst TLR1 (previously known as avian TLR1LB, Velová et al. 2018), TLR10 (previously known as avian TLR1LA, Velová et al. 2018) and TLR4 showed low levels of polymorphism (Table 3.1). Values for Tajima's D were statistically significant only for TLR1 (Table 3.1).

Disease association analysis

Sequences for TLR3 (N = 93, 35 "affected" birds and 58 "unaffected") and TLR5 (N = 49, 17 "affected" and 32 "unaffected") were generated to investigate potential disease response associations in OBP. Although we included "affected" and "unaffected" individuals in both datasets, and these sample sizes represent relatively large proportions of the captive OBP population, we found no variation within the amplified regions of either gene.

Evolution of Neophema TLR sequences

For our OBP alignments, we tested for evidence of selection at the three polymorphic loci (TLR1, TLR4, TLR10). Site models (PAML and MEME/FUBAR) did not detect signals of positive selection at any variable codons (Table 3.2, Supplementary Table S3.5). Negative selection was only detected in TLR1, at one site, by FUBAR and some of the HyPhy methods which it superseded (Table 3.2, Supplementary Tables S3.3 & S3.4).

Phylogenetic evolutionary analysis of 6 TLR loci across the broader taxon revealed moderate levels of sequence divergence among species (Table 3.3). Selection tests on the multispecies alignment showed evidence of negative selection at several sites (Table 3.2). There were also a small number of sites for which signals of positive selection were detected at four loci (TLR1, TLR3, TLR4, TLR5; Table 3.2). Sites identified by various selection tests were broadly consistent with one-another (Table 3.2, Supplementary Tables S3.3 & S3.4). Concordances between MEME and the tests of pervasive selection were also seen; where this was not the case (e.g., *Neophema* TLR5) it may be because MEME tests specifically for episodic, rather than pervasive, positive selection. Lack of concurrence across methods may also result from our low sample size (low number of polymorphic codons).

Phylogenetic trees conformed with currently accepted divisions in the genus (Supplementary Figure S3.1). Because levels of sequence variation between the six *Neophema* species are relatively low, the internal structure of the trees was not well resolved.

Table 3.1: Details of orange-bellied parrot (*Neophema chrysogaster*) Toll-like receptor sequence datasets and diversity indices

Locus	N	Sequence length (bp)	Sequence length (aa)	No. SNPs (synonymous: non-synonymous)	No. estimated haplotypes	Haplotype diversity	Nucleotide diversity	Tajima's D
TLR1	20	615	205	6 (4:2)	4	0.735	0.0046	2.745 **
TLR3	93	951	317	0	1	0	0	n/a
TLR4	20	738	246	3 (1:2)	3	0.501	0.0019	0.542 (ns)
TLR5	49	864	288	0	1	0	0	n/a
TLR7	20	579	193	0	1	0	0	n/a
TLR10	20	924	308	4 (0:4)	5	0.656	0.0016	1.02 (ns)

Abbreviations: N = number of birds, bp = base pairs, aa = amino acids, SNPs = single nucleotide polymorphisms, ns = non-significant
**p < 0.01

Table 3.2: Results of positive selection tests from Toll-like receptor sequence alignments for the orange-bellied parrot (*Neophema chrysogaster*), and representatives of *Neophema* and *Neopsephotus* genera, implemented in HyPhy. Where sites were identified as being under selection, they are labelled in the table by numbers corresponding to codon positions in the sequence alignment. (See Supplementary Table S3.6 for codon positions corresponding to other published sequences). Acronyms in the heading refer to specific selection tests.

Dataset	N	Locus	FUBAR[1] +ve	-ve	MEME[2] +ve	aBSREL[3] +ve
OBP	20	TLR1	None	165 (0.919)	None	None
	20	TLR4	None	None	None	None
	20	TLR10	None	None	None	None
Neophema/ Neopsephotus	7	TLR1	7 (0.968)^	30 (0.903) 173 (0.978)	7 (0.0665)	None
	7	TLR3	259 (0.914)	123 (0.903) 189 (0.913) 264 (0.902) 322 (0.945)	None	None
	7	TLR4	101 (0.998) 183 (0.998)	38 (0.989) 227 (0.926) 254 (0.916) 269 (0.919)	183 (0.0695)	Node leading to scarlet-chested, turquoise and Bourke's parrot
	7	TLR5	None	78 (0.906)	149 (0.072)	None
	7	TLR7	None	23 (0.906) 74 (0.921) 89 (0.921)	None	None
	7	TLR10	None	57 (0.922) 96 (0.910) 256 (0.901) 284 (0.903)	None	None

[1] Only sites are shown that met the posterior probability threshold of 0.9 (value in parentheses)

[2] Only sites are shown that met the *p* value threshold of 0.1 (value in parentheses)

[3] This method uses a likelihood ratio test, with a *p* value (corrected for multiple testing) threshold of 0.05

^This site was the sole site also identified by PAML analyses as showing signs of positive selection, in model comparisons M1a vs. M2a and M7 vs. M8 ($p < 0.001$, BEB > 0.95) (see Supplementary Table S3.5)

Table 3.3: Diversity in the form of polymorphisms/variable sites, and alignment lengths, from Toll-like receptor sequence alignment datasets. Alignments contain sequences from each member of genera *Neophema* and *Neopsephotus* (a single sequence per species).

Locus	Alignment length (nt)	Length (aa)	Variable sites	Synonymous/non-synonymous
TLR1	648	216	34	17/17
TLR3	909	303	31	13/18
TLR4	687	229	41	21/20
TLR5	840	280	30	16/14
TLR7	603	201	12	4/8
TLR10	918	306	37	15/22

DISCUSSION

Here we investigated innate immune diversity at TLR regions in a critically endangered bird and its closest relatives. We assessed diversity across both wild and captive populations of the OBP, and special focus was given to subsets of the populations which have been involved in recent disease outbreaks. Our findings indicate that the OBP has low species-wide diversity at the six TLR loci investigated, consistent with findings in other threatened bird species (Table 3.4). Unfortunately no published data was available for performing comparisons of species-wide TLR diversity in the OBP relative to population diversity within other *Neophema* species.

TLRs are a key component of the innate immune system and part of the first line of immune defence, key for recognising and responding to pathogen threats from viral, bacterial, fungal and other parasitic agents (Uematsu and Akira 2008, Brownlie and Allan 2011). The low general level of diversity observed at these critical immune genes is a concern for OBPs. It indicates that this species may be more vulnerable to disease outbreaks and/or that the species may be slow or unable to adapt to novel pathogens (Ross-Gillespie et al. 2007).

Table 3.4: Published diversity statistics for Toll-like receptor loci from other bird species compared with statistics from this study

Gene	Species	Conservation status	N	Length (bp)	# SNPs (s:ns)	h	Nucleotide diversity (π)
TLR1	OBP [1]	CR	20	617	6 (4:2)	4	0.0046
	White-winged flufftail [2] (*Sarothrura ayresi*)	CR	10	960	5 (3:2)	5	0.0014
	Stewart Island robin [3] (*Petroica australis rakiura*)	severely bottlenecked	10	971	3 (2:1)	2	0.0016
	Seychelles warbler [4] (*Acrocephalus sechellensis*)	bottlenecked	33	750	2 (0:2)	4	0.0011
	Lesser kestrel [5] (*Falco naumanni*)	LC	8	990	16 (12:4)	15	0.0039
	House finch [5] (*Carpodacus mexicanus*)	LC	8	951	25	20	0.0067
TLR4	OBP [1]	CR	20	740	3 (1:2)	3	0.0019
	White-winged flufftail [2]	CR	10	630	0	1	0.0000
	Stewart Island robin [3]	severely bottlenecked	10	649	4 (0:4)	5	0.0027
	Seychelles warbler [4]	bottlenecked	30	648	0	1	0
	Lesser kestrel [5]	LC	8	818	6 (6:0)	7	0.0026
	House finch [5]	LC	8	789	16 (8:8)	14	0.0049
TLR10	OBP [1]	CR	20	924	4 (0:4)	5	0.0016
	White-winged flufftail [2]	CR	10	642	5 (1:4)	4	0.0020
	Stewart Island robin [3]	severely bottlenecked	10	1166	2 (1:1)	2	0.0009
	Seychelles warbler [4]	bottlenecked	22	531	1 (0:1)	2	0.0007
	Lesser kestrel [5]	LC	8	1163	19 (14:5)	11	0.0039
	House finch [5]	LC	51	1161	44 (27:17)	62	0.0058

Abbreviations: N = number of birds; bp = base pairs; SNPs = single nucleotide polymorphisms; s:ns = synonymous and non-synonymous SNPs; h = number of haplotypes; OBP = orange-bellied parrot; CR = critically endangered; LC = least concern (IUCN conservation statuses).
References: [1] This study; [2] Dalton et al. 2016; [3] Grueber et al. 2012; [4] Gilroy et al. 2017; [5] Alcaide and Edwards 2011

The very low level of TLR diversity we observed here precludes formation of conclusive inferences surrounding the role of sequence variation at these genes in two recent disease outbreaks. We focussed our analysis on the TLR regions that were suspected to be the most likely to show patterns, if they exist: TLR3 has been associated with *Beak and feather disease virus* in the red-crowned parakeet (*Cyanoramphus novaezelandiae* [Knafler et al. 2016]) whilst TLR5 has been associated with *P. aeruginosa* in several mammals and at least one reptile (e.g., Hayashi et al. 2001, McIsaac et al. 2012, Voogdt et al. 2016). However, because these genes were monomorphic in sampled OBPs (despite sampling covering relatively large proportions of the population), no association between diversity and disease outcomes could be investigated. Alternative explanations for variation in disease response include genetic variation elsewhere in the genome, or non-genetic factors such as general condition of the individual, presence of secondary infections, or factors relating to the pathogen rather than the host.

There is some uncertainty regarding whether TLR3 is the locus responsible for initiating immune response to BFDV in parrots. We selected this locus because Knafler et al. (2016) found indications of directional selection occurring at TLR3 through an outbreak of BFDV in an island population of the parrot *Cyanoramphus novaezelandiae*, but no further evidence directly linking TLR3 with BFDV has been reported to date. In other species, TLR3 is known to target dsRNA rather than ssDNA, however no other TLR has been identified in parrots that does recognise ssDNA. Alternative candidates for TLR response to this virus may include TLR7, as this has also been associated with responses to viral agents (albeit RNA viruses, Khan et al. [2019]), and this may be an appropriate avenue for future investigations into BFDV and the TLRs.

The lack of diversity at TLR3 and TLR5 in the OBP may be the result of past selection, such as a selective sweep in response to a common pathogen, as has been reported in other species (Teacher et al. 2009, De Groot and Bontrop 2013, Kosch et al. 2016), or may be a result of small population size and genetic drift. As more and more avian reference genomes are published (e.g. the "B10K" project; Zhang 2015), our understanding of the complex avian immunome (Schat et al. 2014) will improve, and

further studies linking immune genotypes to disease resistance or susceptibility phenotypes will be able to be completed.

Selection tests applied to polymorphic TLRs detected few signs of selection within OBPs, probably due to low rates of polymorphism in general. One TLR1 site showed negative selection in the OBP, which was also the predominant form of selection identified to be acting on TLRs across the broader taxon. This result is consistent with observations in other studies of avian TLRs (e.g. Alcaide and Edwards 2011, Grueber et al. 2014). We found no evidence that the factors shaping TLR diversity in the OBP differed from those affecting related species. There was some evidence of episodic diversifying selection at TLR4, specifically impacting the clade containing the scarlet-chested, turquoise and Bourke's parrots. These are likely the species the most divergent from the OBP, though no molecular phylogenies of the complete genus have been published to date to confirm this.

Our findings of low TLR diversity align with observations of low-moderate OBP microsatellite diversity (Miller et al. 2013), although we note that we do not necessarily expect a close relationship between microsatellite and TLR diversity (Grueber et al. 2015). It is likely that OBP functional diversity is low genome-wide, given the species' history of recent small population size. Our Tajima's D results are consistent with this hypothesis, as a statistically significant result for TLR1 indicates above-average representation of haplotypes of intermediate frequency at this locus, and an absence of low- or high-frequency haplotypes. This pattern is characteristic of populations having undergone recent sudden contractions (Tajima 1989). Further analysis of disease interactions using genome-wide markers, such as those obtained via reduced-representation sequencing methods (Miller et al. 2007, Baird et al. 2008) would be useful to further inform species management (Dierickx et al. 2015, Dresser et al. 2017).

As one of the most critically endangered bird species in Australia any data that helps to understand OBP responses to prevailing threats will assist conservation management. Given established links between low immunogenetic diversity and disease threats in other species, our observation of low TLR diversity here supports the current methods of OBP management that are intended to maximise genome-wide

genetic diversity. Existing protocols to limit disease transmission risks are also prudent (Orange-bellied Parrot Recovery Team 2006b, Fogell et al. 2019). Future measurement of genetic diversity at other immunogenetic loci in the species, for comparison with TLR diversity, would be of interest (e.g., Bateson et al. 2016, Knafler et al. 2017).

ETHICS STATEMENT

Blood samples used in this study were collected for management purposes, i.e. disease screening and sexing of fledglings under DPIPWE's Standard Operating Procedures for orange-bellied parrots.

CHAPTER 4: Heterozygosity, reproductive fitness and disease response in a critically endangered Australian parrot

4.1 BACKGROUND

Although conservation management has sought to minimise loss of genetic diversity over time in the OBP, previous studies have indicated that genetic diversity was lost between 1990 and 2010, and questions regarding the impacts of low diversity on OBP population health continue to be raised. Low genetic diversity in small populations can lead to inbreeding depression, which manifests as reduced fitness of inbred individuals, and can include increased susceptibility to disease, and/or low reproductive output. Given the low level of functional diversity found in the OBP at TLR loci in the previous chapter, and the low genome-wide heterozygosity in the chapter before, the work in this chapter investigates whether associations between individual fitness and individual genetic diversity (indicative of inbreeding depression) can be found. Fitness measures investigated included reproductive output, and, in continuity with the previous chapter, individual response to BFDV and *P. aeruginosa*. Improved understanding of the factors underlying documented low reproductive outputs in the OBP and responses to frequent outbreaks of infectious disease is a management priority.

This chapter comprises a manuscript which is in preparation for submission to the journal Animal Conservation. Supplementary material for this chapter is available in Appendix 4.

I led the research in this chapter. Catherine E. Grueber, Carolyn J. Hogg and Rebecca N. Johnson provided guidance on the study design, interpretation of results, drafting of the manuscript and assisted in editing and finalising the manuscript.

4.2 MAIN ARTICLE

Heterozygosity, reproductive fitness and disease response in a critically endangered Australian parrot

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ABSTRACT

Understanding the interactions between inbreeding, genetic diversity and fitness is of growing importance and urgency as the number of species existing in small or fragmented populations increases. These interactions can be studied via heterozygosity-fitness correlations (HFC), and the results used to draw inferences about inbreeding in threatened populations. We examined HFCs in one of Australia's most endangered birds, the orange-bellied parrot (*Neophema chrysogaster*). We tested whether variation in individual heterozygosity (measured as internal relatedness, IR) was associated with variation in reproductive fitness (number of fledglings produced, and number of fledglings to survive to maturity) and/or response to two disease agents: *Pseudomonas aeruginosa* and *Beak and feather disease virus*. No compelling impacts of inbreeding depression in the OBP were found. We did identify a strong correlation between age and reproductive success (number of fledglings produced). We suggest ongoing data collection on earlier stages of reproductive success and on fitness measures from the wild OBP population to continue exploring this area.

Keywords: heterozygosity-fitness correlation, inbreeding depression, Psittacine beak and feather disease, reproductive fitness

INTRODUCTION

Small populations of endangered species are at risk of loss of genetic diversity and increased inbreeding. Increased inbreeding is associated with a decrease in individual heterozygosity, which can cause decreased fitness (inbreeding depression). There are many well-known examples of inbreeding depression in endangered species, including the Florida panther (*Puma concolor coryi*, decreased male fertility; Roelke et al. 1993) and the Californian condor (*Gymnogyps californianus*, increased prevalence of a recessive disease; Ralls et al. 2000). Because inbreeding depression reduces rates of reproduction and survival, it has the capacity to severely hamper conservation efforts, and for this reason studies of inbreeding depression are of critical importance for informing management of endangered species.

Because more inbred individuals from a population have, on average, lower heterozygosity than less inbred individuals, studying the links between individual genome-wide heterozygosity and fitness variation is one approach to investigating inbreeding depression (Hansson and Westerberg 2002). There are many examples of heterozygosity-fitness correlation (HFC) studies in the literature (reviews include David [1998], and Chapman et al. [2009] – and for a detailed discussion of criticisms of, and assumptions underlying, the methodology see Szulkin et al. [2010]). As opposed to studies using non-molecular measures of inbreeding, such as inbreeding coefficients derived from pedigrees (e.g. Kruuk et al. 2002, see Keller et al. 2002 for a review), many past molecular-based studies use heterozygosity values derived from small numbers of microsatellites (Szulkin et al. 2010; see Grueber et al. 2011b, Nietlisbach et al. 2017 and Forstmeier et al. 2012 for comparisons of both methods). Recently, with genotyping of genome-wide markers in non-model organisms becoming increasingly accessible, HFC studies making use of whole genomes or large amounts of SNP data are becoming more common (e.g. Miller and Coltman 2014, Bérénos et al. 2016). Using a greater number of markers enables greater precision in these types of analyses (Hoffman et al. 2010).

In this study, we use SNP data and HFCs to examine inbreeding depression in one of Australia's most endangered birds, the orange-bellied parrot (*Neophema chrysogaster*, OBP). This species has a critically small wild population of fewer than 30 individuals,

and a captive population of c. 400 birds, descended from approximately 30 wild-caught founders (Hockley and Hogg 2013). The captive population is intensively managed, including breeding according to pedigree-based mean kinship (Ballou et al. 2010) to minimise loss of genetic diversity. Small numbers of captive birds are released annually to the wild to supplement that population (Williams and Baker 2015, Troy and Hehn 2019).

There is a high degree of genetic overlap between modern captive and wild OBP populations (Morrison et al. 2019b; Chapter 2), consistent with their management history of repeated translocations (Orange-bellied Parrot Recovery Team 2016). The OBP also exhibits low levels of diversity at innate immune genes the Toll-like receptors (Morrison et al. 2019a; Chapter 3), and microsatellite loci (Miller et al. 2013), similar to other endangered species with small population sizes. Surprisingly, SNP data obtained via DArTseq identified a relatively high number of SNPs compared to other threatened species examined using the same laboratory and bioinformatic protocols (e.g. Tasmanian devil, Wright et al. [2019a]; bilby, Wright et al. [2019b]), and a much larger number of SNPs were detected across the OBP genome than were identified in other avian species with much larger extant population sizes (e.g. myna (Ewart et al. 2019a), cockatoo (Ewart et al. 2019b)) (Morrison et al. 2019b; Chapter 2).

Given the small population size and decline of OBPs in the wild despite intensive and long-term conservation efforts (Orange-bellied Parrot Recovery Team 2006b; 2016), risk of inbreeding depression in the species has been a high priority management concern (Orange-bellied Parrot Recovery Team 2016). Indications of potential inbreeding in the captive OBP population have included past declines in reproductive output: for example, prior to the last major intake of captive founders in 2011, egg fertility and fledgling rates in the captive population had declined considerably relative to historical figures (Hockley and Hogg 2013). Analysis of microsatellite data at this time showed low overall heterozygosity and allelic diversity in both wild and captive populations (Coleman and Weeks 2012). Following the translocation of 21 birds from the wild to captivity in 2011, fecundity improved (Hockley and Hogg 2013, Williams and Baker 2015), consistent with a possible reduction in inbreeding. Nevertheless, infertility and high offspring mortality are still seen in the captive

population (Orange-bellied Parrot Recovery Team 2019), and birds in the wild are now producing fewer offspring than historical averages (current (2019) average is 1.8 fledglings per nest compared to the historical 3.3 [Troy and Hehn 2019]).

The OBP is also faced with several disease challenges (Peters et al. 2014, OBP VRTG 2019, Yang et al. 2019). Two pathogens of interest in this study are *Beak and feather disease virus* (BFDV, the causative agent of Psittacine beak and feather disease [PBFD]), and *Pseudomonas aeruginosa*. In early 2017, a portion of the captive population experienced an outbreak of *P. aeruginosa* in the sprouted seed fed to the population at the main captive OBP breeding site, near Hobart, Tasmania. This outbreak resulted in the death of at least 16 birds (Stojanovic et al. 2018, Yang et al. 2019, J. Clarke *unpublished data*). *P. aeruginosa* is a common pathogen affecting birds. Similarly, BFDV is extremely common among captive and wild parrots globally (Ritchie et al. 2003, Fogell et al. 2016), and there have been several outbreaks of the disease in both captive and wild OBP populations in recent years (Peters et al. 2014, Raidal et al. 2015). OBPs are routinely tested for presence or absence of the virus with a polymerase chain reaction (PCR) test (Shearer et al. 2009). Tests are usually performed every six weeks over the course of some months before release of a bird to the wild, or if feather condition is observed to be poor.

In this study, we explore the relationship between individual heterozygosity and reproductive success in the captive OBP population, and between individual heterozygosity and response to pathogens BFDV and *P. aeruginosa*. We utilised samples available from the extensive sample collection program undertaken by the OBP Recovery Team as part of conservation efforts, and records from long-term disease status monitoring and documentation. The OBP is a particularly interesting species for examining the relationship between heterozygosity and fitness, given its critically small population size, low immunogenetic diversity, and documented fitness declines, yet considerable number of available genome-wide SNP markers.

Better understanding of inbreeding depression via heterozygosity-fitness correlations enhances our ability to manage critically endangered species in similar situations to the OBP into the future, and can contribute to assessments of species' viability in the longer term by providing information about impacts of historic diversity losses.

METHODS

Sample collection and sequencing

OBP samples were collected as part of routine monitoring of wild and captive populations by species management staff. All blood samples were either dried on filter paper or FTA cards, or preserved in Queen's lysis buffer. DNA was extracted using the Bioline Isolate II kit, as described in Morrison et al. (2019b, Chapter 2). Reduced-representation sequencing was performed by Diversity Arrays Technology (DArTseq; Cruz et al. 2013); full methods are described at Morrison et al. (2019b). Briefly, extracted DNA was fragmented using restriction enzymes *PstI* and *SphI*, and libraries sequenced on an Illumina HiSeq2500. Sequencing data were processed using *Stacks* 2.2 (Rochette and Catchen 2017), and filtered on maximum observed heterozygosity = 0.8, and exclusion of SNP loci present in <40% of samples. When more than one SNP occurred at a locus, only one was included in the dataset (selected at random). Remainder of data filtering was performed with a custom R script (Wright et al. 2019a), using minimum read depth ≥ 2.5, minimum allele frequency ≥ 0.03, call rate ≥ 80%, reproducibility (calculated per locus, using technical replicates included by Diversity Arrays Technologies) ≥ 95%, and difference in coverage between reference and alternate alleles ≤ 80%.

Data analysis

In order to determine whether our genetic data are suitable for detecting HFCs that are reflective of inbreeding, we quantified identity disequilibrium using the g2 statistic (David et al. 2008), calculated using R package *inbreedR* (Stoffel et al. 2016). The value of the g2 statistic reflects the variance in inbreeding levels among individuals (Szulkin et al. 2010).

We used internal relatedness (IR, Amos et al. 2001) as our measure of multi-locus heterozygosity, as this is expected to be a particularly suitable metric of heterozygosity for use in inbred populations (Aparicio et al. 2006). IR incorporates allele frequencies: individuals homozygous at rare alleles are treated as more inbred (higher IR) than

individuals who are homozygous for a common allele. This is a useful aspect of this measure, considering that in small inbreeding populations (with low overall heterozygosity) homozygosity at a rare allele is more likely to be caused by inbreeding than homozygosity at a common allele, which happens with high frequency in these populations by chance. IR was calculated with the R package *GenHet* (Coulon 2010). Note that IR is negatively correlated with heterozygosity (i.e. expected to be positively correlated with the inbreeding coefficient, Balloux et al. 2004). Importantly, because IR incorporates allele frequency information, it is interpreted relative to a population of interest. We therefore recalculated IR values for data subsets as relevant to each of our questions (see below).

Heterozygosity fitness correlation

We examined heterozygosity-fitness correlations using generalised linear modelling, with model selection undertaken in an information theoretic framework following Grueber et al. (2011a). We examined four fitness measures/response variables: two reproductive metrics and two disease metrics. For reproduction, we examined 1) number of fledglings (offspring that survived to age one month) produced by genotyped male or female breeders, and 2) fledgling survival (proportion of fledglings that reached maturity, age one year) of genotyped male or female breeders. For disease, we recorded 3) *P. aeruginosa* exposure outcome: whether a bird died/survived the outbreak of *P. aeruginosa* in the captive OBP population in 2017 and 4) BFDV test results: PCR test for the presence of BFDV (positive or negative). All response variables were modelled with a binomial distribution (success/failure) except for number of fledglings produced, which was modelled with a Poisson distribution. When genotyping, efforts were made to include approximately equal numbers of both sexes in all datasets.

Reproductive success analysis

The reproductive success dataset included birds known to have been paired in the captive population 2011-2017. All birds in this dataset were included in both of the

reproductive success models (number of fledglings, and fledgling survival). For the fledgling survival model, the number of events (successes) was the number of fledglings that reached adulthood in a given year from a given breeder, and the number of trials was total number of fledglings produced by the breeder in that year.

For both reproductive success models, we used IR calculated from all genetic data we had for captive birds. IR was included as a fixed continuous variable. Other fixed predictors included the age of the genotyped parent, as well as the age of the partner of that parent (OtherParentAge), as parental age effects on reproductive success have previously been documented in parrots (Young et al. 2012). OBPs can breed from age one to at least a recorded maximum of age 11 in captivity (Holdsworth 2006). Our data included birds breeding up to age 10. In order to determine whether relationships between fitness and age or fitness and IR vary by sex in this species, both reproductive success models also included sex as a fixed predictor, as well as interactions between sex and parental age, and sex and IR.

In our number of fledglings model, to control for the fact that 60% of individuals were included as parents over multiple years, we also incorporated "Individual ID" (of the genotyped parents) as a random factor (random intercepts). In our fledgling survival model, we included "Year" as a random factor, to account for any year-to-year variation in breeding conditions over the period of the study. We were also able to include in this model an additional interaction between sex and age of the genotyped parent's pair ("Sex:OtherParentAge"). Inclusion of "Individual ID" as a random factor in our fledgling survival model prevented model convergence, as did inclusion of "Year" in our number of fledglings model, so these variables were excluded accordingly. In most cases (87 cases out of 119 pairings; 73%) individuals did not breed with the same partner across years so we did not include "Pair ID" as a random factor in either model, as this also hindered model convergence.

Disease response analysis

The disease analyses included birds that were known to have been exposed to either *P. aeruginosa* or BFDV. Birds were designated positive for *P. aeruginosa* based on the

findings of necropsies undertaken by veterinarians associated with OBP management. Birds were only designated negative for *P. aeruginosa* (survivors) if they had known proximity to other birds that died from *P. aeruginosa*, i.e. aviary companions or parents/offspring of affected birds. All *P. aeruginosa* data were collected in 2017. For these models, IR was calculated using all genotyped birds that were alive in captivity in that year. IR was included as a fixed continuous predictor.

Other fixed variables for the *P. aeruginosa* modelling included age, as this is known to sometimes have effects on immunity and disease susceptibility (Dhama et al. 2011), as well as sex (Benskin et al. 2009). No random variables were included in this model. All birds were included only once in the dataset and all observations were from one location over a three month period (Nov 2016-Jan 2017).

For BFDV, birds were designated positive if they received, at any point, a positive qPCR result for presence of the virus in their blood samples. These qPCR tests were performed according to a published protocol (Shearer et al. 2009) by the developers of the method, and coordinated by vets associated with the OBP management program; results were shared with us. Birds were designated negative for BFDV if they had a history of known presence (cohabitation) during an outbreak, but tested negative for virus presence by qPCR.

All BFDV data were collected between 2010 and 2015; we calculated IR for individuals included in this analysis using all genotyped OBPs (N = 165). IR was again included as a fixed continuous predictor, and sex as a categorical predictor. We included age at testing as a continuous predictor, as there is known to be an interaction between age and PBFD severity in most species. We included day of year as a continuous random variable, to account for any seasonal effects that may affect viral presence or infection rates.

A list of all variables included as well as variables considered for inclusion in all of our models are listed in Supplementary Tables S4.2 and S4.3.

Model fitting and model selection

Our overall modelling approach followed the method reported in Grueber et al. (2011a). In brief, a global model was generated for each response variable using *glm* (linear model, *P. aeruginosa* dataset) or *glmer* (linear mixed model, all other datasets) using the package *lme4* (Bates et al. 2015) in R version 3.5.2 (R Core Team 2018). When modest sample sizes are used in development of global models, over-parameterisation can occur and inhibit model convergence. In our study, if the full model did not converge when all variables were input, we eliminated those variables for which our datasets were highly skewed, those variables exhibiting limited variation across our datasets, or those variables for which our biological hypotheses concerning their impacts on fitness were the weakest. This continued until we were able to generate global models that converged (see Results).

Predictors were standardized following Gelman (2008) using R package *arm* (Gelman and Su 2018), to facilitate comparison of effect sizes within and between models, and enable interpretation of main effects where interactions were included. All sub-models were fitted using *MuMIn* (Bartoń 2019), and ranked on the basis of AIC$_C$ (Akaike information criterion with correction for small sample sizes; Hurvich and Tsai 1989). All models within 2 AIC$_C$ of the top model were averaged using the model.avg command in *MuMIn* (natural average method, Bartoń 2019). Inference was based on effect sizes of parameters, their standard errors, and the sum of Akaike weights (also called relative importance, RI), in the final model. Parameters that do not appear in the final model are interpreted as having no evidence of an association with the response variable.

RESULTS

Dataset overview

Our full SNP dataset contained 7768 SNPs for 165 OBPs (Morrison et al. 2019b) (Table 4.1). IR ranged from -0.084 to 0.16 (means in Table 4.1). Higher values of IR indicate higher homozygosity (max. 1, min. -1).

The value of the g2 statistic, calculated using all genotyped birds, was 0.0037 (95% confidence interval 0.0027-0.0047). This is of a similar magnitude to g2 values found in several other studies (Miller and Coltman 2014). Confidence intervals excluding zero indicate that there is identity disequilibrium (i.e. there is inter-individual variance in inbreeding levels) in the group of birds included in this study, and so it is plausible that any HFCs detected herein reflect inbreeding depression.

The number of fledglings produced by a single pair of birds in a single year ranged from 0 to 8 (average 1.96) and the number of fledglings that reached maturity also ranged from 0 to 8 (average 1.60). Sample sizes for the various analyses are in Table 4.1.

Table 4.1: Details of orange-bellied parrot (*Neophema chryosgaster*) individuals included in each heterozygosity-fitness correlation model included in the present study.

Model set	N	n (BFDV -ve | *P. aeruginosa* survived)	n (BFDV +ve | *P. aeruginosa* died)	Average IR
Reproductive fitness	151 (73F, 78M, 3U)	-	-	0.0112
BFDV test result	41 (18F, 22M, 1U)	20 (9F, 11M)	21 (9F, 11M, 1U)	0.0218
P. aeruginosa outcome	30 (18F, 11M, 1U)	15 (11F, 4M)	15 (7F, 7M, 1U)	0.0201

Abbreviations: N, sample size. BFDV, *Beak and feather disease virus*; -ve/+ve, subset of birds with a negative/positive (respectively) PCR test result for presence of *Beak and feather disease virus*; IR, internal relatedness (a measure of homozygosity); F, female; M, male; U, bird of unknown sex

Modelling results: reproductive success analysis

Full details of variables included in all models averaged to produce the final models can be found in in Supplementary Table S4.1. After model averaging, IR was retained in the final models of both measures of reproductive success, but effect sizes were very small, standard error values large, and relative importance was low (Table 4.2). We

therefore infer that IR is not a reliable predictor of reproductive success in our study populations.

There was strong evidence that age is a predictor of reproductive success in our "number of fledglings produced" model (Table 4.2; Fig 4.1). Relative importance of 1 indicates that this factor was included in the top models and all other models <2 AICc from the top model, indicating high model selection certainty. Effect size was -0.355 (SE = 0.141), indicating that older birds produce fewer fledglings than younger birds. The mean age of birds included in our study in the reproductive fitness modelling was 2.50 years for the parent of interest (genotyped bird), and 3.04 for the partners of those individuals (i.e. the other parent of the fledglings counted). Sex and an interaction between age and sex were also included in the final model for this response variable. However, these parameters had low relative importance, indicating poor support for an effect on fitness.

No strong predictors emerged from our modelling of reproductive success: fledgling survival. Age of both parents was included in the final model along with IR, but with low certainty and very high error reported for all.

Modelling results: disease response analysis

IR was not included as a predictor in our final model of response to *P. aeruginosa*. The two other predictors we included, sex and age, were included in the final model but relative importance was low (lower than the null model, with no predictors included), and standard error values large (Table 4.2). Given the low model selection certainty, and the low sample size available for this analysis, we suggest no firm conclusions can be drawn from these results.

Both IR and age were included in the final model for BFDV test result. Age has a relative importance of 1, and the negative coefficient (-21.9; Table 4.2) suggests that older birds are less likely to test positive for BFDV. This may be the case, but we consider it likely to have emerged here as an artefact of data sampling. All positive birds included in the study were found positive before the age of one year, and several older birds included in the dataset were included in the negative test result group.

This is likely due to the testing regimens in the population: routine testing of young birds occurs prior to their potential release to the wild, whereas older birds are only tested if they display feather abnormalities, or have been in contact with other known positive birds. Complete removal of older individuals from the dataset resulted in sample sizes too small for analysis. Although IR appears in the final model, the relative importance value of 0.72 suggests poor model selection certainty in regard to this variable (Table 4.2).
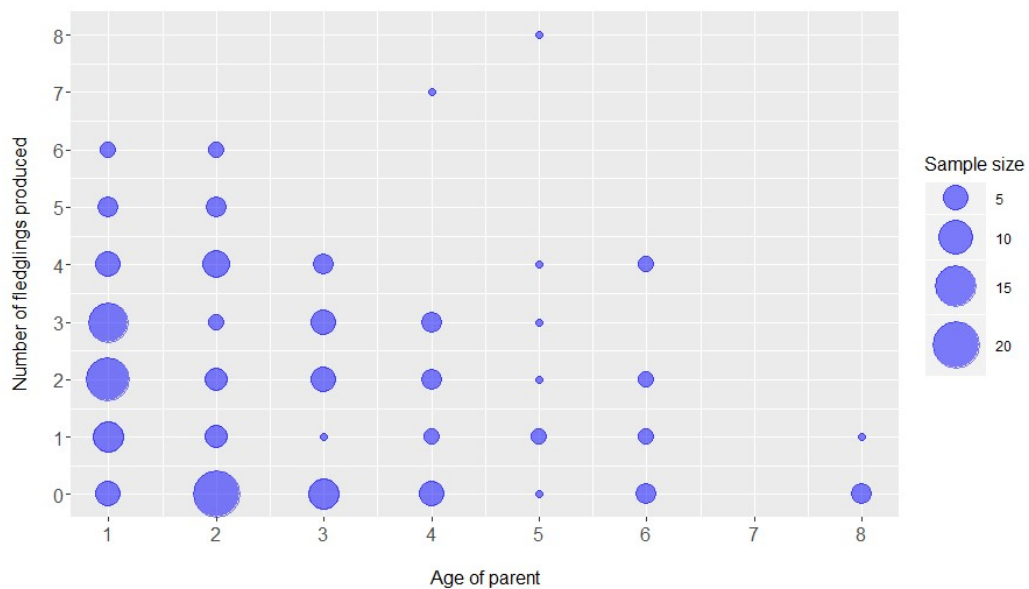
Figure 4.1: Number of fledglings produced by captive orange-bellied parrots (*Neophema chrysogaster*) genotyped in this study, plotted relative to the age of the parent that was genotyped.

Table 4.2: Standardised predictors of fitness from final models of reproductive success and response to pathogenic agents in the orange-bellied parrot (*Neophema chrysogaster*), using a heterozygosity-fitness correlation methodology.

Predictor	Number of fledglings		Number of fledglings survived to maturity		*P. aeruginosa* outcome		BFDV test result	
	β (SE)	RI	β (SE)	RI	β (SE)	RI	β (SE)	RI
Intercept	0.6375 (0.0713)	-	0.1535 (0.2402)	-	0.0019 (0.3702)	-	-3.162 (2.358)	-
IR	0.0774 (0.1309)	0.18	0.1736 (0.3106)	0.19	-	-	0.9032 (1.220)	0.72
Age	-	-	-	-	0.6559 (0.7653)	0.21	-21.90 (9.976)	1
Sex	0.0940 (0.1422)	0.38	-	-	1.020 (0.7729)	0.35	-	-
Age of parent	-0.3554 (0.1407)	1	-0.1052 (0.3425)	0.17	-	-	-	-
Age of other parent	-	-	0.1714 (0.3506)	0.18	-	-	-	-
Sex:Age of parent	0.4697 (0.2944)	0.21	-	-	-	-	-	-

Abbreviations: β, standardised coefficient for model predictors (effect size); SE, standard error; RI, relative importance of each parameter in the final model, IR, internal relatedness, BFDV, *Beak and feather disease virus*. Final models were produced by averaging submodels within 2 AIC_C of the top model generated by generalised linear mixed modelling, or for *P. aeruginosa* outcome, by generalised linear modelling. All predictors were standardised following Gelman (2008). Coefficients reported are conditional averages. Number of fledglings and fledglings matured is a measure of fledglings produced by birds included in our study. BFDV test result refers to results of PCR testing for presence of *Beak and feather disease virus*. *P. aeruginosa* outcome refers to whether a bird survived or died following exposure to *P. aeruginosa* during an outbreak in the orange-bellied parrot captive facility in 2017. The result highlighted in grey was the most informative from all models.

DISCUSSION

Our modelling of correlations between heterozygosity and fitness in the critically endangered OBP found no strong evidence that inbreeding depression is having an effect on reproductive fitness in the population, nor response to infectious agents. In a species as imperilled as the OBP, it is perhaps surprising that we found little to no evidence for association between internal relatedness and fitness measures across any of our datasets. It is, however, possible that inbreeding depression is occurring in the species, but that it was not detected using our datasets or the HFC method. Ability to detect HFCs can be limited by several factors. These include low numbers of individuals included in studies (Coltman and Slate 2003, Chapman et al. 2009), markers not truly representing inbreeding coefficients/genome-wide heterozygosity (Pemberton 2004, Szulkin et al. 2010), and insufficient variance of inbreeding within the study population (low identity disequilibrium; Slate et al. 2004, Miller and Coltman 2014).

As our IR values were derived from 7000+ SNP markers rather than tens of microsatellite markers, we think failure of our marker set to represent genome-wide heterozygosity is unlikely to be the case. The small numbers of individuals included in our study is more likely to affect results, though we had modest numbers (150+) for two of our datasets, which is greater than in many previous studies which did find strong evidence of HFCs (Chapman et al. 2009). In terms of identity disequilibrium, the g2 estimate derived from our dataset was statistically significant, which suggests that there is variance in inbreeding in the populations we studied (i.e. identity disequilibrium *is* present), and so detection of HFCs should be possible. We note that the g2 value was small, however, so the sensitivity of our analysis may have been affected by this. In the captive OBP population, genetic diversity is managed through a mean kinship breeding strategy (Ballou et al. 2010). This minimises loss of genetic diversity over time, but also limits variation in inbreeding (relative to random mating).

In order to detect correlations between fitness and inbreeding, there must not only be inter-individual variation in the extent to which individuals are inbred but also inter-individual variation in fitness (Harrison et al. 2011). A population may be suffering from the negative consequences of inbreeding, but inbreeding depression cannot be

detected using the HFC method if all individuals are suffering those consequences similarly. This was illustrated in a study of light-bellied Brent geese (*Branta bernicla hrota*), where Harrison et al. (2011) noted detection of correlations only in years where there was a large degree of variation in the fitness measure being assessed. It is not a straightforward matter to identify the extent of variation that is required in these measures in order to detect robust correlations, but we note that it is a factor that may affect study outcomes, and a lack of variation in the fitness measures in our data may have contributed to the inconclusive results.

One final factor that may have affected our ability to detect HFCs in the OBP is that our data came from birds reared and held in captivity. These conditions are highly controlled and are not challenging for individuals in the same ways that wild conditions are. It is possible that, if captive conditions contain fewer stressors than wild conditions, fitness differences may be more moderate in a captive environment and harder to measure. Previous studies have observed that fitness differences manifest more strongly across a range of taxa (including birds, e.g. Keller et al. 2002, Marr et al. 2006) when individuals are in stressful conditions (for reviews see Armbruster and Reed 2005, Fox and Reed 2010).

In this case, if we were to collect data on individual reproductive fitness from the wild population, homozygosity may show a stronger relationship with reproductive success or disease response. It would be an interesting avenue for future analyses to collect such data, particularly as fitness in the wild population is crucial to the ultimate success of efforts to conserve the OBP as a species. Additionally, collection of data on future disease events, and standardised recording of reasons for testing and history of individual exposure to BFDV-positive birds, would enable analysis of more robust datasets regarding responses to infectious agents. The infectious disease analyses herein were somewhat constrained by small sample sizes and incomplete data.

The most robust fitness predictor to emerge from any of our analyses was age, in our model of reproductive fitness measured as the number of fledglings produced. We found that older birds are predicted to produce fewer fledglings. Avian patterns of age-related reproductive variation have been well-characterised in the literature (Forslund and Pärt 1995), and in many cases, reproductive output has been shown to increase

over early life, to an optimum in middle age, before senescence occurs in old age (Fowler 1995). The increase in productivity over early life stages is mostly attributed to increasing experience with age (Martin 1995), suggesting that there is a strong learnt component to many parenting behaviours. Given our findings, it would be interesting to further investigate the relationship between age and reproductive output in the OBP. Our modelling predicts a reproductive decline in the species with age, but this does not preclude an initial increase in fitness over the first few years of life, akin to other birds which reach reproductive optima at age two years (e.g. the barn swallow [*Hirundo rustica*], Møller and de Lope 1999), followed by a decline thereafter. More precise determination of the age at which OBPs reach the prime of their reproductive fitness could assist with design of best-practice breeding strategies for the captive population.

We did not observe an equivalent relationship between age of parents and our second measure of reproductive fitness, the rate of fledgling survival into adulthood. Once OBP fledglings have left the nest they no longer depend directly on their parents for survival, so perhaps our result is a reflection of fledgling survival being more independent of parental fitness at this point than at earlier stages of offspring life, and more heavily influenced by characteristics of the offspring themselves.

Although we found no strong evidence for an influence of inbreeding on reproductive success at the two specific stages we measured, this does not mean that reproductive success overall is not affected by inbreeding depression. Grueber et al. (2010) concluded from a study in another endangered bird, the takahe (*Porphyrio hochstetteri*) that although the effects of inbreeding on reproductive success were weak at any single life-stage, they accumulated to become significant over multiple life stages. If this is the case for the OBP, collection of data on other reproductive stages (i.e. egg laying, egg hatching) and collection of genetic data across generations would assist in better refining our understanding of any influence heterozygosity may be having on reproductive output in the species. The captive OBP population has suffered from documented fertility declines in the past (Hockley and Hogg 2013) which had some hallmarks of inbreeding depression, and the fall of reproductive output in the wild population below historical averages has been a cause of concern

for some time (Troy and Hehn 2019). It is therefore important to continue to explore possible subtleties of the relationship between heterozygosity and reproductive success as thoroughly as possible. The results could identify vulnerable life-history stages to target in conservation interventions, in both captivity and in the wild.

In this study, we investigated inbreeding depression in one of Australia's most endangered birds. Somewhat surprisingly, we found no conclusive evidence of inbreeding depression in the species (in the form of correlations between variations in fitness and variations in individual genetic diversity). It remains a possibility, however, that low species-wide genetic diversity may be compromising fitness of all individuals within the species similarly. This would need to be explored through comparison of genetic diversity in the OBP relative to genetic diversity in other closely-related species. We believe this area deserves further exploration, including making such interspecies comparisons, in the future.

CHAPTER 5: Phylogenetic position of the critically-endangered orange-bellied parrot (*Neophema chrysogaster*) within the genus *Neophema*

5.1 BACKGROUND

Previous chapters presented in this thesis have reported low genetic diversity in the OBP, which prompts concern over the future of the species, its adaptive potential and the capacity of management strategies to postpone indefinitely the appearance of deleterious effects of inbreeding depression. There has been interest from the OBP Recovery Team in exploring the viability of outbreeding the OBP with one of its close relatives to improve species genetic diversity. In order to undertake a thorough assessment of this option, information regarding the evolutionary history of the OBP's genus, and identification of the closest genetic relative of the OBP, will be necessary. No molecular phylogenies of the full genus have previously been published. This chapter presents a phylogeny of *Neophema* parrots based on two mitochondrial loci, as a starting point for addressing these questions.

This chapter is based on research that I presented at the Society for Molecular Biology and Evolution Conference 2016:

Phylogenetic position of the critically-endangered orange-bellied parrot within the genus *Neophema*.

Morrison CE, Grueber CE, Frankham GJ, Hogg CJ, Johnson RN, Gold Coast Convention and Exhibition Centre, Queensland, Australia. 3-7 July 2016

This work will contribute to a future, larger analysis of the *Neophema* phylogeny. It has been formatted here for consistency with this thesis. Supplementary material for this chapter is available in Appendix 5.

I led the research presented in this chapter. Rebecca N. Johnson, Catherine E. Grueber, Carolyn J. Hogg and Greta J. Frankham provided guidance in the study design, interpretation of results, drafting of the manuscript and assisted in editing and finalising the manuscript.

5.2 MAIN ARTICLE

Phylogenetic position of the critically-endangered orange-bellied parrot (*Neophema chrysogaster*) within the genus *Neophema*

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ABSTRACT

Neophema is a genus of small Australian grass parrots. It currently contains six species, including several popular aviary birds, as well as one of Australia's most endangered species, the orange-bellied parrot (*Neophema chrysogaster*). The current phylogenetic structure of the *Neophema* genus contains two suspected subgenera (*Neonanodes* and *Neophema*) and two pairs of subspecies, however, no molecular phylogeny of the entire genus has been published to date. Available partial molecular phylogenies dispute the current distribution of species between the two subgenera. Due to the critically endangered status of *N. chrysogaster*, and threats posed by its low population genetic diversity, the phylogenetic, evolutionary and genetic contexts of the species are of conservation interest. This study investigated the position of *N. chrysogaster* within its genus using sequences of two fragments of mitochondrial genes, cytochrome oxidase I (COI) and cytochrome B (CytB). Phylogenetic analysis supported division of the genus into two subgenera, but resolution within phylogenetic trees for the placement of *N. chrysogaster* was poor. We recommend that additional sequencing data from all *Neophema* and *Neopsephotus* species be produced in order to develop a more robust phylogeny.

INTRODUCTION

Neophema (order Psittaformes, family Psittaculidae, subfamily Platycercinae) is a genus of small parrots also known as the grass parakeets, all of which are endemic to Australia (Higgins 1999; Thomas et al. 2011). There is currently a total of six species in the genus (Figure 5.1): *N. chrysogaster* (orange-bellied parrot); *N. chrysostoma* (blue-winged parrot); *N. elegans* (elegant parrot); *N. splendida* (scarlet-chested parrot); *N. petrophila* (rock parrot), and *N. pulchella* (turquoise parrot). The next most closely related species to the genus, Bourke's parrot (*Neopsephotus bourkii*), was previously also included in *Neophema*, but is now classified in the monotypic sister genus *Neopsephotus* (Christidis and Boles 2008).

Neophema species have varying distributions across Australia (Table 5.1), and many are reclusive, migratory, or nomadic in wild populations. They are generally ground feeders, eating seeds and grasses, and frequently occur in the wild in small flocks (Higgins 1999). Many of the genus are also popular aviary birds, with attractive plumage (Campagne 2008, Figure 5.1).

According to some classifications, the genus *Neophema* can be separated into two subgenera. The subgenus *Neonanodes* encompasses the coastal-dwelling *N. petrophila*, *N. elegans*, *N. chrysostoma* and *N. chrysogaster*, which lack red colouration, have a frontal blue band on their faces, and exhibit minimal sexual dimorphism, whilst the subgenus *Neophema* encompasses *N. splendida* and *N. pulchella* (Schodde and Mason 1997, Forshaw 2010). Furthermore, *N. petrophila* and *N. elegans* are often classified as having two subspecies each (*N. petrophila petrophila* and *N. petrophila zieti*; and *N. elegans elegans* and *N. elegans carteri*), which occur in discrete populations (Clayton et al. 2006). However, no molecular-based phylogeny of the genus including all six species has been produced to date. The partial molecular phylogenies that do exist are inconsistent in their division of the genus into the two subgenera currently hypothesised (Schweizer et al. 2010, Schirtzinger 2011, Schweizer et al. 2012).
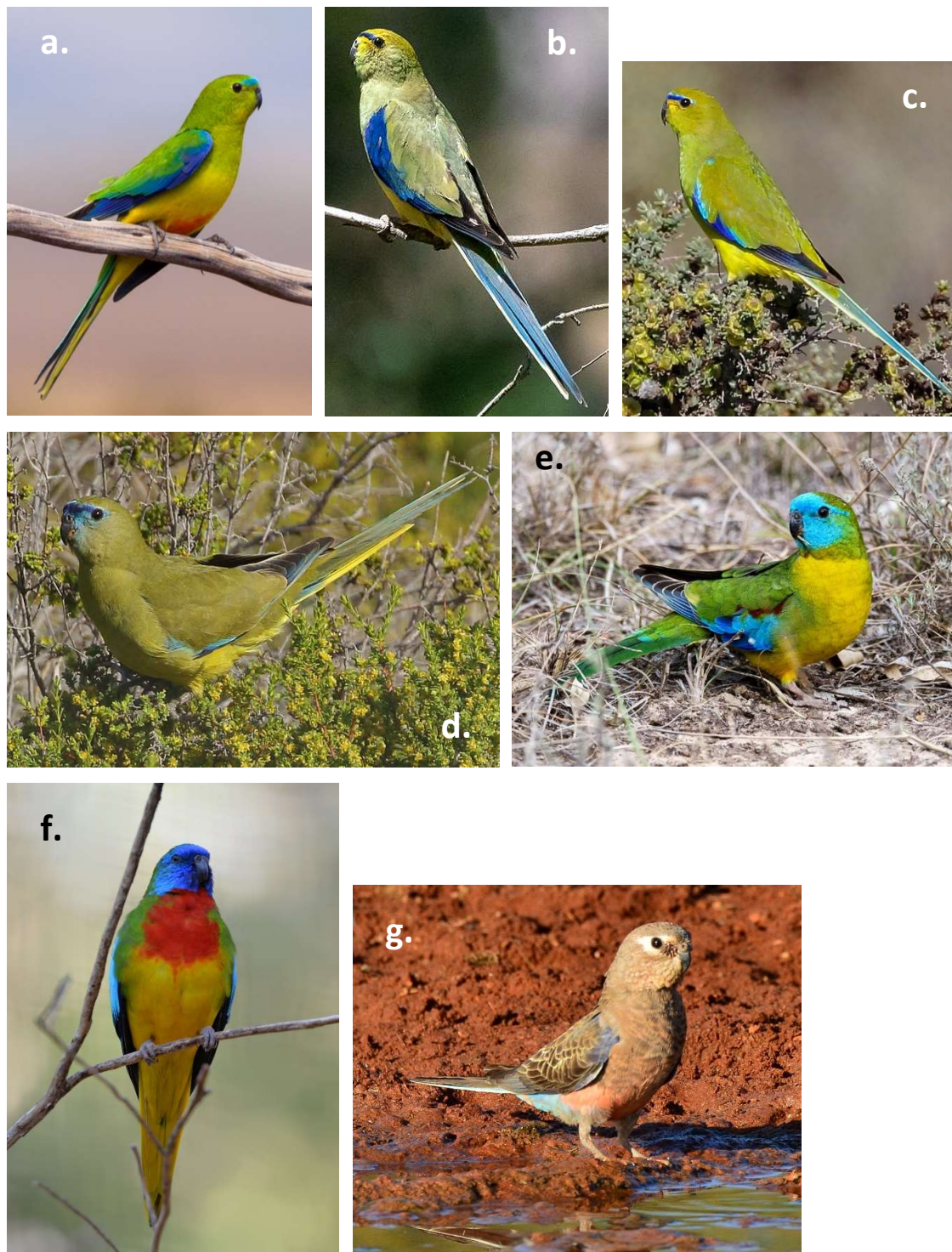
Figure 5.1: Images of all parrots in genera *Neophema* and *Neopsephotus*. a) Orange-bellied parrot, *Neophema chryosgaster*; b) Blue-winged parrot, *N. chrysostoma*; c) Elegant parrot, *N. elegans*; d) Rock parrot, *N. petrophila*; e) Turquoise parrot, *N. pulchella*; f) Scarlet-chested parrot, *N. splendida*; g) Bourke's parrot, *Neopsephotus bourkii*. Details of photograph sources listed in Supplementary Table S5.1.

Table 5.1: Species names and distributions of all species in genera *Neophema* and *Neopsephotus*

Common name	Scientific name	Distribution
Orange-bellied parrot	*Neophema chrysogaster*	SW Tasmania (nesting) / south coast Victoria/South Australia (winter)
Blue-winged parrot	*Neophema chrysostoma*	SE Australia and Tasmania
Elegant parrot	*Neophema elegans*	SW WA and SE SA/Vic (discrete populations)
Rock parrot	*Neophema petrophila*	WA and SA (discrete populations)
Turquoise parrot	*Neophema pulchella*	SE Australia
Scarlet-chested parrot	*Neophema splendida*	Across southern Australia
Bourke's parrot	*Neopsephotus bourkii*	Australia-wide

The genus *Neophema* also contains one of Australia's most endangered birds, *N. chrysogaster*, the orange-bellied parrot (OBP). There are currently only approximately 20 OBPs remaining in the wild, whilst an additional c. 450 individuals constitute a captive breeding program (Hogg and Everaardt 2019). *N. chrysogaster* is one of only two obligate migratory parrots in the world, and was the first species in Australia to have a single-species recovery plan developed (Smales et al. 2000).

In order to best conserve species and the genetic diversity they represent, it is necessary to have a clear understanding of their phylogenetic context (Moritz 1994). *N. chrysogaster* is known to have low diversity, but little is currently known about its demographic or genetic history. This study aimed to address these topics through generation of a complete molecular phylogeny of *Neophema* using data from representatives of all six species, plus the closely-related *Neopsephotus bourkii*.

Resolving taxonomies of species and delineating evolutionarily significant units is an important foundational component of conservation work, required for measurement and assessment of biodiversity as well as for outlining, legislating and enforcing protections of biological entities (Moritz 1994). Resolution of phylogenetic trees is also

important from a conservation management perspective: delineating where species boundaries begin and end can have significant impacts on conservation management outcomes (e.g. Rhymer and Simberloff 1996, Banes et al. 2016). In the case of *N. chrysogaster*, where the population size is critically small, genetic diversity is low, and effects of inbreeding are a concern (Hemmings et al. 2012, Orange-bellied Parrot Recovery Team 2016), species managers are seeking avenues for introduction of novel genetic diversity into the species (Magrath et al. 2019). The limited range of options for achieving this includes outbreeding of the species with one of its close relatives, as has been undertaken with some success in other bird species (e.g. boobook owl, (*Ninox novaeseelandiae undulata*) Garnett et al. 2011b). In order to properly assess this option, the phylogenetic relationships within *Neophema* need to be better understood.

We used fragments from mitochondrial protein-coding genes, cytochrome oxidase I (COI) and cytochrome B (CytB), to undertake a phylogenetic analysis of the *Neophema*. Mitochondrial sequences have a long history of use in phylogenetic research (Avise 1986). This is because mitochondrial DNA is relatively easy to obtain from samples (mitochondria being highly abundant in most tissue types) and has several genetic characteristics that make it highly informative and suitable for resolving phylogenetic relationships, including lack of recombination and rapid sequence evolution, yet high levels of sequence conservation (Birky Jr. 1995, Allendorf et al. 2013). COI and CytB are mitochondrial genes that have been well-characterised across a range of organisms.

MATERIALS AND METHODS

Regions of cytochrome oxidase I (COI) and cytochrome B (CytB), were sequenced from representatives of all six species of *Neophema* as well as their next closest relative *Neopsephotus bourkii*. Genomic DNA was extracted from museum-accessioned material (Table 5.2) using the Isolate II genomic DNA kit (Bioline, NSW, Australia). DNA was amplified by PCR (primer sequences in Supplementary Table S5.2, PCR conditions in Supplementary Table S5.3). PCR reactions were carried out in 25 μL with 1x MyTaq™ Reaction Buffer Red (Bioline, NSW, Australia), 0.5 U MyTaq™ Red DNA

Polymerase and 200 nM of each primer. Forward and reverse sequences were obtained by Sanger sequencing by the Australian Genome Research Facility (Sydney, NSW, Australia). Sequencing chromatograms were analysed, and consensus sequences generated, using Sequencher® version 5.2 (Gene Codes Corporation, Ann Arbor, MI USA), and alignments were produced using MEGA X (Kumar et al. 2018).

A maximum likelihood (ML) approach was used to produce phylogenetic trees using MEGA X. Outgroup sequences, from *Agapornis roseicollis* and *Melopsittacus undulatus* (both from the family Psittaculidae), were obtained from GenBank (Tavares et al. 2006, Pratt et al. 2009; Table 5.2). Phylogenetic trees were built using alignments of COI and CytB data separately, as well as from a combined dataset produced by concatenating the two sets of data. Alignments were partitioned by codon position and best-fit nucleotide substitution, and evolutionary rate models were selected for each dataset separately, based on the Bayesian Information Criterion as implemented in MEGA X. Statistical support for node placement was determined by bootstrapping over 1000 iterations.

RESULTS

Sequencing generated high-quality individual sequences of CytB from 10 samples. This included at least one representative of each species included in the study except for *N. chrysogaster*, from which CytB sequences were difficult to generate. For this species, the CytB sequence was obtained from GenBank (Table 5.2). High-quality sequences for COI were produced from 12 samples, including at least one sequence from all seven species included in the study. Alignment length was 591 bp. The concatenated dataset included only sequences where both high-quality CytB and COI sequences had been produced from the same individual, except for *N. chrysogaster*, where COI sequence from this study was concatenated with the CytB sequence for this species from GenBank. Total concatenated alignment length was 785 bp (591 bp COI, 192 bp CytB). Indications are that there is very limited variation across *N. chrysogaster* mitogenomes population-wide (Miller et al. 2013) so it is unlikely that using sequences from a different individual should affect results, providing sequence quality is good.

Table 5.2: Details of samples used to build phylogenetic trees in the present study

Code from tree	Sample source	Museum number/GenBank accession number	Sample source location	Associated publication	Sequence data used in this study
Neophema chrysogaster (i)	AM	AM127-182	Taroona	N/A	COI
Neophema chrysogaster (ii)	AM	AM127-195	Taroona	N/A	COI
Neophema chrysogaster	GenBank	NC_019804.1	Healesville Sanctuary	Miller et al. 2013	CytB
Neophema chrysostoma (i)	MV	Z5947	VIC	N/A	COI
Neophema chrysostoma (ii)	MV	Z11285	VIC	N/A	COI and CytB
Neophema elegans (i)	ANWC	B31737	WA	N/A	CytB
Neophema elegans (ii)	ANWC	B48294	SA	N/A	CytB
Neophema petrophila (i)	MV	Z3881	WA	N/A	COI and CytB
Neophema petrophila (ii)	MV	Z41415	SA	N/A	COI and CytB
Neophema pulchella (i)	ANWC	B41912	NSW	N/A	COI and CytB
Neophema pulchella (ii)	ANWC	B49205	NSW	N/A	COI and CytB
Neophema splendida (i)	ANWC	B52084	SA	N/A	COI and CytB
Neophema splendida (ii)	ANWC	B54179	WA	N/A	COI
Neopsephotus bourkii (i)	ANWC	B32776	SA	N/A	CytB
Neopsephotus bourkii (i)	ANWC	B33374	WA	N/A	COI and CytB
Melopsittacus undulatus	GenBank	DQ143295.1	N/A	Tavares et al. 2006	COI and CytB
Agapornis roseicollis	GenBank	EU410486.1	N/A	Pratt et al. 2009	COI and CytB

Abbreviations: Taroona, Taroona OBP captive breeding facility, Hobart Tasmania, AM, Australian Museum; MV, Museum Victoria; ANWC, Australian National Wildlife Collection; VIC, Victoria; WA, Western Australia; SA, South Australia; NSW, New South Wales.

Best-fit substitution models as determined by MEGA X (Kumar et al. 2018) varied for each alignment. For the CytB alignment, the Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution with a gamma distribution (G) of evolutionary rate differences among sites was used. For COI, a general time-reversible (GTR) substitution model with an invariant sites (I) rate model was used, and for the concatenated dataset the GTR model plus gamma distribution of rates was applied. The remainder of analysis in each case used default parameters.

In all instances where more than one sequence from the same species was included in an alignment, these sequences were grouped together with strong support. Of all trees produced, the CytB tree (Figure 5.2) had the lowest bootstrap supports, and a structure the least reminiscent of current divisions within the genus/genera, including placement of *Neopsephotus bourkii* within the genus *Neophema* and placement of *N. chrysostoma* outside of it. Considering the low bootstrap support, it is unlikely that this tree is reliable. This is not unexpected as the fragment used to build this tree was only 192 bp.
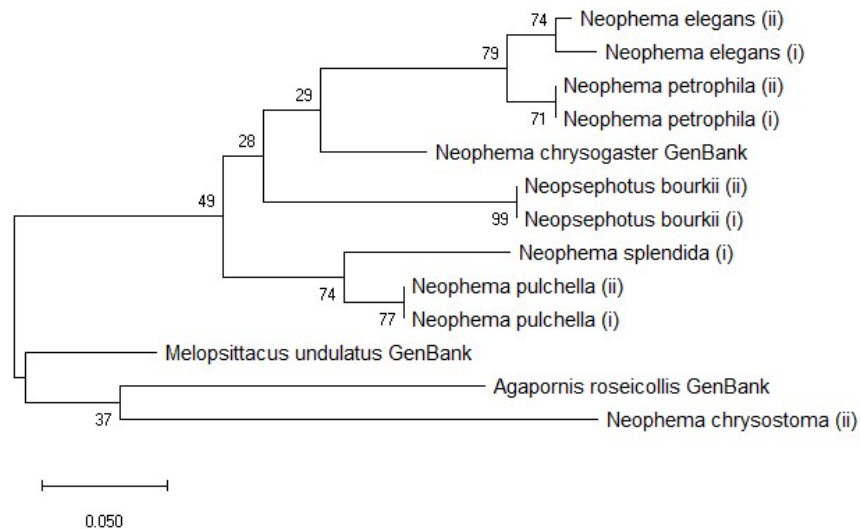
Figure 5.2: Maximum likelihood tree produced from an alignment (194 bp in length) of a fragment of a mitochondrial gene, cytochrome B (CytB) using HKY+G models of nucleotide substitution and rate differences across sites. Bootstrap support values are displayed next to nodes. Species included are all *Neophema* or *Neopsephotus* parrots, plus an outgroup (*A. roseicollis*). Roman numerals are provided to distinguish between sequences from different individuals of the same species.

Both the tree based on COI data (Figure 5.3) and the tree based on the concatenated data (Figure 5.4) placed *Neopsephotus bourkii* outside of the *Neophema* genus, alongside the two outgroups included in our analysis. These trees also split *Neophema* into two subgenera, with *N. petrophila, N. chrysostoma* and *N. elegans* in one well-supported clade, and *N. pulchella* and *N. splendida* in a second clade. The only qualitative difference between these two trees is in the position of *N. chrysogaster*. The contribution from the CytB data in the concatenated tree appears to have been sufficient to cause *N. chrysogaster* to be placed closer to *N. petrophila, N. chrysostoma* and *N. elegans* rather than in the other clade, where it is placed in the COI tree. The remainder of the topography within the COI and concatenated trees were the same. In general, bootstrap support within the COI tree was slightly higher than within the concatenated tree.
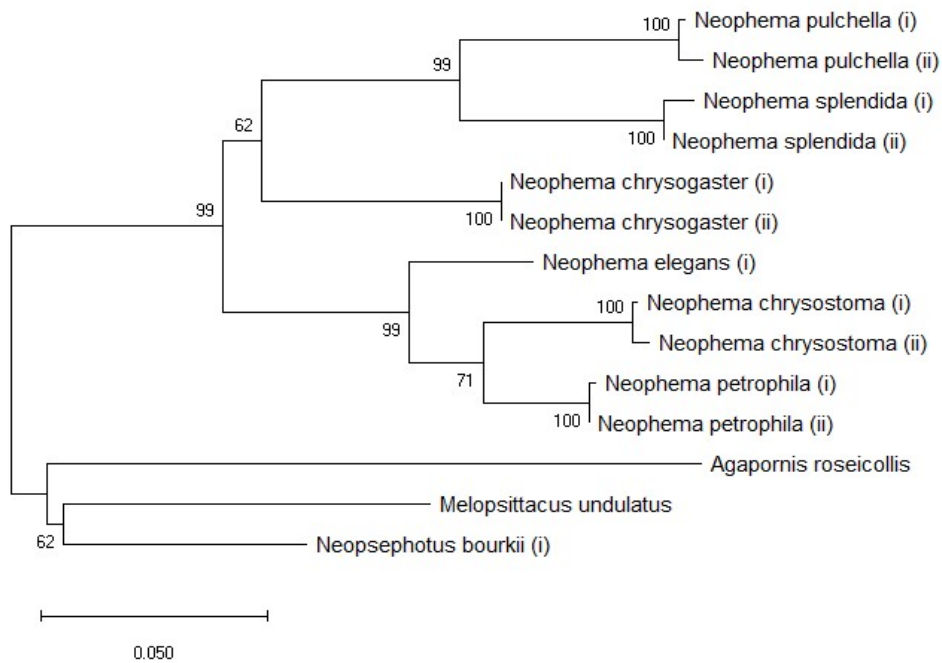
Figure 5.3: Maximum likelihood tree produced from an alignment (591 bp in length) of a fragment of a mitochondrial gene, cytochrome oxidase I (COI) using GTR+I models of nucleotide substitution and rate differences across sites. Bootstrap support values are displayed next to nodes. Species included are all *Neophema* or *Neopsephotus* parrots, plus outgroup species (*A. roseicollis*/*M. undulatus*). Roman numerals are provided to distinguish between sequences from different individuals of the same species.
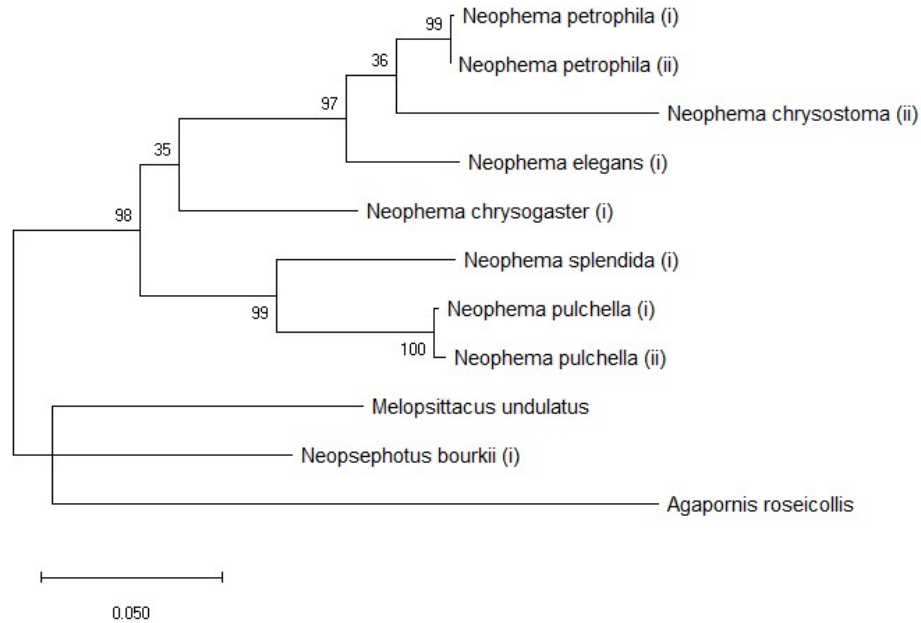
Figure 5.4: Maximum likelihood tree produced from an alignment (785 bp in length) of concatenated sequences from two mitochondrial gene fragments, Cytochrome B (CytB) and Cytochrome oxidase I (COI). GTR+G models of nucleotide substitution/rate differences across sites were used. Bootstrap support values are displayed next to nodes. Species included are all *Neophema* or *Neopsephotus* parrots, plus outgroup species (*A. roseicollis*/*M. undulatus*). Roman numerals are provided to distinguish between sequences from different individuals of the same species.

DISCUSSION

The most robust trees produced within this study supported the placement of *Neopsephotus bourkii* outside the genus *Neophema* and supported the division of *Neophema* into two subgenera. However, trees differed over placement of *N. chrysogaster* within these subgenera. All trees grouped *N. pulchella* and *N. splendida* together with high support, which matches their alignment in current non-molecular phylogenies, as well as behavioural/distribution data (Higgins 1999). *N. petrophila* and *N. chrysostoma* were also grouped as close relatives in the trees with the best support (COI and concatenated) with *N. elegans* being in a clade with these, though diverging earlier. No molecular phylogeny of *Neophema* previously published has included *N. petrophila* and as such its close association in our trees with *N. chrysostoma* and *N. elegans* is an important finding.

The tree with the highest bootstrap support for placement of *N. chrysogaster* is the COI gene tree, where *N. chrysogaster* is placed in a clade with *N. pulchella* and *N. splendida*. Such a result contradicts the current placement of *N. chrysogaster* in subgenus *Neonanodes*. Behaviourally, *N. chrysogaster* has been associated with the other coastal-dwelling species in *Neophema* (i.e. *N. petrophila, N. elegans* and *N. chrysostoma*), which have similar life histories, behaviour (in particular, at least some populations of *N. chrysostoma* also undertake migration) and colouration (Forshaw 2010; Figure 5.1). However, placement of *N. chrysogaster* in a grouping with *N. splendida* and *N. pulchella* does concur with the results of some of the previous phylogenetic studies on subsets of this genus (Schweizer et al. 2010, Schirtzinger 2011). Regardless, it must be noted that the placement of *N. chrysogaster* in all trees in our study receives low bootstrap support (no higher than 62). We conclude that the data reported here is insufficient for full resolution of *N. chrysogaster*'s placement within this genus.

This low level of bootstrap support for the placement of *N. chrysogaster* in these trees could be the result of several phenomena. These include lack of variation across the genus (so lack of phylogenetic signal), homoplasy, a history of introgression between species, signals of selection, or incomplete lineage sorting. Without further study of the phylogeny, including addition of data from other mitochondrial, and likely nuclear, loci, it is not possible to tell which of these scenarios is more probable. Interbreeding between *Neophema* species in captivity has been reported anecdotally.

Debates exist in the literature around the merits of mitochondrial sequences versus nuclear genes for the generation of phylogenies (e.g. Ballard and Whitlock 2004). It is likely the case that each approach has its benefits and drawbacks (Rubinoff and Holland 2005). There is a greater consensus within the literature on the benefits of using sequences from multiple genetic loci, rather than single genes or small numbers of gene fragments (Nichols 2001). In broad terms, and within limits, the use of more sequencing data can be expected to produce more robust phylogenetic trees, and we anticipate this to be the case with the genus *Neophema*. It is likely that the failure of our data to produce a well-resolved and well-supported phylogeny, particularly with

regard to placement of *N. chrysogaster*, is due (at least in part) to the short sequence lengths we generated in this preliminary study and their co-location on the mitochondrial genome. It is therefore our recommendation that further data be produced to better resolve phylogenetic questions surrounding this genus.

CONCLUSION

This study is the first to have produced molecular data to support inclusion of all six currently-accepted *Neophema* species within the genus *Neophema*, and to simultaneously support the placement of *Neopsephotus bourkii* in its own sister genus *Neopsephotus*. The current subdivision of *Neophema* into two subgenera was also supported, but the precise placement of *N. chrysogaster* within the phylogeny received poor support in all cases. The placement of *N. petrophila*, which has never before been included in a published molecular phylogenetic study of *Neophema*, in a clade with *N. elegans* and *N. chrysostoma* received strong statistical support and confirms the current phylogenetic placement of this species. It appears that the two mitochondrial fragments assessed here are insufficient to confirm the positioning of *N. chrysogaster* within its genus, or to identify its closest living relative. Further work will be required in order to do so. One recommended course of action is the building of a phylogeny from more complete mitochondrial data; this approach has been successfully applied in resolution of other avian phylogenies (e.g. Powell et al. 2013, Barker 2014). We recommend sequencing of a small number of nuclear genes also.

ACKNOWLEDGEMENTS

CHAPTER 6: GENERAL DISCUSSION

6.1 Key findings

This thesis set out to characterise genetic diversity in the orange-bellied parrot (*Neophema chrysogaster*) and use molecular genetic tools to inform conservation management of the species. Areas addressed included genome-wide diversity, functional immunogenetic diversity, population differentiation, phylogenetic structure and impacts of genetic diversity on disease response and reproductive success. The findings of this research, and their implications for the conservation of the OBP, are discussed in more detail below.

The main conclusions of this thesis are as follows:

1. Toll-like receptor (TLR) diversity, and genome-wide heterozygosity measured using a large number of SNPs, are both low, comparable to other endangered avian species. Unfortunately, direct comparison with diversity in other *Neophema* species closely related to the OBP was not possible, as genetic data on birds in this genus is scarce.

2. There was no indication that sequence variation at TLR loci is responsible for differential OBP responses to *Beak and feather disease virus* nor *P. aeruginosa*. However, a better understanding of which TLR locus is specifically responsible for responding to BFDV in birds is necessary before definitive claims can be made in this regard. Further, I found no compelling evidence that differences in genome-wide heterozygosity could explain the diversity observed in OBP responses to these disease agents, either. It remains a possibility that low genetic diversity in the OBP as a species is having an effect on disease interactions and susceptibilities that is species-wide (i.e. that all OBP individuals are more susceptible to disease than individuals of a comparable species) – this is a topic for future studies.

3. SNP data revealed no significant genetic differences between wild and captive OBP populations through 2013-2017. Correspondingly, TLR diversity in wild fledglings transferred to captivity in 2010/11 was equivalent to TLR diversity in captive OBPs in 2016.

4. Heterozygosity of captive-released birds was similar on average to heterozygosity of the captive OBP population as a whole, which was stable through 2013-17, and higher than heterozygosity of wild birds during this time. Genome-wide heterozygosity declined in the wild OBP population following transfer of several wild juveniles into captivity in 2010/11, but has increased since captive releases started in 2013.

5. Captive release birds can successfully breed in the wild with either wild birds or other captive release birds.

6. No definitive evidence of inbreeding depression affecting reproductive output in the OBP was found using heterozygosity-fitness correlations, with heterozygosity measured by genome-wide SNPs. There was strong evidence of an impact of age on reproductive output.

6.2 Characterising genetic diversity: chapters 2 and 3

Genetic diversity is essential for the long-term persistence of populations because it provides adaptive potential and reduces the likelihood of a species suffering from the harmful effects of inbreeding depression. Therefore, assessment and monitoring of genetic diversity are important components of species conservation (Frankham et al. 200). Genetic diversity in the critically endangered orange-bellied parrot was first reported in a study of microsatellite loci provided to the species Recovery Team in 2012 (Coleman and Weeks 2012, Miller et al. 2013). That study found that genetic diversity in the species in 2011 was 25% lower than diversity present in 1992, and overall allelic diversity was low (Coleman and Weeks 2012). Given this background, the known population history of the species, its small size, and the widely-characterised relationship between high risk of endangerment and low genetic diversity (e.g. Willoughby et al. 2015), I hypothesised that I would find low levels of

functional and genome-wide diversity in this study species, whilst developing molecular tools for addressing more specific questions regarding its management.

6.2.1 Genome-wide diversity

In this thesis I assessed two types of genetic diversity in the OBP: genome-wide diversity and functional diversity. I set out to gain a picture of genome-wide genetic diversity in the OBP using markers produced via reduced-representation sequencing (RRS). These markers do not necessarily reflect functional diversity (Ljungqvist et al. 2010), but do provide a good overview of the combined demographic and evolutionary forces affecting diversity across the genome, including genetic drift (Fischer et al. 2017, Leroy et al. 2017).

Although a great deal of data suggest that both laboratory and bioinformatic choices can influence SNP diversity estimates from RRS data (e.g. Herrera et al. 2015, Torkamaneh et al. 2016, Shafer et al. 2017), few controlled studies have addressed the degree to which obtaining higher numbers of SNPs *per se*, during marker development, reflects actual species diversity. Nevertheless, it is noteworthy that my SNP marker yields from the OBP (>7000) are considerable compared to those achieved using the same development pipeline (both laboratory and bioinformatic methods) in other endangered species (e.g. Wright et al. 2019a, Reid-Anderson et al. 2019). Given that greater numbers of molecular markers yield greater statistical power (Hoffman et al. 2014, Nazareno et al. 2017), the high number of SNP markers obtained in my study suggests that these data production methods will be very useful for genetic studies of the OBP into the future.

A more reliable way to compare diversity among species is to compare summary statistical measures of average individual diversity. One statistic used for this purpose is heterozygosity. Heterozygosity has been widely used to compare results across species in other studies, using various markers (e.g. Barrowclough and Corbin 1978, Evans and Sheldon 2008, Pinsky and Palumbi 2014), though there are to date fewer published analyses of interspecies SNP heterozygosity comparisons specifically (though examples exist, e.g. Baute et al. 2016). In my study, comparison of SNP

heterozygosity measures to equivalent data for other species confirmed that the levels of genetic diversity found using my markers in the OBP were similar to, or in some cases slightly higher than, other species of similar population size or threat status. For example, heterozygosity averaged over 11,059 SNPs in the critically endangered helmeted honeyeater (*Lichenostomus melanops cassidix*) was 0.22 (Harrisson et al. 2019), compared to the value for OBP of 0.29 (Chapter 2). In future, whole-genome sequencing of the OBP would enable arguably the most sensitive measurement through which to contrast genetic diversity of the OBP with other avian species (e.g. Lei et al. 2016, Irestedt et al. 2019). Whole-genome sequencing is becoming increasingly cost-effective and accessible, particularly for birds. It is a reasonable prospect that whole-genome sequencing of the OBP could take place in the very near future, as a result.

Completion of further genetic research on other species closely related to the OBP, including the other members of the *Neophema* genus, would also be a valuable further area of research. This would improve understanding of the significance of levels of genetic diversity observed in the OBP: whether these levels are commonly the case across the genus, or if the OBP is genetically depauperate relative to other species with similar life histories. Such an understanding would assist in efforts to anticipate the future impacts of low genetic diversity in the species.

6.2.2 Functional diversity

Having assessed genome-wide diversity in the OBP, I set out to gain a more specific picture of functional diversity in the OBP, with a focus on immunogenetic diversity. This component of my thesis focused on six Toll-like receptors, a group of immune genes important for the recognition of pathogens and subsequent activation of the immune system (Brownlie and Allan 2011). I found a total lack of genetic diversity at three of these loci, including TLR3 and TLR5, which I investigated for disease associations in a larger subset of samples. I found low numbers of haplotypes from the OBP at the other three TLR loci. This level of TLR diversity is very similar to reports from other endangered bird species (e.g. Dalton et al. 2016, Gilroy et al. 2017). Those

birds with much larger, non-bottlenecked populations generally have much higher TLR diversity (e.g. Alcaide and Edwards 2011).

The low level of diversity seen across the six OBP TLR loci I investigated suggests that the OBP as a species may be vulnerable to disease, with restricted pathogen resistance, given the limited array of TLR alleles present. Different TLR variants have been shown to be differentially protective against various pathogens in humans (Skevaki et al. 2015), suggesting that a population with a greater number of alleles is likely to be more resilient to disease than a population with low numbers of alleles. The same is likely to be true in non-human organisms.

6.2.3 Interpretation of diversity levels

One of the challenges for interpreting genetic diversity statistics is that there must be another population to act as a reference point, in order to draw firm conclusions (Ottewell et al. 2016). My measurements of OBP genetic diversity from Chapters 2 and 3 will play this role in future diversity assessments for the species: acting as snapshots to which future measures of OBP population diversity can be compared.

Given that small population size is known contribute to loss of diversity (Frankham 1996), and the OBP has undergone a recent population decline (Orange-bellied Parrot Recovery Team 2006a), it is likely that the causes of the moderately low diversity I found in this species are a recent decrease in population size, and increased inbreeding and genetic drift, as they are for other endangered populations (Frankham et al. 2017). Consistent with this assertion, a genetic signature of demographic reduction (such as that caused by a recent bottleneck) was detected in Chapter 3 using data from locus TLR1 (measured with Tajima's D; Tajima 1989).

That measurable, albeit low, genetic diversity remains in the OBP suggests that the species likely still has some capacity for adaptation to future conditions (Harrisson et al. 2014). This area deserves continued careful attention and management in the species, through continued tracking of genetic changes, investigation of any additional functional loci suspected to play a role in individual fitness, and continued

implementation of breeding strategies designed to preserve diversity as best possible. Investigation of more historic genetic diversity, through the study of older samples, or through genomics (e.g. as in Irestedt et al. [2019]), would also be very useful for interpreting the significance of the findings of low genetic diversity presented here.

My data cannot be used to confidently predict the outlook for the OBP with any certainty. However, following my analyses of genetic diversity in Chapters 2 and 3, I was able to investigate the relationship between genetic diversity and fitness in the species at present. Assessments of whether current OBP populations are experiencing negative impacts from low genetic diversity provide an indication of whether things are likely to worsen into the future, and how to prioritise actions for management now.

6.3 Effects of genetic diversity on fitness: Chapter 3 and Chapter 4

I chose TLR loci as targets for my assessment of functional diversity in the OBP because these genes are well-characterised in other species, their functional roles are well understood, and because those functional roles directly relate to disease, which is a known threatening process affecting OBP conservation (Orange-bellied Parrot Recovery Team 2016). Several pathogens have been identified as causing disease in the OBP, particularly in the captive population (where monitoring is most intensive; OBP VRTG 2019, Yang et al. 2019). In several instances over the past decade, valuable breeding birds, or birds flagged for release, were not able to perform their intended functions in the population due to disease (C. Hogg, *unpublished data*). This includes birds which died from disease and therefore no longer participate in the breeding population, as well as birds that test positive as carriers of pathogens such as BFDV, even in the absence of clinical disease.

6.3.1 Disease response and TLR diversity

Studies linking TLR diversity and disease often do so by investigating the relationships between specific TLR alleles and responses to specific diseases or pathogens (e.g. in

chickens, Leveque et al. 2003; wildlife, Tschirren et al. 2013, Kloch et al. 2018; for a review, Vinkler and Albrecht 2009). I undertook such a study in Chapter 3, where I surveyed diversity at two TLR loci in a wider sample of OBPs, chosen for their involvement in recent disease outbreaks. The two loci were selected due to their reported association with two pathogens that have recently impacted the OBP population, *Pseudomonas aeruginosa* and *Beak and feather disease virus* (BFDV; Keestra et al. 2008, Peters et al. 2014, Knafler et al. 2016, Yang et al. 2019). I investigated whether any particular alleles at either of these loci were more strongly associated with one type of response to these pathogens than another. However, both loci exhibited a total lack of diversity in the OBP, so ultimately, I could not assess the relationship between their variants and OBP disease response. Only limited conclusions can be drawn about the impact of diversity at these loci in the OBP: whether the single allele fixed at either locus is more beneficial for fighting these or other pathogens (as may be the case if fixation occurred through positive selection), or whether these alleles are detrimental to resistance against these particular pathogens (which could account for the susceptibility of the population to outbreaks of the pathogens) cannot be concluded from these data.

The fact that not all individuals are responding similarly to pathogen exposure, with some BFDV-exposed birds (those in proximity to BFDV-positive birds) testing negative, and most captive birds not succumbing during the *P. aeruginosa* outbreak, suggests that there is still some potential for resilience in the species in regard to these, and perhaps other, pathogens. Whether this variation has an immunogenetic basis or not remains unknown. It is possible that diversity at other immune genes, or regulation of gene expression, could compensate for the species' deficit in TLR sequence diversity. Investigation of functional diversity at other genes in the OBP would be useful to further investigate links between disease and diversity, given the significant risks and impacts of disease outbreaks in this species.

It is likely that, in addition to any genetic effects in the OBP, different pathogen and environmental factors interact to mediate variation in disease severity and response between individuals. In the case of infection with *P. aeruginosa*, for example, infections can occur in healthy hosts if large bacterial doses are received which

overwhelm the host's immune system (Fujitani et al. [n.d.]). Individual responses following exposure to the bacterium can therefore vary in a dose-dependent manner, independent of host characteristics.

6.3.2 Disease response and genome-wide heterozygosity

In a follow-up to my investigation of the role of genetic diversity in disease response in Chapter 3, I addressed a similar question in a complementary study presented in Chapter 4. In the latter, I examined correlations between disease response and diversity using the genome-wide data first presented in Chapter 2, using a heterozygosity-fitness correlation (HFC) methodology.

Overall response to pathogens may be affected by particular alleles, in specific cases (e.g. Skevaki et al. 2015, similar to the investigations in my TLR study), or, alternatively, responses may be more strongly associated with diversity across multiple loci, which cumulatively influence fitness (Lvovs et al. 2012, more likely to be detected in my HFC study). Exploring disease response in an HFC study was thus intended to cover subtly different ground from my TLR approach. Correlations between heterozygosity and disease susceptibility or response have been found in other animal populations using such an approach (e.g. Acevedo-Whitehouse et al. 2003, Valsecchi et al. 2004, Hoffman et al. 2014) and this, combined with recent methods for investigating HFCs through modelling (outlined in Grueber et al. 2011a), provided the rationale for the study presented in Chapter 4. Using my SNP dataset, I explored the potential for relationships between genome-wide heterozygosity measures and OBP response to the two pathogens identified above: *P. aeruginosa* and BFDV.

My HFC analysis of *P. aeruginosa* found no correlation between disease response and diversity. Power to detect a correlation in this case may have been reduced by the small sample size of birds involved in the outbreak, by the relatively small degree of inbreeding variance in the dataset (though the g2 value was significant), or it may simply be that no correlation exists between (specifically) host genome-wide diversity and the disease response. Factors affecting the life or death outcomes following OBP exposure to *P. aeruginosa* were not able to be identified in this study, but may include

factors separate from host genetic factors, e.g. degree of exposure/amount of bacteria consumed (Fujitani et al. [n.d.]), or presence of secondary conditions.

Application of the same method for detection of a genetic influence on BFDV status was slightly more ambiguous, but ultimately also inconclusive. The disease (PBFD) caused by the virus (BFDV) is complicated by a variety of outcomes: sometimes it results in death, whilst other times individuals maintain a chronic infection, and in other cases the viral agent is successfully cleared by the immune system (Pass and Perry 1984, Ritchie 1995, Todd 2000). BFDV infection (at times resulting in PBFD) has plagued the OBP for as long as efforts to conserve the species have been occurring (Smales et al. 2000). PBFD is a global disease which affects other parrot species (Raidal et al. 2015, Fogell et al. 2018), a vast number of which are also endangered or critically endangered (Olah et al. 2016). Given the significance of this disease in the OBP, the genetic underpinnings of responses to the virus warrant further study. This could include use of greater sample sizes, alternative genomic regions (e.g. other specific functional loci, including - potentially - other TLR loci, as it has not been conclusively determined that TLR3 is the locus involved in BFDV-response) or higher resolution/greater numbers of genetic markers (e.g. genome-wide association studies).

Studies addressing the genetic basis of the host immune response to BFDV in species other than the OBP would also be of great interest in informing our understanding of the disease dynamic in this species. This is particularly important given the complexity of the possible outcomes of viral exposure, which include 1) no infection; 2) infection with no clinical disease (no symptoms), which can remain chronic or result in an antibody response leading to viral clearance; or 3) infection and manifestation of clinical disease, followed by recovery from disease, chronic illness, or death due to disease, as outlined above. The different means of monitoring BFDV in a species, including by PCR for virus presence and through detection of antibody responses, could be of assistance in exploring this complex area.

Overall, my two studies of disease and genetic diversity in the OBP found no evidence to support two of the hypothesised genetic bases for variable individual responses to disease in the species: TLR allelic diversity, and overall individual heterozygosity. Low population genetic diversity may be playing a general role in disease outbreak, but

there is limited evidence thus far suggesting that inter-individual variation in diversity within this range is having any great impact on individual outcomes for the birds I studied.

My studies of OBP and disease largely focused on the captive population, as this was where data on disease, as well as samples, were best available. Other studies of the fitness impacts of low genetic diversity have found that these manifest more strongly when populations are in more stressful conditions (e.g. Keller et al. 2002, Marr et al. 2006, for reviews see Armbruster and Reed 2005, Fox and Reed 2010). Given this pattern, I predict that if inbreeding depression were to be detectable in the OBP at all, it would be more likely to be detected in data from the wild population. I assessed TLR diversity in wild individuals that were present during an outbreak of BFDV in 2015 as part of my TLR study, and as stated above unfortunately found no genetic diversity between any individuals (thus could not test for a correlation with disease outcomes).

Any future data on disease outbreaks in the OBP, in the wild or captivity, should be examined to build on my analyses of associations between disease and genetic diversity in the species. Building good datasets for future study of this issue would include detailed recording of individual responses, preferably including results from molecular tests for presence of pathogens (and immune responses to those, if possible), recording of the extent of clinical disease manifested in infected birds, and recording of which birds have been in proximity to others/likely exposed to the same pathogens. In order for genetic data to be examined, DNA samples from individuals would also be required.

6.3.3 Reproductive success in the captive population

In addition to the significant threat that loss of individuals through disease outbreak poses to the OBP, low reproductive fitness (and associated slowing of population growth) poses another. I explored the relationship between genetic diversity and reproduction, also in Chapter 4.

In 2010, the fertility rate in the captive OBP population (as evidenced by hatching and fledging success) was falling, and analysis of historical pedigree records indicated that three individuals were vastly over-represented in the population as a whole (Hockley and Hogg 2013). Around the same time, results from an unpublished study of microsatellite diversity in the OBP concluded that diversity had been lost since the early 1990s (Coleman and Weeks 2012). Taking these findings together, it was hypothesised that the fall in reproductive rate in captivity may be a consequence of inbreeding depression (Hockley 2011, Orange-bellied Parrot Recovery Team 2016). The recovery of fecundity and survival rates in the captive population after addition of 21 wild birds in 2010/11 (Williams and Baker 2015) appeared to support this assertion (Hockley and Hogg 2013). I could not investigate molecular signals of inbreeding depression in captive birds from pre-2010, as samples were not available, so I am unable to confirm whether the reproductive phenomena observed at this time were due to inbreeding depression or not. However, I was able to assess whether this is the case in the current population.

Although fertility rates have improved relative to their low in 2010, and indications through 2016-2018 have been positive, there are still many infertile eggs laid by captive OBPs, including both eggs that are unfertilised and eggs which fail to hatch due to early death of embryos (as explored in Hemmings et al. [2012]). In addition, both hatching and juvenile survival rates are, at times, poor (Orange-bellied Parrot Recovery Team 2019). Using my SNP data and the HFC method, I tested the relationship between inbreeding depression and reproductive success in captive OBPs, spanning 2012-2017. I found no evidence that reproductive success was associated with individual heterozygosity over this time (Chapter 4).

Perhaps the lack of evidence I found for inbreeding in the current population is due to intake of the 2010/11 founders improving the captive genetic situation. Alternatively, it is possible that inbreeding depression *is* causing low reproductive fitness in the current captive population, but that my study design did not capture enough information to detect it. Previous studies have shown that inbreeding depression can sometimes be associated with only weak effects when assessed at any individual stages

of the reproductive cycle, but when those stages are assessed cumulatively, larger effects are revealed (Grueber et al. 2010).

The only information available to me for measurement of reproductive success in the captive population was the number of fledglings produced by each breeding pair, and the number of those fledglings that then survived until their first breeding season (1 year of age). This dataset does not capture any possible influence of inbreeding depression in the number of eggs laid or hatched. Improved protocols for systematic collection of information at these earlier reproductive stages in captive OBPs - including determination of reasons underlying hatch failure, which can include laying of unfertilised eggs as well as early embryonic death - would expand the scope of future inbreeding analyses. This is particularly important given that both low egg fertility and hatching rate are still recorded in captive OBPs (Orange-bellied Parrot Recovery Team 2019). Comparison of reproductive metrics e.g. rates of egg fertility, brood sizes and fledgling survival between the OBP and related *Neophema* species would be another useful way to explore this issue.

6.3.4 Reproductive success in the wild

OBP reproductive success is also poor in the wild, and this has been lower in recent years (2014-2019) than historical averages (Troy and Hehn 2019). Inbreeding depression is a plausible explanation for this phenomenon. It is possible that inbreeding depression would be detectable in the wild population even though it wasn't in the captive because, as noted above, the effects of inbreeding depression are more likely to manifest under stressful (wild) conditions than benign (potentially, captive) conditions. This has been suggested to be the case for another intensively managed species, the Tasmanian devil (*Sarcophilus harrisii*; Gooley 2018), and another critically endangered bird, the pink pigeon (*Columba mayeri*, Swinnerton et al. 2004).

In order to undertake an assessment of whether inbreeding depression is affecting reproductive output in the wild OBP population, more intensive monitoring and record keeping including numbers of eggs laid, hatched, reasons for hatch failure, and offspring survival, as well as conclusive identification of parentage, would be required.

Some monitoring of wild nests occurs already, with both camera monitoring and visitation of nests by research and conservation management teams having taken place. However, nests are not easily accessible and such efforts are intensive. Whether there is currently the capacity for extra data collection in this way is uncertain.

There may also be other factors impacting wild reproduction, including habitat quality, or the high proportion of breeders that are captive-release birds in recent years. Given the low numbers of wild females recently surviving migration (Troy and Hehn 2019), captive release birds have comprised the majority of breeding females in the population over at least the last four years (2015/16-2018/19). Data from captive release birds therefore has a strong influence on overall breeding rates in the wild.

Other studies of captive release programs suggest that overall health, or vigour, of captive individuals often decreases upon release to the wild, and they experience high mortality (Griffith et al. 1986, Wolf et al. 1996). Reproductive outputs of these individuals can be limited due to a variety of causes (reviewed in Berger-Tal et al. 2019 and Wolf et al. 1998), including stress (Teixeira et al. 2007). Broadly, studies on the health impacts of reintroduction are mixed, but several have documented increased individual stress during reintroductions which diminishes over time as individuals acclimatise to their new surroundings (e.g. in the Chukar partridge, Dickens et al. 2009; whooping crane, Hartup et al. 2005; and Grevy's zebra, Franceschini et al. 2008). In the OBP, monitoring of reintroduced birds has shown that after release to the wild, ex-captive birds have poorer feather condition than wild birds (Stojanovic et al. 2018). Becoming accustomed to release sites may put strain on captive release birds, and these individuals are generally preparing to breed at the same time as adjusting to their new environment. Releases of birds are managed to minimise stress as much as possible, with soft release strategies and supplementary feed provided, but it is likely difficult to eliminate stress completely.

An alternative explanation for the decreased wild population productivity (Troy and Hehn 2019) is low heterozygosity in the wild OBP population generally (relative to historical values) resulting in decreased individual fitness population-wide. Coleman and Weeks (2012) reported declines in microsatellite diversity between 1990 and 2010, prior to my study. Evaluation of genome-wide heterozygosity in samples from

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historical individuals (pre-2000s) could be of interest to determine whether genome-wide heterozygosity has also declined over this time. Given a lack of records regarding individual reproductive output from this era, however, it would still be difficult to determine whether changes in reproductive output in the wild population over time were specifically related to changes in genetic diversity, or not.

6.4 Genetic impacts of past species management actions: Chapters 1 & 2

The unpublished microsatellite study of OBP genetics (Coleman and Weeks 2012) also suggested that the OBP populations at two captive breeding facilities, Taroona (Tasmania) and Healesville (Victoria), were distinct from each other as well as distinct from the wild population, due to the presence of 1-3 private alleles in each population. Additionally, in 2010, predictions were that the wild population was likely to decline to extinction within 3-5 years (Orange-bellied Parrot Recovery Team 2016). Faced with these two pieces of information, 21 of the wild fledglings from 2010/2011 were incorporated into the captive population (Orange-bellied Parrot Recovery Team 2010). It was hoped that this action would minimise genetic differentiation between captive and wild populations, although this question was not directly assessed until the work constituting this PhD was started.

My study did not include genetic analysis of captive individuals hatched prior to the 2010/11 founder intake, due to a lack of available samples. Furthermore, because integration of the 2010/11 founders into the breeding program was rapid, widespread and effective, many of the more modern birds assessed in my study included in their ancestry at least some heritage from the 2010/11 founder birds. This largely precluded my verifying specifically what genetic changes occurred in the captive population as a result of addition of these founder individuals. However, I was able to investigate, through my TLR and SNP studies, the patterns of genetic diversity that have occurred in the years *following* the founder intake event.

6.4.1 Wild vs captive diversity over time

My thesis focused on assessment of diversity from 2010 to 2017. Population differentiation tests using F_{ST} values derived from my SNP data (Chapter 2) showed no large differences between wild and captive genetic diversity in any of the years assessed (2013-2017), as F_{ST} values were very low. However, these values were nevertheless significantly different from zero, suggesting some small differences in allele frequencies may distinguish the populations. Given our small sample sizes (at minimum, N = 3, average 23 per population), this result should not be interpreted too strongly, as it may be due to the stochastic nature of sampling (Kalinowski 2005). Other measures of population distinctiveness (e.g. PCA plots, Appendix 1; TLR diversity, Chapter 3) indicated overwhelming similarity between wild and captive populations. These results together indicate that wild and captive populations have not been significantly genetically different in recent history, and that diversity from 2010/11 wild juveniles has been preserved in the captive population via their integration into the breeding strategy.

There is a long history of captive releases to the wild in this species (Orange-bellied Parrot Recovery Team 2006a), including releases at Melaleuca occurring 1991-1994 and 2013-present (2019). Because of the critically small numbers of wild females present in the population at the beginning of the most recent breeding seasons, the majority of wild nests have at least one captive release parent (Stojanovic et al. 2018, Troy and Hehn 2019). My results regarding a lack of distinctiveness between wild and captive populations are consistent with this history.

6.4.2 Captive diversity 2010 to present

As well as exploring differences in diversity *between* wild and captive populations over time, I assessed the dynamics of diversity over time *within* each population. Overall, my data suggest that diversity has been stable in the captive population in recent years, subsequent to the founder intake in 2010/11.

My TLR study, for example, found that all alleles detected in 2010 remain in the modern captive population (c. 2017). Because concerns existed surrounding the

presence of private alleles in different OBP populations in the past, it is illuminating that none were found in this study of TLRs. However, it should be noted that sample sizes were relatively low, limiting power to detect rare alleles, and that TLR allelic diversity was very low, which limits sensitivity in use of these genes as a measure of genetic change. Nevertheless, these results provide an indication that breeding strategies integrating the 2010/11 founders into the captive population, and subsequent management of the captive population, have effectively conserved at least some remaining functional diversity in the species.

My SNP study addressed maintenance of diversity in the form of heterozygosity, rather than allelic diversity: in this component of my study I found that mean individual heterozygosity has been stable in captivity through 2013-2017. Relative to 2010/11 founder heterozygosity, modern captive diversity is slightly lower. This pattern is consistent with the integration of 2010/11 founder birds into a population with, on average, lower individual heterozygosity than that represented amongst the founders. However, I could not confirm this hypothesis by directly measuring diversity of captive birds in 2010 due to lack of samples. As OBPs breed annually, genetic change can accumulate rapidly. The stability of heterozygosity in captive OBPs over recent years, evident even when only juvenile cohorts are assessed (Figure 2.2a), suggests that the current captive management strategy of breeding by minimising mean kinship has been successfully preserving diversity over this time.

It would be useful to assess diversity in the OBP between 2010 and the present using other specific functional genes, particularly if there are any that have greater allelic diversity than TLR loci in this species. SNP markers are not ideal for investigating rare alleles, nor private alleles between populations, but those can be functionally significant for species (Burke 2012, Hoffmann et al. 2017). A more detailed assessment of where and at what frequencies rare alleles exist across captive and wild OBPs would therefore be a useful complement to the other results presented in this thesis. Such an assessment could include production of whole genome data from a number of individuals (e.g. Zhao et al. 2013, Li et al. 2014), or exome sequencing (e.g. George et al. 2011, Bi et al. 2019). However, given the long history of combining captive and wild OBP populations, through captive releases and incorporation of wild founders into the

captive population (Orange-bellied Parrot Recovery Team 2006a), it is considered unlikely that major differences between captive and wild populations would be detected using even these more sensitive measures.

6.4.3 Wild diversity post-2010

I found, in Chapter 2, that wild diversity, in the form of heterozygosity, underwent a marked decline after the founder harvest of 2010/11. I speculate that removal of these birds from the wild, which shrank the population, resulted in decreased genetic diversity in the years following, and increased inbreeding. However, we cannot be certain of the extent to which removal of those founders *per se* was responsible for subsequent wild population size reductions and increased inbreeding. The wild population was in decline prior to this action, such that projections from the management team predicted wild extinction in 3-5 years (Orange-bellied Parrot Recovery Team 2016). This suggests that the wild population may have declined whether the 2010/11 juveniles were removed or not. It is impossible to know how many of the 21 juvenile birds transferred to captivity would have survived to breed in the wild the following year, were they not removed into captivity.

Conversely, removal of these founders appears to have had an advantage for the captive population in that breeding rates improved (Hockley and Hogg 2013), and (possibly as a result) the enhanced captive breeding population has been able to sustain a release strategy since 2013. The wild population has avoided extinction thus far, though it is still not self-sustaining and critically low numbers of wild female birds suggests that the population would likely have achieved functional extinction in recent years without assistance from the captive release program (Troy and Hehn 2019).

6.4.4 Captive release impacts on genetic diversity

Although my SNP marker study documented a negative genetic trend in the wild population following 2010/11, it also documented a reversal of this trend in juvenile

wild cohorts since captive releases began in 2013 (Chapter 2). Furthermore, my parentage analysis confirmed that captive release birds successfully breed in the wild (Chapter 2). Based on this information, I speculate that the trend towards increased heterozygosity in the wild population is fuelled by genetic contributions from released captive birds. Given the previous heterozygosity decline in the wild, and stability of higher levels of heterozygosity in the captive population, if release of captive birds was not currently included in the management strategy of the species, results from my study would recommend it.

My results further suggest that removal of additional wild birds into the captive population is not likely to be useful or necessary at this point, as there is no evidence that diversity is present in the wild that has not already been captured in the captive insurance population. This saves managers from considering placing the wild population under further strain through any additional removal of birds in the near future.

6.5 Parentage: study tools and conclusions

In addition to assessing genetic diversity in the OBP, my study provides genetic tools that will be valuable for answering other questions in the species. My SNP study revealed that subsets of SNPs can be used to confidently identify individuals, and to identify close genetic relationships between individuals. This was illustrated with my results on parentage.

The parentage results described in Chapter 2 detected extra-pair paternity occurring in the species, but at very low rates. This is useful knowledge for species management, particularly regarding release strategy, where it is important to consider breeding dynamics and social structure (Allen et al. 1993, Sigg et al. 2005, Gregory et al. 2012). The current captive release strategy involves equalising the sex ratio in the wild by releasing greater numbers of females than males (correcting the male bias of recent years). This approach is likely to be effective given the mostly monogamous OBP breeding behaviours.

Results from the parentage analysis also showed that captive release females form pairs in the wild (separately) with both captive release males and wild males. The possibility that captive release females (the majority of females breeding in the wild population) may selectively choose captive release males to pair with has been raised as a hypothetical point of concern in the past. That my results indicate that release of captive OBP males does not prevent wild males from being able to pair and breed is therefore reassuring.

The successful assignment of parentage in the wild OBP population using a subset of c. 900 of my SNPs, and the congruence of these assignments with studbook records and nest observations, indicates that genetic parentage assignment is a viable monitoring strategy for the OBP. Further exploration of parentage data generated using this approach could assist in gaining a greater understanding of species demographic and evolutionary dynamics in the wild, and enable analysis of reproductive fitness in the wild similar to my study of reproductive fitness in captivity. Parentage identification could also be used to improve estimates of relatedness in the studbook, if samples are available for any of the birds maintained in group housing at the beginning of the captive program, whose parentage is uncertain. My study has already resolved one of these uncertainties.

My HFC study also revealed that older birds, though able to breed, on average have fewer offspring than younger birds. The current breeding strategy in place for captive birds, which prioritises younger birds, is endorsed by this information from my study. Replicating the study using data on reproductive outputs from the wild OBP population could help ascertain if this phenomenon is a characteristic of the species or if it is a consequence of captive conditions in some way.

6.6 Future directions for the conservation of the OBP: Chapter 5

My study found low diversity in the OBP across both wild and captive populations, over approximately the last decade. Given these observations, and the critically small population size, I performed a preliminary study to investigate options for more

radical breeding strategies in the future, as raised previously by the OBP management team for discussion (Magrath et al. 2019).

My study of the phylogeny of the *Neophema* (Chapter 5) suggested that current taxonomic divisions of the genus are probably justified, but that more data are required to determine which of the other grass parrots is the closest genetic relative of the OBP. Work is already underway to sequence complete mitochondrial genomes of all *Neophema* to build a more robust phylogeny. Additionally, as phylogenetic patterns revealed by mitogenomic data can be different from those of nuclear genetic data (Moore 1995, Rubinoff and Holland 2005), especially where interbreeding between species is known to be possible (introgression; Rheindt and Edwards 2011; but also as a result of incomplete lineage sorting; Ottenburghs et al. 2017), sequencing of nuclear material will accompany this to strengthen conclusions. If limited, controlled outbreeding were to become part of the OBP's conservation future, molecular phylogenetic information would be combined with other biological information, including life history traits and knowledge of habitat types and wild population behaviours, in order to determine which would be the best outbreeding candidate.

Preservation of migration behaviours, in particular, is a priority for conservation of the OBP and continued viability of the species. In captive conditions, where birds do not undertake migration, selection on the ability to do so successfully is relaxed. There is thus a possibility that any genetic drift (or selection based on captive conditions) occurring in the captive population could be compromising the ability of the species to successfully undertake migration. Captive-release birds are known to have a lower migration success-rate than wild birds (Troy and Hehn 2019), but without the means to successfully track birds and identify at what stage mortality is occurring in the period between OBPs leaving the breeding site one year and returning the next, this is difficult to study. Further study of the *Neophema* phylogeny may also shed light on the evolutionary history of the OBP, and its highly specialised behaviours, including migration.

My study of TLR diversity, which included representatives of the other *Neophema* (Chapter 3) suggested that the genus contains variation where the OBP doesn't, which

is an interesting possibility for enhancing the future of resistance to disease in the OBP at loci where there is currently no adaptive potential due to monomorphism.

6.7 Additional future genetic opportunities

The OBP is a species with a critically small wild population, which is currently being sustained only through repeated releases of captive individuals. Although I found no clear evidence in my study to suggest that low genetic diversity is having a deleterious impact on the species, the low overall level of genetic diversity encountered indicates that, if this is not already compromising the fitness of the species, it may do so in the near future. Proposals to outbreed the OBP with a closely-related parrot species may go some way towards ameliorating further genetic erosion in the OBP, and this could be of assistance in improving species outlook. I would suggest that, if it does occur, any outbreeding of the OBP be undertaken with caution, and considered attention be paid to the phylogenetics of the genus, and the closeness of relationships between species (i.e. if the species are found to be very closely related, the greater the support for such an intervention would be).

Despite a growing tradition of population genetics studies, it remains difficult to reliably predict in what direction and with what severity low genetic diversity may impact a species in the long term, especially in a changing world. Given the results of my study provide no firm indication that genetic diversity is affecting OBP fitness to date (though this possibility was not conclusively ruled out, either), it is not possible to draw any strong conclusions regarding the prognosis for the species until further information is gathered.

This study has characterised a range of genetic measures in the OBP and provides a baseline for future studies that expand upon these findings. Valuable and promising avenues of future enquiry include the following:

1. It will be important to more fully characterise functional diversity in the species as genetic sequencing capacity improves, to best capture the status and rarity of functional genetic variants in the OBP across the wild population and in

captivity. Results from such a study would contribute to a higher-resolution picture of diversity in the OBP and dynamics of change in diversity over time, as well as providing further opportunities to examine associations between individual fitness and genetics in the species.

2. Exploration of the relationship between functional diversity at other candidate genes and disease response would result in better understanding of infectious disease dynamics in this species, and others with similar challenges, and provide guidance on management priorities regarding disease control.

3. Investigation of parentage in the wild, offspring survival, and survival of wild birds during the migratory period would provide the opportunity to assess the role that individual genetic diversity plays in these areas of fitness. Survival in the wild is one of the most important impediments to OBP recovery at present, so further understanding of these dynamics would be highly valuable for ranking management options, such as genetic rescue versus habitat-focussed conservation.

4. Further investigation of breeding dynamics in the wild using parentage assignment could enable better understanding of the reproductive biology of the species, and identify ideal demographic conditions in order to set biologically meaningful conservation targets.

5. Developing a record-keeping strategy whereby eggs laid and hatched (and reasons for hatch failure) are documented in a uniform manner across all institutions in the captive population would help fully characterise reproductive fitness and finalise investigation of any links between genetics and inbreeding in this population. Collection of such data from the wild population would be extremely valuable also.

6. Further mitochondrial and nuclear sequencing data would help resolve the *Neophema* phylogeny and address the management team's questions regarding suitability of outbreeding candidates from an evolutionary phylogenetic perspective.

7. Measurement of genetic diversity within and between populations of the other *Neophema* parrots, and comparison of these with the OBP, would provide

valuable information about levels of genetic diversity expected in the OBP based on its life history and evolutionary past.

8. Sequencing of a complete reference genome and ideally accompanying transcriptomes for the OBP would provide opportunities to look in greater detail at the evolutionary history of the species, and to further utilise results from my SNP studies by identifying the locations of these markers in the genome, as well as providing a resource which could be the starting point for several further investigations of the species.

6.8 GENERAL CONCLUSIONS

Overall, this study has investigated genetic diversity in one of Australia's most endangered birds more thoroughly than has ever previously been achieved. I found that low genetic diversity is a concern, as for all critically endangered species, but that recent management strategies in the absence of genetic information have been largely favourable for making the most of what genetic diversity remains. Although my study did not conclusively identify any negative effects of inbreeding currently impacting the OBP, the low genetic diversity I identified in the species, and the dynamics of its change over recent years, support the current intensive management of breeding in the captive population and the careful selection of captive individuals for release to the wild, to minimise loss of genetic diversity over time. My results also support the continued high-priority efforts to monitor, control and understand infectious disease in the species.

This study acts as an example of the utility of different types of genetic information for evaluating various aspects of endangered species management plans and actions. I have identified possible outcomes for wild populations when significant numbers of individuals are removed to found or supplement a captive program: in at least some cases, this leads to declines in genetic diversity, and population size, in the wild over the following generations. Likewise, the preservation of wild genetic diversity in a captive context, and reintroduction of diversity into wild populations at a later date through captive release events, was also explored, and was found to be a feasible

possibility when conditions are suitable (i.e. when captive release individuals breed effectively with wild individuals). Although the OBP has been considered a promising model candidate for exploration of the interactions between infectious disease susceptibility/response and genetic diversity, or reproductive output and genetic diversity, the information which was yielded in these areas of my study is unlikely to be informative when assessing other species, as no conclusive evidence of any interactions was found.

My research findings from this thesis were directly communicated to the Recovery Team annually throughout the course of my candidature. This permitted the Recovery Team to have the most up-to-date and relevant genetic data to incorporate into their decision-making processes. My SNP data, showing that there is no difference genetically between the captive and wild populations, was particularly relevant to decisions regarding releases and the potential development of a metapopulation strategy in 2018. Finally, the findings of this thesis will act as a useful reference point for any future studies of diversity in this critically endangered species.

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APPENDIX 1: **SAMPLE DETAILS**

Supplementary Table S1.1: Details of samples used in this study

Species[1]	Category of study	DArTseq	Subset of population	TLR loci sequenced [2]	Specimen ID[3]
OBP	BFDV	Y	Captive	TLR3	O.74917
OBP	BFDV	Y	Captive	TLR3	O.74919
OBP	BFDV	Y	Captive	TLR3	O.74923
OBP	BFDV	Y	Captive	TLR3	O.74925
OBP	BFDV	Y	Captive	TLR3	O.74929
OBP	BFDV	Y	Captive	TLR3	O.74931
OBP	Pop gen	Y	Wild	None	O.74933
OBP	BFDV	Y	Captive	TLR3	O.74938
OBP	BFDV	Y	Captive	TLR3	O.74939
OBP	BFDV	Y	Captive	TLR3	O.74940
OBP	BFDV	Y	Captive	TLR3	O.74944
OBP	BFDV	N	Captive	TLR3	O.74946
OBP	BFDV	Y	Captive	TLR3	O.74949
OBP	Pop gen	Y	Captive	None	O.74950
OBP	Pop gen	Y	Captive	None	O.74951
OBP	BFDV	Y	Captive	TLR3	O.74956
OBP	BFDV	Y	Captive	TLR3	O.74957
OBP	BFDV	Y	Captive	TLR3	O.74958
OBP	TLR	Y	Captive	All six loci	O.74959
OBP	TLR	Y	Captive	All six loci	O.74960
OBP	BFDV	Y	Captive	TLR3	O.74965
OBP	BFDV	Y	Captive	TLR3	O.74967
OBP	BFDV	Y	Captive	TLR3	O.74971
OBP	Pop gen	Y	Captive	None	O.74972
OBP	TLR	Y	Captive	All six loci	O.74973
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.74978
OBP	Pop gen	Y	Wild	None	O.74983
OBP	BFDV	Y	Captive	TLR3	O.74984
OBP	Pop gen	Y	Wild	None	O.74988
OBP	Pop gen	Y	Captive	None	O.74990
OBP	TLR	Y	Captive	All six loci	O.74991
OBP	BFDV	Y	Wild	TLR3	O.74993
OBP	BFDV	Y	Captive	TLR3	O.74999
OBP	BFDV	Y	Wild	TLR3	O.76338
OBP	BFDV	Y	Wild	TLR3	O.76339
OBP	BFDV	Y	Wild	TLR3	O.76340
OBP	BFDV	Y	Wild	TLR3	O.76341
OBP	BFDV	Y	Wild	TLR3	O.76342
OBP	BFDV	Y	Wild	TLR3	O.76343

OBP	BFDV	Y	Wild	TLR3	O.76344
OBP	BFDV	Y	Wild	TLR3	O.76345
OBP	BFDV	Y	Wild	TLR3	O.76346
OBP	BFDV	Y	Wild	TLR3	O.76347
OBP	BFDV	Y	Wild	TLR3	O.76348
OBP	BFDV	Y	Wild	TLR3	O.76349
OBP	BFDV	Y	Wild	TLR3	O.76350
OBP	BFDV	Y	Wild	TLR3	O.76351
OBP	BFDV	Y	Wild	TLR3	O.76352
OBP	BFDV	Y	Wild	TLR3	O.76353
OBP	BFDV	Y	Wild	TLR3	O.76354
OBP	BFDV	Y	Wild	TLR3	O.76355
OBP	BFDV	Y	Wild	TLR3	O.76356
OBP	BFDV	Y	Wild	TLR3	O.76357
OBP	BFDV	Y	Wild	TLR3	O.76358
OBP	BFDV	Y	Wild	TLR3	O.76359
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76361
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76362
OBP	BFDV	Y	Captive	TLR3	O.76364
OBP	BFDV	Y	Captive	TLR3	O.76365
OBP	Pop gen	Y	Captive	None	O.76366
OBP	BFDV	Y	Captive	TLR3	O.76367
OBP	BFDV	Y	Captive	TLR3	O.76371
OBP	BFDV	Y	Captive	TLR3	O.76373
OBP	BFDV	Y	Captive	TLR3	O.76385
OBP	BFDV	Y	Captive	TLR3	O.76393
OBP	BFDV	Y	Captive	TLR3	O.76399
OBP	BFDV, *P. aeruginosa*	Y	Captive	TLR3 and TLR5	O.76400
OBP	Pop gen	Y	Captive	None	O.76401
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76402
OBP	BFDV	Y	Captive	TLR3	O.76403
OBP	BFDV	Y	Captive	TLR3	O.76404
OBP	Pop gen	Y	Captive	None	O.76407
OBP	BFDV	Y	Captive	TLR3	O.76408
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76410
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76413
OBP	Pop gen	Y	Captive	None	O.76416
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76422
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76424
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76425
OBP	BFDV	Y	Captive	TLR3	O.76431
OBP	BFDV	Y	Captive	TLR3	O.76432
OBP	BFDV	Y	Wild	TLR3	O.76434
OBP	BFDV	Y	Wild	TLR3	O.76436
OBP	BFDV	Y	Wild	TLR3	O.76438

OBP	BFDV	Y	Wild	TLR3	O.76439
OBP	BFDV	Y	Wild	TLR3	O.76441
OBP	BFDV	Y	Wild	TLR3	O.76442
OBP	BFDV	Y	Wild	TLR3	O.76443
OBP	BFDV	Y	Wild	TLR3	O.76445
OBP	BFDV	Y	Captive	TLR3	O.76447
OBP	TLR	Y	Captive	All six loci	O.76449
OBP	BFDV	Y	Captive	TLR3	O.76450
OBP	Pop gen	Y	Captive	None	O.76451
OBP	Pop gen	Y	Captive	None	O.76453
OBP	Pop gen	Y	Captive	None	O.76457
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76458
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76460
OBP	TLR	Y	Captive	All six loci	O.76461
OBP	Pop gen	Y	Captive	None	O.76466
OBP	TLR	Y	Captive	All six loci	O.76468
OBP	Pop gen	Y	Captive	None	O.76472
OBP	TLR	Y	Captive	All six loci	O.76475
OBP	Pop gen	Y	Captive	None	O.76477
OBP	Pop gen	Y	Captive	None	O.76479
OBP	BFDV, *P. aeruginosa*	Y	Captive	TLR3 and TLR5	O.76480
OBP	BFDV	Y	Captive	TLR3	O.76481
OBP	TLR	Y	Captive	All six loci	O.76482
OBP	BFDV	Y	Captive	TLR3	O.76483
OBP	BFDV	Y	Captive	TLR3	O.76487
OBP	TLR	Y	Captive	All six loci	O.76488
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76489
OBP	*P. aeruginosa*	Y	Captive	TLR5	O.76491
OBP	BFDV	Y	Captive	TLR3	O.76493
OBP	BFDV	Y	Wild	TLR3	O.76495
OBP	BFDV	Y	Wild	TLR3	O.76497
OBP	Pop gen	Y	Captive	None	O.76506
OBP	Pop gen	Y	Captive	None	O.76515
OBP	BFDV	Y	Captive	TLR3	O.76521
OBP	*P. aeruginosa*	Y	Founder	TLR5	1341
OBP	*P. aeruginosa*	Y	Founder	TLR5	1342
OBP	TLR	Y	Founder	All six loci	1343
OBP	Pop gen	Y	Founder	None	1345
OBP	TLR	Y	Founder	All six loci	1346
OBP	TLR	Y	Founder	All six loci	1347
OBP	TLR	Y	Founder	All six loci	1349
OBP	TLR	Y	Founder	All six loci	1353
OBP	*P. aeruginosa*	Y	Founder	TLR5	1397
OBP	TLR	Y	Founder	All six loci	1398
OBP	*P. aeruginosa*	Y	Founder	TLR5	1399

OBP	TLR	Y	Founder	All six loci	1400
OBP	TLR	Y	Founder	All six loci	1401
OBP	TLR	Y	Founder	All six loci	1402
OBP	TLR	Y	Founder	All six loci	1403
OBP	*P. aeruginosa*	Y	Founder	TLR5	1404
OBP	Pop gen	Y	Captive	None	1591
OBP	*P. aeruginosa*	Y	Captive	TLR5	1649
OBP	Pop gen	Y	Captive	None	1779
OBP	*P. aeruginosa*	Y	Captive	TLR5	1799
OBP	Pop gen	Y	Captive	None	2005
OBP	Pop gen	Y	Captive	None	2006
OBP	*P. aeruginosa*	N	Captive	TLR5	2177
OBP	*P. aeruginosa*	Y	Captive	TLR5	2325
OBP	*P. aeruginosa*	Y	Captive	TLR5	2327
OBP	*P. aeruginosa*	Y	Captive	TLR5	2357
OBP	*P. aeruginosa*	Y	Captive	TLR5	2361
OBP	*P. aeruginosa*	Y	Captive	TLR5	2380
OBP	*P. aeruginosa*	Y	Wild	TLR5	MP56-C1
OBP	Pop gen	Y	Wild	None	WF2017.01
OBP	Pop gen	Y	Wild	None	WF2017.02
OBP	Pop gen	Y	Wild	None	WF2017.04
OBP	Pop gen	Y	Wild	None	WF2017.05
OBP	Pop gen	Y	Wild	None	WF2017.06
OBP	Pop gen	Y	Wild	None	WF2017.07
OBP	Pop gen	Y	Wild	None	WF2017.08
OBP	Pop gen	Y	Wild	None	WF2017.09
OBP	Pop gen	Y	Wild	None	WF2017.12
OBP	Pop gen	Y	Wild	None	WF2017.13
OBP	Pop gen	Y	Wild	None	WF2017.14
OBP	Pop gen	Y	Wild	None	WF2017.15
OBP	Pop gen	Y	Wild	None	WF2017.16
OBP	Pop gen	Y	Wild	None	WF2017.17
OBP	Pop gen	Y	Wild	None	WF2017.18
OBP	Pop gen	Y	Wild	None	WF2017.19
OBP	Pop gen	Y	Wild	None	WF2017.20
OBP	Pop gen	Y	Wild	None	WF2017.21
OBP	Pop gen	Y	Wild	None	WF2017.22
OBP	Pop gen	Y	Wild	None	Red Red P
BP	TLR/Phylogeny	N	N/A	All six loci	B33374
BP	Phylogeny	N	N/A	None	B33374
BWP	TLR/Phylogeny	N	N/A	All six loci	Z11285
BWP	Phylogeny	N	N/A	None	Z5947
EP	TLR/Phylogeny	N	N/A	All six loci	B31737
EP	Phylogeny	N	N/A	None	B48294
RP	TLR/Phylogeny	N	N/A	All six loci	Z41415
RP	Phylogeny	N	N/A	None	Z3881

SCP	TLR/Phylogeny	N	N/A	All six loci	B52084
SCP	Phylogeny	N	N/A	None	B54179
TP	TLR/Phylogeny	N	N/A	All six loci	B49205
TP	Phylogeny		N/A	None	B41912

[1] OBP = orange-bellied parrot, BP = Burke's parrot, BWP = blue-winged parrot, RP = rock parrot, SCP = scarlet-chested parrot, TP = turquoise parrot

[2] "All six loci" refers to sequencing of fragments of TLR1, TLR3, TLR4, TLR5, TLR7 and TLR10

[3] Museum numbers with starting letter O were sourced from the Australian Museum; starting letter B from the Australian National Wildlife Collection; starting letter Z from Museum Victoria. Four-digit numbers refer to OBP studbook identification numbers or sample names; these samples are pending accession into the Australian Museum collection

Abbreviations: TLR, Toll-like receptor study (Chapter 3); BFDV, *Beak and feather disease virus* association study (Chapters 3 and 4); *P. aeruginosa, Pseudomonas aeruginosa* association study (Chapters 3 and 4); Pop gen, population genetics study (Chapters 2 and 4); Phylogeny, phylogenetic study (Chapter 5)

APPENDIX 2: **SUPPLEMENTARY MATERIALS CHAPTER 2**
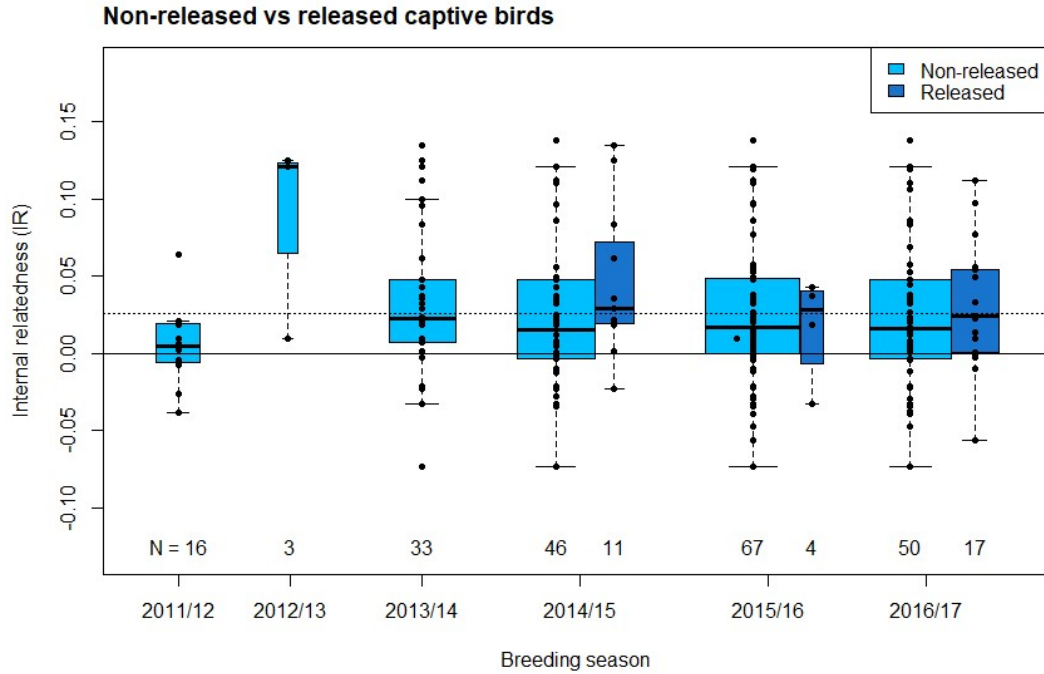
Supplementary Figure S2.1: Internal relatedness (IR) over time in captive orange-bellied parrots *(Neophema chryosgaster)* separated into released birds and those retained in captivity, between breeding seasons spanning years 2010/11 to 2016/17. Mean IR is represented by a dotted horizontal line at 0.0257. Width of boxes corresponds to sample size and dots represent individual IR values. "N =" refers to sample sizes. Boxes are bounded by quartile (Q)1 and Q3, with the median value in each box indicated by the bold bar. The whiskers represent +/- 1.5 times the interquartile range. The solid horizontal line at x = 0 is provided as a reference point. 2010/11 founder data was excluded from all years subsequent to 2011/12. There is overlap of individuals between years. Fourteen of the 16 2010/11 founders sampled here survived their first year in captivity, hence N = 14 for the 2011/12 sample set in this graph.

APPENDIX 3: **SUPPLEMENTARY MATERIALS CHAPTER 3**

SUPPLEMENTARY METHODS

TLR primer development, amplification, and sequencing

Primers developed previously for use in other bird species (Alcaide and Edwards 2011, Grueber and Jamieson 2013) for amplification of extracellular domains of six TLR genes (TLR1, TLR3, TLR4, TLR5, TLR7 and TLR10) were the starting point for this study (Table S2). Primer pairs were initially trialled on a single sample each from *Neophema* and *Neopsephotus* parrot species. All primer pairs successfully amplified material from at least one species. PCRs were performed in a total volume of 15 uL containing either 1 µL (for samples of concentration >10ng/µL) or 5 µL (for samples <10ng/µL) of sample DNA, 1x concentration of MyTaq Red Buffer (Bioline, Eveleigh, NSW, Australia), 500 nM of both forward and reverse primers, 9 U MyTaq DNA polymerase (Bioline, Eveleigh, NSW, Australia) per reaction, and purified water to reach final volume. PCR amplification conditions followed published protocols (Grueber & Jamieson 2013), with annealing temperatures as given in Supplementary Table S2, on a Bio-Rad T100™ thermal cycler (Bio-Rad, Hercules, CA, USA).

Amplification of the target band was confirmed by 1% TBE agarose gel electrophoresis, and target bands were excised under UV light and purified using either QiaExII Gel Extraction Kit (Qiagen, Hilden, Germany) or Bioline Isolate II PCR and Gel Purification Kit (Bioline, Eveleigh, NSW, Australia), as per manufacturers' recommendations. Purified amplicons were Sanger sequenced at the Australian Genomic Research Facility, Sydney, using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and BigDye Terminator chemistry v.3.1 (Applied Biosystems, Foster City, CA, USA). Data were processed and aligned using Geneious v. 9.1.5 (https://www.geneious.com, Kearse et al. 2012). Sequence data produced in this way from *Neophema*/*Neopsephotus* parrots were used to design more specific primers, using the program Oligo 6.71 (Molecular Biology Insights, Inc., Colorado Springs, CO, USA; Supplementary Table S3). Newly designed primers showed greater specificity than cross-species PCR, so gel clean-up was no longer required. Instead, later

amplifications were purified using an Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA) bead clean-up protocol, as per manufacturer's recommendations. All unique polymorphic sites were confirmed by repeat PCR and sequencing. All sequences were translated into protein sequences using Geneious v. 9.1.5 and investigated for stop codons or other evidence of pseudogene amplification. Sequences were exported in FASTA format for further analysis.

Testing for selection

Signatures of positive selection across both the OPB and *Neophema* datasets were investigated using HyPhy (Kosakovsky Pond et al. 2005) on the Datamonkey web server (Weaver et al. 2018) as well as CodeML within PAML 4.0 (Yang 2007; *Neophema* datasets only, as analysis depends on phylogenetic difference between samples). Alignments were first investigated for signals of recombination events using GARD (a Genetic Algorithm for Recombination Detection: Kosakovsky Pond et al. 2006), in HyPhy. No evidence of recombination was found at any of our loci. Models of nucleotide substitution were determined using the model selection tool (Delport et al. 2010) implemented on the Datamonkey webserver for each locus, and these were applied for all tests run on that platform. Phylogenetic trees used in positive selection tests implemented on Datamonkey were generated by the Datamonkey system using only the alignment for the locus under analysis at the time. The phylogenetic tree used in PAML analyses was produced from our concatenated TLR dataset using a maximum likelihood method implemented in MEGA v.7.0.18 (Kumar et al. 2016).

Several different tests for positive and negative selection were implemented, some of which are targeted at identification of different types of selection and thus employ slightly different methods. Such tests classify sites into groups based on the ratio of non-synonymous vs. synonymous mutation (dN/dS, ω) modelled at the site, where $\omega>1$ is interpreted as positive selection; $\omega=0$ as neutral evolution; and $0<\omega<1$ as negative selection. We explored our data with both site-based and branch-site tests. Site-based tests seek to identify where individual amino acids across a phylogeny have been subject to selection, and assume consistent selection across all branches of a tree.

These tests include FUBAR (Murrell et al. 2013) in HyPhy and model comparisons M1a vs. M2a and M7 vs. M8 in PAML. Branch-site tests allow ω to vary both between sites and between branches of a phylogeny, and thus are more suitable for detection of episodic selection, that is, where selection pressures have varied spatiotemporally, with the type of selection differing over time and differing between branches of a phylogenetic tree. Branch-site tests include MEME (Murrell et al. 2012) and aBSREL (Smith et al. 2015) in HyPhy and Branch-site A vs. Branch-site null models implemented in PAML. Episodic selection is more likely to be the model of evolution for immune genes such as avian TLRs (e.g., Grueber et al. 2014, Raven et al. 2017) and so it is of particular interest here.

Phylogenetic trees

Phylogenetic trees were built using neighbour-joining methods implemented in MEGA-X (Kumar et al. 2018), from *Neophema* sequence alignments for each locus investigated. Branch support values were derived from bootstrapping over 1000 iterations. Outgroup sequences were sourced from GenBank, and were sequences from *Cyanoramphus novaeseelandiae* in each case (published in Grueber and Jamieson 2013). Some selection tests rely on phylogenetic trees built from the sequence data being investigated. Therefore building of phylogenetic trees was completed to check assumptions built into selection tests, and as another means of visualising sequence data variation.

(a) TLR1

Elegant parrot, N. elegans

74

Orange-bellied parrot, N. chrysogaster

99

Rock parrot, N. petrophila

Blue-winged parrot, N. chrysostoma

Bourke's parrot, Neopsephotus bourkii

48

Turquoise parrot, N. pulchella

93

Scarlet-chested parrot, N. splendida

KF265268.1 Cyanoramphus novaezelandiae isolate 201732

0.0020

(b) TLR3

Rock parrot, N. petrophila

65

Elegant parrot, N. elegans

89

Blue-winged parrot, N. chrysostoma

100

Orange-bellied parrot, N. chrysogaster

Scarlet-chested parrot, N. splendida

83

Turquoise parrot, N. pulchella

Bourke's parrot, Neopsephotus bourkii

KF265284.1 Cyanoramphus novaezelandiae isolate 201759

0.0020

Supplementary Figure S3.1: (continues on next page)

(c) TLR4

(d) TLR5

Supplementary Figure S3.1: (continues on next page)

(e) TLR7

Orange-bellied parrot, N. chrysogaster
Elegant parrot, N. elegans
Rock parrot, N. petrophila
Blue-winged parrot, N. chrysostoma
Turquoise parrot, N. pulchella
Scarlet-chested parrot, N. splendida
Bourke's parrot, Neopsephotus bourkii
KF265318.1 Cyanoramphus novaezelandiae isolate 201783

96
88
86

0.0020

(f) TLR10

Orange-bellied parrot, N. chrysogaster
Elegant parrot, N. elegans
Blue-winged parrot, N. chrysostoma
Rock parrot, N. petrophila
Scarlet-chested parrot, N. splendida
Turquoise parrot, N. pulchella
Bourke's parrot, Neopsephotus bourkii
KF265258.1 Cyanoramphus novaezelandiae isolate 201759

62
91
99
67
90

0.0050

Supplementary Figure S3.1: Neighbour-joining phylogenetic trees of the *Neophema* genus and nearest relative *Neopsephotus bourkii,* based on sequence alignments of fragments of six Toll-like receptor genes. Branch support was derived from bootstrapping over 1000 iterations. GenBank reference numbers are included in outgroup sequence labels. "N." = *Neophema*

Supplementary Table S3.1: Primers used for PCR amplification of Toll-like receptor sequences in Australian grass parrots *Neophema* and *Neopsephotus sp.* (note product length includes primers)

Target	Primer pair	Reference	T_a (°c)	Product length (bp)
TLR1	PcaTLR1LBF/PcaTLR1LBR	Grueber & Jamieson 2013	60	971
	NboTLR1LBF/NboTLR1LBR	current study	56	720
TLR3	avTLR3F/avTLR3R	Alcaide & Edwards 2011	52	1,267
	PcaTLR3F/PcaTLR3R	Grueber & Jamieson 2013	54	1,080
	NspTLR3F/NspTLR3R	current study	46	1,034
	NchTLR3F/NchTLR3R	current study	59	1,044
TLR4	MunTLR4F/MunTLR4R	Grueber & Jamieson 2013	55	943
	NchTLR4F/NchTLR4R	current study	56	794
TLR5	ShaTLR5F/ShaTLR5R	Grueber & Jamieson 2013	54	983
	ShaTLR5F/AalTLR5R	Grueber & Jamieson 2013	50	1,073
	NchTLR5F/NchTLR5R	current study	47	952
	NchTLR5F.v2/NchTLR5R.v2	current study	56	927
TLR10	avTLR1LAF/avTLR1LAR	Alcaide & Edwards 2011	58	1,267
	NchTLR1LAF/NchTLR1LAR	current study	56	1,025

Abbreviations: T_a = PCR annealing temperature; bp = base pairs; OBP/Nch = *Neophema chrysogaster*; BWP = *Neophema chrysostoma*; RP = *Neophema petrophila*; EP/Nel = *Neophema elegans*; TP = *Neophema pulchella*; SCP/Nsp = *Neophema splendida*; BP/Nbo = *Neopsephotus bourkii*

Supplementary Table S3.2: Sequences for primers designed in this study for the amplification of Toll-like receptor fragments from species in genera *Neophema* and *Neopsephotus*

Target locus	Primer name	Primer sequence (5'-3')
TLR1	NboTLR1LBF	TGAAGGCACTGATAATGGAGA
	NboTLR1LBR	CCCACGGATAAAGTCTCG
TLR3	NspTLR3F	CTTTCATACATTGGCAAGTCA
	NspTLR3R	GAGTTCAGAGAGGCTTGGTC
	NchTLR3F	CTTTCATACATTGGCAAGTC
	NchTLR3R	GAAGGTTCAGTGAGTTCAGA
TLR4	NchTLR4F	AACAGACTTAACCTCACGCT
	NchTLR4R	ACATGAGTTCTGGTATGGGAA
TLR5	NchTLR5F	TTCATATAATGGGTTCTG
	NchTLR5R	TGTGTTATATCCAAAATAGTC
	NchTLR5F.v2	GCTTTCATATAATGGGTTCT
	NchTLR5R.v2	TCATAAAGATTTCAGGATCAG
TLR7	NelTLR7F	ACTGCCCACGCTGCTATAATG
	NelTLR7R	GCCCGTACTCATCATACTTGA
TLR10	NchTLR1LAF	TTTCCTGCTTATTTGGTATTCAC
	NchTLR1LAR	CCAGCACCAGCGTCAGTAT

Supplementary Table S3.3: Results from alternative pervasive selection tests implemented in HyPhy. Numbers refer to positions in our sequence alignment. These position numbers relative to published chicken sequences are listed in Table S7.

Dataset	N	Model	GARD [1]	SLAC [2]		FEL [2]		IFEL [2]		REL [3]	
Type of selection		n/a	n/a	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
OBP TLR1	20	000000	none	none	62 (0.019) 80 (0.012) 165 (0.077)	none	63 (0.041) 80 (0.036)	none	165 (0.097)	none	all
OBP TLR4	20	000000	none	none	232 (0.080)	none	none	none	none	none	all
OBP TLR10	20	000000 (F81)	none	none	none	none	none	none	none	210 sites	none
Neophema TLR3	7	010010 (HKY85)	none	none	77 (0.0023) 187 (0.017) 238 (0.017)	none	184 (0.085) 189 (0.030) 222 (0.037)	none	none	259 (0.93)	none
Neophema TLR4	7	010110	none	none	38 (0.054) 106 (0.085) 142 (0.017) 160 (0.017) 189 (0.029) 200 (0.013) 249 (0.025) 251 (0.038)	101 (0.086)	36 (0.089) 38 (0.014) 86 (0.086) 121 (0.081) 165 (0.043) 227 (0.045) 254 (0.053) 257 (0.080) 269 (0.080)	183 (0.053)	38 (0.060)	101 (1.0)	36 (0.096) 38 (0.99) 54 (0.96) 65 (0.95) 86 (0.96) 116 (0.96) 121 (0.96) 136 (0.96) 165 (0.96) 166 (0.96) 227 (0.97) 231 (0.96) 254 (0.97) 257 (0.96) 269 (0.97)

Neophema TLR5	7	010110	none	none	23 (0.015) 123 (0.015) 205 (0.010) 206 (0.015) 287 (0.029) 289 (0.029) 96 (0.025)	none	15 (0.076) 78 (0.088) 249 (0.076)	149 (0.027)	none	none	none
Neophema TLR7	7	010010 (HKY85)	none	none	96 (0.025)	none	23 (0.081) 89 (0.0097)	none	23 (0.081) 89 (0.024)	none	none
Neophema TLR1	7	010000	none	none	41 (0.023) 63 (0.012) 80 (0.0048) 161 (0.017) 165 (0.017) 182 (0.0055) 212 (0.017)	none	30 (0.065) 76 (0.10) 227 (0.088)	7 (0.044)	none	7 (1.0)	17 (1.0) 30 (1.0) 32 (0.99) 40 (0.99) 76 (1.0) 126 (0.96) 173 (0.99) 227 (0.99)
Neophema TLR10	7	010010 (HKY85)	none	none	98 (0.040) 110 (0.037) 167 (0.017) 209 (0.040) 263 (0.168) 280 (0.172) 286 (0.168) 359 (0.091)	none	57 (0.070) 96 (0.077) 256 (0.067) 284 (0.099)	none	none	none	all

[1] Any evidence of recombination
[2] Only sites are shown that met the *p* value threshold of 0.1 (value in parentheses)
[3] Only sites are shown that met the posterior probability threshold of 0.9 (value in parentheses)
[4] Only sites are shown that met the *p* value threshold of 0.05 (value in parentheses)

Supplementary Table S3.4: Results from alternative episodic selection tests implemented in HyPhy, applied to alignments of Toll-like receptor sequences from species in genera *Neophema* and *Neopsephotus*

Dataset	N	GA-branch [1] +ve (episodic)	Branch-site REL [2] +ve (episodic)
OBP TLR1	20	n/a	n/a
OBP TLR4	20	n/a	n/a
OBP TLR10	20	n/a	n/a
Neophema TLR1	7	none	none
Neophema TLR3	7	none	none
Neophema TLR4	7	none	node leading to BP, SCP, TP (0.05)
Neophema TLR5	7	none	none
Neophema TLR7	7	none	none
Neophema TLR10	7	branch of BP (0.973)	none

[1] Only sites are shown that met the posterior probability threshold of 0.9 (value in parentheses)

[2] Only sites are shown that met the *p* value threshold of 0.05 (value in parentheses)

Abbreviations: TP = *Neophema pulchella*; SCP = *Neophema splendida*; BP = *Neopsephotus bourkii*

Supplementary Table S3.5: Details of PAML analysis applied to alignments of Toll-like receptor sequences from all *Neophema* and *Neopsephotus* parrots

Locus	M1a vs M2a	M7 vs m8	Branch-site null vs a	Branch-only 2-ratio vs 1-ratio
TLR1	p<0.001 7S (0.995)	p<0.001 7S (0.988) 9F (0.950) 42S (0.951) 68E (0.957) 85 (0.950) 88 (0.950) 101 (0.950) 113V (0.950) 141V (0.950) 160V (0.950) 175R (0.952) 177A (0.950)	ns	ns
TLR3	ns	ns	ns	ns
TLR4	0.1<p<0.5 (ns)	0.1<p<0.5 (ns)	ns	ns
TLR5	ns	ns	ns	ns
TLR7	ns	ns	ns	ns
TLR10	ns	ns	ns	ns

Each model (e.g. M1, M2) within PAML permits a different range of possibilities for the value of ω (dN/dS) in the data being analysed. M1a is a nearly neutral model, allowing two classes of sites, $\omega = 1$ (neutral evolution) and $0 \leq \omega < 1$ (negative selection). M2a allows in addition a third class of sites, $\omega > 1$ (positive selection). M7 models a β-distribution of 10 classes of sites with differing values within $\omega \leq 1$ (neutral/negative selection), and M8 models the same plus one further class of sites with $\omega > 1$. PAML assesses how well each model fits the data and produces a likelihood output score. A likelihood ratio test (with *p* value significance determined using χ^2 distributions) can then be applied to determine, for each set of nested models tested, whether one fits the data significantly better than the other. A signal of positive selection consists in a significant likelihood ratio test value for comparisons where M2a and M8 are found to fit the data significantly better than M1a or M7 respectively. PAML then uses Bayes Empirical Bayes methods to try to identify individual sites where this positive selection may have occurred. Sites with BEB posterior probabilities greater than 0.9 are listed here as identified positively selected sites; posterior probabilities are listed in parentheses.

Supplementary Table S3.6: Codon numbers from Toll-like receptor (TLR) sequence alignments produced in the current study, relative to reference sequences for *Gallus gallus* TLR sequences available on GenBank. OBP = alignments of groups of orange-bellied parrot sequences. *Neophema* = alignments containing sequences from all *Neophema* and *Neopsephotus* parrots.

Locus	Dataset	Chicken codon # corresponding to codon 1 in dataset from this paper	Chicken protein reference sequence used (genbank number)
TLR1	*Neophema*	16	ABF67957.1
	OBP	165	
TLR3	*Neophema*	253	NP_001011691.3
	OBP	275	
TLR4	*Neophema*	193	AAL49971.1
	OBP	212	
TLR5	*Neophema*	274	CAF25167.1
	OBP	272	
TLR7	*Neophema*	238	NP_001011688.1
	OBP	272	
TLR10	*Neophema*	234	BAD67422.1
	OBP	280	

APPENDIX 4: **SUPPLEMENTARY MATERIALS CHAPTER 4**

Supplementary Table S4.1: Details of submodels within 2 AIC_C of the top model generated by generalised linear mixed modelling (or for *P. aeruginosa* outcome, by generalised linear modelling), which were averaged to produce final models

Response variable	Predictor variables included	Degrees of freedom	log likelihood	AIC_C	ΔAIC_C	Weights (Akaike weights)
P. aeruginosa outcome	Intercept	1	-20.79	43.73	0	0.45
	Intercept + Sex	2	-19.89	44.22	0.49	0.35
	Intercept + Age	2	-20.42	45.28	1.54	0.21
Number of fledglings produced	Intercept + Age of parent	3	-287.81	581.78	0	0.44
	Intercept + Age of parent + Sex + Sex:Age of parent	5	-286.41	583.22	1.44	0.21
	Intercept + Age of parent + IR	4	-287.63	583.54	1.76	0.18
	Intercept + Age of parent + Sex	4	-287.71	583.7	1.92	0.17
Proportion of fledglings matured	Intercept	2	-90.05	184.17	0	0.46
	Intercept + IR	3	-89.89	185.94	1.77	0.19
	Intercept + Age of other parent	3	-89.93	186.01	1.84	0.18
	Intercept + Age of parent	3	-90	186.16	1.99	0.17
BFDV test result	Intercept + Age	3	-9.6	25.85	0	0.72
	Intercept + Age + IR	4	-9.33	27.76	1.91	0.28

Abbreviations: BFDV, *Beak and feather disease virus*; IR, internal relatedness (measure of individual homozygosity).

Supplementary Table S4.2: Predictors included in global models for orange-bellied parrot (*Neophema chrysogaster*) heterozygosity-fitness correlations of disease outcome and reproductive output

Dataset	Variables included	Variable type	Variable in final model?
P. aeruginosa outcome	Age	fixed	Y
	Sex	fixed	Y
	IR	fixed	N
BFDV test result	Age	fixed	Y
	IR	fixed	Y
	Sex	fixed	N
	Time of year tested	random	n/a
Number of fledglings produced	Age of parent	fixed	Y
	Sex	fixed	N
	IR	fixed	Y
	IR:sex	fixed	N
	Individual ID	random	n/a
	Sex: Age of parent	fixed	N
	OtherParentAge	fixed	Y
Proportion of fledglings matured	Age of parent	fixed	Y
	Sex	fixed	N
	IR	fixed	Y
	IR:sex	fixed	N
	Year	random	n/a
	OtherParentAge	fixed	Y
	Sex: Age of parent	interaction	N
	Sex: OtherParentAge	fixed	N

Abbreviations: BFDV, *Beak and feather disease virus*; IR, internal relatedness (measure of individual homozygosity). Year refers to year data collected. Age of parent refers to age of bird included in our study, whose fitness (number of fledglings produced/proportion of fledglings matured) is being assessed. OtherParentAge refers to age of the bird that was paired with the bird of interest. ID, arbitrary number specific to each individual included in the study. Pair ID, arbitrary number representing both birds included in a pairing. Location refers to institution, or wild, where birds were located at time fitness measurements were taken.

Supplementary Table S4.3: Predictors for which data were available but which were not included in building global models for heterozygosity-fitness correlations relating to disease outcome and reproductive output in the orange-bellied parrot (*Neophema chrysogaster*)

Dataset	Variables excluded	Variable type	Reason for variable exclusion
P. aeruginosa outcome	none	-	-
BFDV test result	Individual ID	fixed	No individuals were included in the dataset more than once
	Location	random	Data was dominated by one location
Number of fledglings produced	Year	random	Inclusion of this factor prevented model convergence
	Pair ID	random	Most birds did not pair with the same individual across years
	Location	random	Data was dominated by one location
	Sex: OtherParentAge	fixed	Model failed to converge so this was excluded
Proportion of fledglings matured	Individual ID	random	Many individuals appeared in this list, which overcomplicated the model and prevented convergence
	Pair ID	random	Most birds did not pair with the same individual in multiple years
	Location	random	Data was dominated by one location

Abbreviations: BFDV, *Beak and feather disease virus*. Year refers to year data collected. ID, arbitrary number specific to each individual included in the study. Pair ID, arbitrary number representing both birds

APPENDIX 5: **SUPPLEMENTARY MATERIALS CHAPTER 5**

Supplementary Table S5.1: Details of sources for the *Neophema* and *Neopsephotus* parrot images included in Figure 5.1

Photo	Species (common name)	Photographer	Photo title	Link	Creative Commons License
a.	orange-bellied parrot	Ron Knight	Orange-bellied Parrot (Neophema chrysogaster)	https://flic.kr/p/diY6BN	CC BY 2.0
b.	blue-winged parrot	David Cook	Blue-winged Parrot (Neophema chrysostoma)	https://flic.kr/p/PDxWhV	CC BY-NC 2.0
c.	elegant parrot	Brian McCauley	Elegant Parrot (Neophema elegans)	https://flic.kr/p/Pmf5Ct	CC BY-NC 2.0
d.	rock parrot	Brian McCauley	Rock Parrot (Neophema petrophila)	https://flic.kr/p/P8zz3m	CC BY-NC 2.0
e.	turquoise parrot	David Cook	Turquoise Parrot (Neophema pulchella)	https://flic.kr/p/H9XGtA	CC BY-NC 2.0
f.	scarlet-chested parrot	Aaron Fellmeth Photography	Australia 2018 - South Australia - Scarlet-Chested Parrot 1a	https://flic.kr/p/2bU2noW	CC BY-NC-ND 2.0
g.	Bourke's parrot	Laurie Boyle	Bourke's Parrot.	https://flic.kr/p/yGzUXK	CC BY-SA 2.0

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Images were cropped but otherwise unmodified

Supplementary Table S5.2: Sequences of primers used for amplification of fragments of Cytochrome oxidase I and Cytochrome B used in this study to amplify sequences from *Neophema* and *Neopsephotus* parrots

Primer target gene	Primer name	Primer sequence	Expected product length	Relevant publication
Cytochrome oxidase I (COI)	BAK1490	CTCAACCAACCACAAAGACATCGG	658 bp	Neaves et al. 2018
	BAK2198	TAGACTTCTGGGTGGCCGAAGAATCA		
Cytochrome B (CytB)	L14841	AAAAAGCTTCCATCCAACATCTCAGCATG ATGAAA	307 bp	Kocher et al. 1989
	H15149	AAACTGCAGCCCCTCAGAATGATATTTGT CCTCA		

Supplementary Table S5.3: PCR conditions for amplification of fragments of Cytochrome oxidase I and Cytochrome B. Used in this study to amplify sequences from *Neophema* and *Neopsephotus* parrots, with the primers from Supplementary Table S5.2

PCR step	Temperature (°C)	Length (seconds)	Number of cycles
Initial denaturation	94	180	1x
Denaturation	94	20	38x
Annealing	55	40	
Extension	72	40	
Final extension	72	300	1x