The Ecology and Evolution of Beak and Feather Disease

Virus in *Platycercus elegans*

by Justin Eastwood

B. Sc. (Hons)

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Deakin University

March, 2015
Title page image: crimson rosella (*Platycercus elegans*).

Courtesy of Dr Mathew L. Berg
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Preface

All data chapters (2-6) within this PhD thesis were intended to be submitted separately to peer-reviewed scientific journals. Deakin University offers candidates the option of submitting a “thesis by publication”, whereby each chapter is presented in publication format with a single reference list placed at the end. Consequently, there is some overlap between the five data chapters. Chapters 2 and 3 have already been published and chapters 4-6 will be submitted shortly. All chapters have benefited from the many contributions made by my supervisory team and collaborators. The published chapters have also benefited from anonymous reviewer comments. A list of publications and intended submissions from this thesis are presented below:

Chapter 2:


Chapter 3:

Chapter 4:


Chapter 5:


Chapter 6:

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Abstract

The links between free-living animals and emergent infectious diseases have important implications for mitigating disease impacts on agriculture, human health, and conservation efforts. Generally, pathogen infection dynamics and their impact on free-living host populations are not well understood, particularly in relation to biodiversity. Infectious disease outbreaks may reduce biodiversity by driving rapid mortality rates and subsequently species extinction. Investigation of the variables that influence the spread of infection in free-living populations is needed, particularly in important yet poorly understood host-pathogen systems. Beak and feather disease virus (BFDV) is a single-stranded DNA circovirus that infects Psittaciformes (parrots, lorikeets and cockatoos). BFDV is highly transmissible and typically lethal due to its severe disease signs, including feather loss, beak deformity and immune suppression. Currently, BFDV is of conservation concern on a global scale, but few studies have investigated the ecology and evolution of this virus in the wild. In this thesis, the crimson rosella (Platycercus elegans) species complex was utilised to examine variables that affect BFDV infection in wild, free-living birds. P. elegans is a common and widespread Australian parrot; it has generally been considered a rare example of a ring-species and consists of several phenotypically and genetically distinct populations, which encircle a geographic barrier. Due to this unusual population structure, P. elegans is a ‘natural laboratory’ to elucidate patterns of infection, and to investigate viral differentiation in response to host divergence. Samples were collected over a period of eight years across the four main subspecies populations including: P. e. elegans, P. e. flaveolus, P. e. adelaidae, and a hybrid between P. e. elegans × P. e. flaveolus (Western Slopes hybrid). A quantitative real-time PCR detection method was developed to determine BFDV prevalence and viral load in these samples. Subspecies was found to be the most important predictor of BFDV prevalence and viral load, with hybrid and P. e. adelaidae populations less infected. Additionally, Julian date, sex and age were also found to be important predictors of BFDV infection. Geographic location, intraspecific host density, and susceptible host community diversity and composition appeared to have no effect on BFDV infection dynamics. In addition, there was no variation in seroprevalence (BFDV specific antibodies) levels between the subspecies and hybrid populations. Possible host genetic diversity effects on BFDV infection were also identified. Microsatellite heterozygosity was higher in non-infected
individuals, and viral load was predicted by genotype rarity. These data suggest that the evolved host response was important in determining the spread of infection, whilst ecological effects appeared to be less important. My research also indicates that BFDV may have played a role in the maintenance of diversity in *P. elegans* by influencing the rate of gene flow between subspecies, via differentially affecting hybrid populations. Host population structure may have influenced BFDV evolution. BFDV was found to share phylogeographic structure with its host *P. elegans*, resulting in BFDV displaying properties analogous to those postulated for the ring-species hypothesis. This thesis also offers insight into *P. elegans* mating structure, suggesting that breeding birds changed partners and nest boxes regularly. Moreover, parentage analysis revealed that there was no extra-pair paternity in *P. elegans*, but there were low levels of conspecific brood parasitism. Finally, this study investigated transmission dynamics within *P. elegans* breeding pairs and their offspring. Transmission did not appear to be dependent on direct contact within breeding pairs. However, mating patterns did seem to vary according to BFDV infection, with few infected males found breeding. Parental infection did not appear to influence offspring infection, but broods were found infected despite both parents not being infected. Therefore, it was inferred that environmental contamination could be a likely transmission route in natural populations. My study not only underlines the variables important in predicting BFDV infection in wild populations, but demonstrates more broadly that host co-evolutionary dynamics are key factors in explaining the spread of infection. Furthermore, my results have important conservation implications and potential application for disease management in a threatened and declining order of birds.
CHAPTER 1: Introduction

Photo: yellow roSELLA (*Platycercus elegans flaveolus*).

Courtesy of Dr Mathew L. Berg
1.1 Introduction overview

Over recent decades, researchers have begun to realise the importance of wildlife in mediating the epidemiology of emerging infectious disease in agriculture and human populations (Daszak et al. 2000; Keesing et al. 2010). Disease in wildlife has also been linked to a reduction in biodiversity, with several diseases causing conservation concern (Altizer et al. 2003). Host-pathogen relationships in wildlife have also received attention from ecologists and evolutionary biologists, who are increasingly aware of the importance of pathogens in explaining phenomena such as genetic diversity, sexual reproduction, or the maintenance of extravagant sexually selected traits (Haldane 1949; Hamilton & Zuk 1982; Lively et al. 2014). The study of wildlife host-pathogen model systems can also help elucidate new pathways explaining the spread of disease. In this chapter, I first provide a brief introduction into the field of wildlife disease ecology and evolution, and then summarize the literature relevant to the main topics addressed in chapters 2-6. This consists of describing the ecological complexity of host-pathogen interactions and the subsequent co-evolutionary dynamics. Furthermore, I discuss the pathogen of study (Beak and Feather Disease Virus), the study species (Platycercus elegans species complex), and finally, outline the aims of this thesis.

1.2 Ecological predictors of infection

Parasites are ubiquitous and highly diverse in nature (Schmid-Hempel 2011). Macroparasites are relatively large organisms with long generation times and include helminths and parasitic arthropods. Microparasites are smaller, have shorter generation times and exist in larger numbers within their host, and typically include viruses, bacteria, fungi and protozoa (Schmid-Hempel 2011). Micro- and macro-parasites negatively affect their hosts in the form
of disease, and then are collectively termed as pathogens. Not all host individuals within a population may be equally infected and typically a small proportion of hosts harbour most of the population’s pathogens (Woolhouse et al. 1997; Hudson 2002). Heterogeneity in infection risk is diverse and complex, but there are many examples in wild animals that demonstrate how essential individual characteristics are for predisposing infection risk (Hudson 2002). Individual host demographics are important, with many studies identifying sex and age related variation in prevalence and infection load (Poulin 1996; Zuk & McKean 1996; Hudson 2002; Sol et al. 2003). However, individuals often also vary in body condition, genetic predisposition, and behaviour making them more or less susceptible to infection (Thomas et al. 2009; Schmid-Hempel 2011). Similarly, heterogeneity in infection prevalence and load may vary according to pathogen characteristics including genetics, virulence, and transmission pathways (direct and indirect) (Hudson 2002).

While individuals differ in their susceptibility, population level effects may escalate infection risk by increasing exposure levels (Hudson 2002). Studies have shown that higher host density increases host exposure to pathogens (Arneberg et al. 1998; Bell et al. 2006; Donnelly et al. 2013). Furthermore, community characteristics (e.g. species composition, richness, and density) can also impact host-pathogens interactions, which subsequently influence pathogen prevalence and load (Johnson et al. 2008; Keesing et al. 2010; Johnson et al. 2013). Additional to these biotic effects, abiotic factors such as temperature, seasonality, chemical background, pH, and humidity can influence infection (Hudson 2002; Altizer et al. 2006). Hence, environmental variables (biotic and abiotic), as well as individual host and pathogen variables all interact to shape the spread and severity of infection, both spatially and temporally. In attempts to gain general insights into the ecological complexity of host-
pathogen interactions, epidemiologists and disease ecologists can utilize mathematical models. The most common and simple model is the SIR-model, which is a function of the number of Susceptible, Infected, and Recovered hosts (Anderson & May 1979; Schmid-Hempel 2011). SIR-models account for births and deaths (naturally and pathogenically induced), pathogen transmission, pathogen clearance (e.g. immunity), and are typically expanded to include two important concepts: $R_0$ and the “force of infection” (Anderson & May 1979, 1985). $R_0$ is referred to as the basic reproductive number of a pathogen, in other words, the number of new infections transmitted from an infected individual (Anderson & May 1985). For a pathogen to spread and not become extinct within a population, $R_0$ must be greater than or equal to one. The force of infection is not independent of $R_0$, nor most of the variables discussed above, and is defined as the rate at which an uninfected individual becomes infected within a population (Anderson & May 1985). Such models form the basis of our understanding into the ecology of wildlife disease and are important in describing the spread of infection.

1.3 Evolutionary predictors of infection

In the previous section, I outlined some of the main factors that account for the variation in pathogen infection within host individuals and populations, and their interconnectedness. It is therefore not surprising that this variability could be open to host-pathogen reciprocal selection, resulting in host-pathogen evolution. Hosts have evolved a variety of defences against infection, namely avoidance, resistance and tolerance (Medzhitov et al. 2012). Resistance to pathogens involves the reduction and removal of pathogens once infected; in animals, this task is handled by the immune system (innate and adaptive) (Schmid-Hempel 2011). Immunity is a costly mechanism and its efficacy can be reduced by a suite of
environmental factors such as poor nutrition, environmental contaminants and stress (Thomas et al. 2009). Moreover, when investment in immunity is required, important life history factors can be negatively affected including growth, reproduction, or the expression of certain traits important for attracting a mate (Sheldon & Verhulst 1996; Thomas et al. 2009). The costly nature of the immune system leads to a trade-off between the effects of the pathogen, immunity and reduced resources for other traits. For example, traits under sexual selection can reflect an individual’s infection status by changing according to available resources (Able 1996). However, other host physiological factors such as steroid hormones can influence both sexually selected traits and suppress the immune system – a concept encapsulated by the Immunocompetence Handicap Hypothesis (Folstad & Karter 1992; Roberts et al. 2004). Tolerance mechanisms are also important for pathogen defence and aim to decrease fitness related costs and/or damage to tissues from either the pathogen or the immune system (Medzhitov et al. 2012). Variability in these three host defences appear to be genetically determined, particularly resistance and tolerance (Medzhitov et al. 2012). The severity of an infection may also be attributed to the evolved level of pathogen virulence (Schmid-Hempel 2011). Use of the term virulence varies between fields (Alizon et al. 2009), but as a general definition it typically refers to a pathogen’s capacity to negatively affect its host, either by damaging host tissues and/or reducing host fitness (Read 1994). In the short term, evolution can result in selection for high pathogen virulence (Levin & Bull 1994). However, high virulence can also result in associated high host mortality rates, which consequently reduce transmission opportunities. In a condition of low virulence, the host can have a fast recovery rate, again reducing transmission (Anderson & May 1982). This is known as the virulence-transmission trade-off hypothesis and suggests that pathogens should evolve intermediate levels of virulence (Shaw & Dobson 1995). However, this
The antagonistic, yet co-evolutionary dynamic between host and pathogen can be mediated by micro-evolutionary processes leading to local adaptation, potentially leading to speciation. Given that pathogens have the capacity to influence host fitness, susceptibility can vary within and between host species, and host defence can be heritable, it is hypothesised that pathogens can influence host divergence and potentially speciation (Karvonen & Seehausen 2012). Pathogens may not only drive divergence between populations that are allopatric (Buckling & Rainey 2002), but are also capable of promoting divergence in sympatric host populations (Sage et al. 1986; Bordenstein et al. 2001; Moulia & Joly 2009; Brucker & Bordenstein 2013). Alternatively, pathogens could also constrain host divergence by selecting for introgression and therefore maintaining gene flow between populations (Fritz et al. 1999; Wolinska et al. 2008; Moulia & Joly 2009). However, host-pathogen selection is reciprocal and hosts exert a strong selective force on their pathogens resulting in a co-evolutionary process (Schmid-Hempel 2011). Selection in pathogens and hosts has been found to result in strong host-pathogen phylogenetic associations, with particular pathogens being found only on particular hosts (Hafner & Nadler 1988; Huyse et al. 2005). Furthermore, as some pathogens have larger population numbers, faster generation times and higher mutation rates (e.g. influenza, human immunodeficiency virus).
when compared to their hosts, pathogens have even been associated with recent host population demographics and population structure (Sugimoto et al. 1997; Falush et al. 2003; Biek et al. 2006).

Underlying these macro-evolutionary patterns, micro-evolutionary dynamics such as arms races and frequency-dependent selection exist (Woolhouse et al. 2002; Schmid-Hempel 2011). In arms race dynamics, selection is directional with hosts and pathogens developing resistance and infectivity respectively (Schmid-Hempel 2011). Arms race dynamics are typically characterised by a continuing series of selective sweeps. In this scenario, novel resistance alleles/genes arise within a host population and increase in frequency over time, until they reach fixation or become extinct as pathogens adapt (Gandon et al. 2008). The competing co-evolutionary force is that of frequency-dependent selection, where allele or genotype frequencies in both the host and pathogen are under selective pressure to increase and decrease over time and space (Gandon et al. 2008). If the pathogen also evolves more rapidly (e.g. larger population size, faster generation times, and higher mutation rates), both genotypes interact, and variation exists, then pathogens are likely to rapidly adapt to the most common host genotype within a population (Schmid-Hempel 2011). This gives a selective advantage to rare genotypes or alleles within a population and therefore, protection from extinction. This mechanism may explain the maintenance of genetic diversity (Schmid-Hempel 2011; King & Lively 2012), polyandry (Baer & Schmid-Hempel 1999), polygyny (Liersch & Schmid-Hempel 1998; van Baalen & Beekman 2006), and has led some to propose that pathogen selection could explain the evolution of sex (Hamilton 1980). This phenomenon has been demonstrated in various invertebrates, resulting in a time-lagged negative frequency-dependent scenario. This model is well
supported theoretically (Woolhouse et al. 2002; King & Lively 2012) and experimentally (Lively & Dybdahl 2000; Koskella & Lively 2009; Hall et al. 2011; Betts et al. 2014; Gómez et al. 2015), but evidence for negative frequency-dependent selection in natural populations has predominantly been found in asexual invertebrates (Decaestecker et al. 2007; Jokela et al. 2009; Wolinska & Spaak 2009; King et al. 2011), asexual fish (Lively et al. 1990), plants (Thrall et al. 2012), bacteria (Gómez & Buckling 2011), or in studies investigating allelic frequency within a population (Trachtenberg et al. 2003; Kamath et al. 2014). Both the arms race and frequency-dependent selection processes underlie the Red Queen hypothesis (Van Valen 1973), thus named after Lewis Carroll’s Alice in Wonderland, “…it takes all the running you can do, to keep in the same place…” and both may operate across multiple loci within a population (Gandon et al. 2008). In addition, the dominant co-evolutionary force (arms race or fluctuating selection) may depend on the environment or population structure (host and pathogen), thus explaining the variability of these mechanisms presented between species across time and space (Gómez et al. 2015). At a population level, host genotype diversity can limit the evolution of virulence; within monocultures, pathogens are adapted and can readily infect individuals. However, when multiple genotypes exist within a population, pathogens are constrained adaptively and are less virulent (Kubinak et al. 2012; Kubinak et al. 2015). Consistent with this finding, modelling shows that increasing genetic diversity can also decrease $R_0$ (Lively 2010).

Host genetic diversity in terms of heterozygosity at the individual or population level may also be important in host susceptibility to infection (Altizer et al. 2003). Low heterozygosity can arise through inbreeding. Inbred individuals are more likely to be homozygous at a specific locus and could therefore have a lower capacity for coding defence
mechanisms for a wide range of pathogens (Schmid-Hempel 2011). Considerable evidence within natural populations has shown that inbred individuals tend to display a higher prevalence (Acevedo-Whitehouse et al. 2003; MacDougall-Shackleton et al. 2005; Ortego et al. 2007a; Townsend et al. 2009; Isomursu et al. 2012) and load of pathogens (MacDougall-Shackleton et al. 2005; Calleri et al. 2006; Luikart et al. 2008; Isomursu et al. 2012). There are exceptions to this trend (Cote et al. 2005; Ortego et al. 2007b), but these tend to relate to low pathogen selective pressure or in some cases the methodology used for measuring genetic diversity (Luikart et al. 2008; Gompper et al. 2011; Loiseau et al. 2011). Analysing genetic diversity in functional regions involved in immunity (e.g. major histocompatibility complex or toll-like receptors) also demonstrates that host genetic diversity is negatively related to pathogen infection (Westerdahl et al. 2005; Westerdahl 2007; Tschirren et al. 2013). Evidence for stronger immune function in genetically diverse individuals is also well supported (Reid et al. 2003; Hawley et al. 2005; Reid et al. 2007; Fossøy et al. 2009; Gompper et al. 2011; Varsani et al. 2011). At a population level, pathogen-mediated removal of inbred and homozygous individuals (diversifying selection) is thought to contribute to increasing population level heterozygosity (Coltman et al. 1999). Hence, pathogens are a strong selective force in nature that have the capacity to influence host evolution.

1.5 This study – the pathogen, Beak and Feather Disease Virus

Beak and feather disease virus (BFDV) is the causative agent of a debilitating disease known as psittacine beak and feather disease (PBFD). This virus is exclusive within the order Psittaciformes, encompassing all lorikeet, parrot and cockatoo species (Paré & Robert 2007). The first possible indication of this disease might be traced to a number of red rump parrots (Psephotus haematonotus) in 1887, reportedly in the Adelaide Hills of South Australia (Ashby
Currently, BFDV is considered one of the most common viral infections in psittacine birds (Paré & Robert 2007) and has so far been detected in over 60 different species, however, it is assumed that all of the approximated 372 psittacine species are susceptible (Harkins et al. 2014). PBFD may present as either a chronic, acute or peracute infection (Paré & Robert 2007). The signs of disease vary according to species, age and individual health status, but typically manifest as feather malformation and/or loss, beak deformity, claw deformity (Pass & Perry 1984), and immuno-suppression (Todd 2000; Kiatipattanasakul-Banlunara et al. 2002; Ortiz-Catedral 2010). Conversely, individuals may die from infection without displaying any signs of the disease (Ritchie 1995; Paré & Robert 2007). PBFD disease progression may also be determined by BFDV’s long incubation period (3 weeks to a year).

BFDV is characterised as a single-stranded DNA (ssDNA) circovirus from the family circoviridae (Bassami et al. 1998; Varsani et al. 2011), and its genome consists of approximately 2000 nucleotides enveloped within an icosahedral protein structure (Ritchie et al. 1989; Niagro et al. 1998; Bassami et al. 2001). The genome has up to seven open reading frames (ORF) one of which has been identified as the capsid ORF and another referred to as the replication associated ORF (Bassami et al. 1998; Bassami et al. 2001). The BFDV genome also contains a stem loop structure that contains a nonanucleotide motif (TAGTATTAC) that is involved in rolling circle replication (Bassami et al. 1998; Bassami et al. 2001). Nucleotide substitution rates of BFDV are amongst the highest in ssDNA viruses (Ritchie et al. 2003; Harkins et al. 2014), and the virus is susceptible to high levels of recombination (Heath et al. 2004; Julian et al. 2013).

Both phylogenetic and anecdotal evidence suggest that BFDV originated in Australia and was disseminated internationally via the pet trade (Varsani et al. 2011; Harkins et al. 2014).
Currently, there is some evidence to suggest that BFDV is genotypically associated with particular psittacine species and that geographic location on a continental scale also explains some of the phylogenetic variation (Ritchie *et al.* 2003; Varsani *et al.* 2011; Harkins *et al.* 2014; Sarker *et al.* 2014a). However, it does appear that spill over can occur between highly divergent host psittacine species (Harkins *et al.* 2014; Peters *et al.* 2014; Sarker *et al.* 2014a). Although there is some suggestion that BFDV is a generalist among Psittaciforme species (Sarker *et al.* 2014a), it is unclear whether this is simply an artefact of the high proportion of sequences arising in captive breeding/rescue centres, or whether BFDV lineages can indeed infect all species. In captivity, birds are typically confined to small cages at high density, therefore facilitating horizontal transmission (Varsani *et al.* 2011; Harkins *et al.* 2014). Horizontal transmission occurs by direct contact with infected individuals, via shedding large amounts of virus (usually in feather dust, crop secretions and faeces) or with the virus persisting in the environment (Ritchie *et al.* 1991a). Vertical transmission has also been suggested, evidenced by PCR detection of the virus in eggs and reproductive organs (Rahaus *et al.* 2008). Individuals can harbour multiple genetic variants of BFDV, some of which are likely due to multiple infections and not necessarily multiple divergent variants (Sarker *et al.* 2014a; Sarker *et al.* 2014b). Multiple BFDV variants within individuals create the opportunity for genetic exchange between variants by viral recombination (Varsani *et al.* 2011; Harkins *et al.* 2014).

Much research has centred on BFDV in captive psittacine birds because of its devastating effect in commercial and domestic aviaries (Rahaus & Wolff 2003; Bert *et al.* 2005; Piasecki & Wieliczko 2010; Zhuang *et al.* 2012). In captivity, BFDV prevalence is often higher in juvenile or young (< 3 years) birds (Bert *et al.* 2005), and there appears to be no
difference between males and females (Rahaus & Wolff 2003). Notably, some species
appear to be less susceptible or less likely to be found with BFDV; cockatiels are rarely found
to have the virus (Shearer et al. 2008a). BFDV is increasingly receiving attention from
conservationists who are concerned about the effects that this virus could have on already
threatened and endangered species in the wild (Ortiz-Catedral et al. 2009; Kundu et al. 2012;
Jackson et al. 2014; Peters et al. 2014). The order Psittaciformes represent one of the most
heavily threatened orders of birds, with 27% worldwide classified between vulnerable to
critically endangered (Baillie et al. 2004; IUCN 2014). An 11 year study on Echo parakeets
(Psittacula echo) in Mauritius demonstrated that an outbreak of BFDV had severely reduced
population size (Kundu et al. 2012). This was attributed to a selective sweep of a series of
mutations within the BFDV genome which are now predominant within this small
population. In New Zealand and New Caledonia there are grave concerns about the
conservation implication of BFDV spill-over into endemic species, for example, red-fronted
parakeet (Cyanoramphus novaezelandiae), yellow-fronted parakeet (Cyanoramphus
auriceps), kakapo (Strigops habroptila), Ouvea parakeet (Eunymphicus uvaeensis) and
Malherbe’s parakeet (Cyanoramphus malherbi) (Ha et al. 2009; Ortiz-Catedral et al. 2009;
Massaro et al. 2012; Jackson et al. 2014). BFDV appears to be introduced into both New
Zealand and New Caledonia via the introduction of Australian psittacines (Julian et al. 2012;
Massaro et al. 2012), particularly, eastern rosellas (Platycercus eximius) which are
considered to be a BFDV reservoir (Ha et al. 2007; Ha et al. 2009; Ortiz-Catedral et al. 2009;
Massaro et al. 2012). In Australia, BFDV is considered a “key threat to biodiversity” by the
Commonwealth Government because the virus has the potential to cause extinction to many
Australian species (Act EPBC 1999; Australian Department of the Environment and Heritage
2005; Orange-bellied Parrot Recovery Team 2006). For example, the orange bellied parrot
(Neopbema chrysogaster) has less than 50 individuals remaining in the wild. Over the course of one year, viral prevalence in this population reached 29% (Sarker et al. 2014b). This is concerning due to the fact there is evidence to suggest that this population does not maintain its own co-evolved strain, but is susceptible to spill over events from other species (Peters et al. 2014; Sarker et al. 2014a; Sarker et al. 2014b). Furthermore, some species can be regularly exposed to BFDV infection, as evidenced by a high BFDV seroprevalence (41-94%) (McOrist et al. 1984; Raidal et al. 1993b). Despite posing such a critical threat to endangered Psittaciformes, the ecology and evolution of BFDV in wildlife is not well understood.

1.6 This study – the host, Platycercus elegans

Highly variable and hybridising species complexes offer a natural laboratory for the identification of factors or processes that are important in host divergence (Hewitt 1988; Barton & Hewitt 1989; Arnold & Martin 2010). Hybrids are the result of two divergent forms meeting and reproducing (Barton & Hewitt 1989). Pathogens potentially represent an important selective force within hybridising species complexes, because they have the capacity to influence gene flow between diverging host species (Wolinska et al. 2008; Moulia & Joly 2009). Our understanding of pathogens in hybridizing communities is drawn primarily from studies in plant taxa, and surprisingly only a few animal models exist (Fritz et al. 1999; Wolinska et al. 2008). Four main scenarios have been proposed to reflect prevalence and infection load across hybrid zones: (1) susceptibility, (2) resistance, (3) additive (hybrid infection is intermediate), and (4) dominance (hybrid infection is similar to one parental species) (Wolinska et al. 2008). Similar to hybrid species complexes, host ring-species offer a unique opportunity to elucidate predictors of infection and their potential for manipulating
host divergence. Additionally, ring-species may provide insight into pathogen divergence in response to host speciation. Ring-species are rare in nature, as evidenced by a mere 25 species that have been proposed worldwide (Irwin et al. 2001a). Such species complexes typically consist of two phenotypically and genetically distinct subspecies (terminal populations), that are connected through a chain of intermediate populations that encircle a geographic barrier (Mayr 1942; Irwin et al. 2001a; Alcaide et al. 2014).

*Platycercus elegans* represents one example of a putative ring-species and consists of four main populations surrounding unsuitable semi-arid habitat of Mallee heathland (Figure 1.1) (Cain 1955). The crimson rosella (*P. e. elegans*) represents one terminal subspecies which inhabits mesic Australian woodlands and forests along the south eastern side of the distribution. The yellow rosella (*P. e. flaveolus*) constitutes the other terminal subspecies, being most phenotypically distinct from the crimson rosella with a characteristic yellow plumage (Figure 1.2). *P. e. flaveolus* occurs in the drier inland riparian zones, primarily distributed along the Murray and Murrumbidgee River systems (Figure 1.1). The *P. e. flaveolus* and *P. e. elegans* distributions converge along the Western Slopes of the Great Dividing Range (Figure 1.1). Interm breeding within this contact zone has resulted in what has been termed the Western Slopes hybrid (in sensu, Joseph et al. 2008). At the western margins of the distribution, a phenotypic intermediate (Figure 1.2; orange plumage), the Adelaide rosella (*P. e. adelaidae*), is geographically continuous with *P. e. flaveolus*. 200 kilometres separates *P. e. adelaidae* from *P. e. elegans*, although it appears that gene flow can still occur across this gap (Joseph et al. 2008).
Figure 1.1. Distribution map of *Platycercus elegans*, which is based on atlas data collected by members of Birdlife Australia. (A) represents the crimson rosella (*P. e. elegans*), (B) the yellow rosella (*P. e. flaveolus*), (C) the Adelaide rosella (*P. e. adelaidae*), (D) the Kangaroo Island population (*P. e. melanoptera*), (E) the far North Queensland population (*P. e. nigrescens*), and (F) the Western Slopes hybrid population formed between A and B [WS hybrid in sensu Joseph et al. (2008)].

*P. e. adelaidae* can be broken into two additional subspecies *P. e. fleurieuensis* and *P. e. subadelaidae*. *P. e. fleurieuensis* is a dark orange, almost red phenotype, however in the northern range *P. e. subadelaidae* is closer to the yellow phenotype (Forshaw & Cooper 2002). In reality, genetic evidence (microsatellite assignment) supports *P. e. adelaidae* being a single subspecies, with a phenotypic gradient (yellow to dark orange) running north to south (Joseph et al. 2008). This cline has been suggested to exist in response to...
environmental variation, specifically rainfall (drier in the north and wetter in the south) (Joseph et al. 2008). Two other subspecies belong to P. elegans, P. e. melanoptera on kangaroo island and P. e. nigrescens in far north Queensland (Forshaw & Cooper 2002). However, these populations are not the focus of this study. Although P. elegans was once considered a ring-species, genetic testing of this hypothesis suggests a more complex evolutionary history that is still unresolved (Joseph et al. 2008). Previously, this system has been utilised for investigating acoustic call structure (Ribot et al. 2009; Ribot et al. 2011; Ribot et al. 2012), avian vision (Carvalho et al. 2010; Knott et al. 2010; Knott et al. 2013), olfaction (Mihailova et al. 2014), and colouration (Berg & Bennett 2010). No study has investigated the spread of infection within a ring-species complex, excluding my preliminary work, which indicates that BFDV is a widespread and prevalent infection within this P. elegans complex (Eastwood 2010).
Figure 1.2. Shows the three main *Platycercus elegans* subspecies that are the focus of this study. (A) denotes *P. e. adelaïdae*, (B) *P. e. flaveolus* and (C) *P. e. elegans*. *P. e. flaveolus* and *P. e. elegans* meet on the Western Slopes of the Great Dividing Range and hybridise, forming the phenotypic intermediate population WS hybrid. WS hybrids resemble *P. e. adelaïdae* in plumage colour.
1.7 Chapter outline and study aims

In this thesis, I report my investigation of a pathogen in a putative ring-species (the *P. elegans* species complex). To my knowledge this is the first time a pathogen has been investigated in a putative ring-species. There are several justifications for conducting my research. First, BFDV is a key threatening process to biodiversity in Australia and understanding the specific variables important for infection in wild populations will aid conservation efforts (Australian Department of the Environment and Heritage 2005). Second, understanding host-pathogen interactions in wild populations can provide insight into the spread of infection. Third, by utilising a host ring-species complex we can better understand host-pathogen co-evolution.

In chapter 2, I describe a method to detect BFDV using quantitative real-time PCR and validate this method using samples collected from wild *P. elegans*. In addition, I assess three sample types (blood, muscle tissue and feathers) from the same individuals for BFDV detection and test whether the results from each are comparable.

In chapter 3, I address the paucity of BFDV knowledge in wild Psittaciforme species utilising the common parrot species *P. elegans*. Specifically, I aim to (1) determine important host factors (subspecies, age, sex) that predict BFDV infection (viral prevalence and load), (2) determine the ecological factors (host population density, host community diversity and composition, temporal and spatial location) that predict BFDV infection, (3) determine how BFDV isolates differ in response to host divergence, and (4) test the ring-species concept using BFDV isolates as a proxy for host population structure. This study not only contributes to our understanding of a global conservation problem but provides insight into host-pathogen ecology and co-evolution in a natural environment.
In chapter 4, I investigate the role genetic diversity plays in explaining BFDV prevalence and load. There is a wealth of evidence supporting the hypothesis that genetic diversity influences pathogen susceptibility, notably heterozygote advantage (diversifying selection) and rare genotype advantage (fluctuating selection). However, the relative importance of these distinct types of genetic diversity is unknown. In the chapter, I report measures of genetic diversity at the individual and subspecies level, and tests of whether they are associated with BFDV prevalence and load. In addition, host genetic distance was compared to BFDV viral genetic distance to test the hypothesis that the host and pathogen are antagonistically co-evolving.

The original aim of chapter 5 was to test the hypothesis that infected females increase their fitness by increasing brood genetic diversity through extra pair paternity (Johnsen et al. 2000; Foerster et al. 2003; Soper et al. 2014). Higher offspring genetic diversity may reduce pathogen susceptibility. First, I determined the level of extra-pair paternity or conspecific brood parasitism in P. elegans. Second, I tested whether social mate fidelity continues across breeding seasons, and whether breeding pairs maintain nest site fidelity across years. The results of this study did not allow one to test if infected influenced females extra pair paternity rates, but did offer novel insight into Psittaciforme mating systems.

BFDV is transmitted directly (horizontal and vertical transmission) and indirectly (via the environment) between susceptible hosts. These transmission routes are all likely to be maximised between family members, therefore BFDV infection could manipulate infection patterns between breeding pairs and their nestlings. In chapter 6, I report sampling of breeding pairs and their nestlings for BFDV in three P. elegans populations (P. e. elegans, P.
I then test for non-random infection (prevalence and load) patterns among breeding pairs, and determine whether parental infection status influences that of their nestlings.

Finally in chapter 7, I integrate these data chapter findings and summarise the contribution this work has made to our understanding of host-pathogen interactions and the evolution of a virus within an avian ring-species complex.
CHAPTER 2: Prevalence of beak and feather disease virus in wild *Platycercus elegans*: comparison of three tissue types using a probe-based real-time qPCR test

Photo: crimson rosella (*Platycercus elegans elegans*).

Courtesy of Dr Raoul F. H. Ribot
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<td>Australian Journal of Zoology</td>
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J. Eastwood made a significant contribution to all aspects of the project. Including: conception of the project, the design of methodology, data collection, analysis, interpretation of the data, drafting the manuscript, and manuscript revision.

I declare that the above is an accurate description of my contribution to this paper, and the contributions of other authors are as described below.

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<td>Justin Eastwood</td>
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<td>Conception of the project, the design of methodology, data collection, analysis, interpretation of the data, drafting the manuscript, and manuscript revision.</td>
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<td>The design of methodology, data collection, interpretation of the data, drafting the manuscript, and manuscript revision.</td>
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<td>Ken R. Walder</td>
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<td>Mathew Berg</td>
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2.1 Abstract

The detection of avian viruses in wild populations has considerable conservation implications. For DNA-based studies, feathers may be a convenient sample type for virus screening and are, therefore, an increasingly common technique. This is despite recent concerns about DNA quality, ethics, and a paucity of data comparing the reliability and sensitivity of feather sampling to other common sample types such as blood. Alternatively, skeletal muscle tissue may offer a convenient sample to collect from dead birds, which may reveal viraemia. Here, we describe a probe-based quantitative real-time PCR for the relative quantification of beak and feather disease virus (BFDV), a pathogen of serious conservation concern for parrots globally. We used this method to test for BFDV in wild crimson rosellas (Platycercus elegans), and compared three different sample types. We detected BFDV in samples from 29 out of 84 individuals (34.5%). However, feather samples provided discordant results concerning virus presence when compared with muscle tissue and blood, and estimates of viral load varied somewhat between different sample types. This study provides evidence for widespread infection of BFDV in wild crimson rosellas, but highlights the importance of sample type when generating and interpreting qualitative and quantitative avian virus data.
2.2 Introduction

A number of avian pathogens represent major conservation concerns (Newton 1998; Dazsak et al. 2000; Altizer et al. 2003). Noteworthy examples include malaria in Hawaiian forest birds, cholera in albatross, and beak and feather disease virus in psittacine birds (van Riper III et al. 1986; Weimerskirch 2004; Australian Department of the Environment and Heritage 2005). For this reason, considerable time and economic resources are devoted to the detection of pathogens such as viruses in wild bird populations. Due to the limited resources available and the vast scale of the problem that pathogens pose, sampling techniques that allow for the rapid and reliable PCR-based detection and quantification of pathogen loads from a range of host sample types are desirable. In particular, non-invasive sampling methods provide practical advantages and have been extensively used to draw general conclusions about pathogen levels (e.g. Davidson & Skoda 2005; Yamamoto et al. 2008; Busquets et al. 2010). For birds, feather sampling is increasingly being used as a source of DNA because feathers are often considered easier (less training required), quicker and less invasive to obtain than tissue biopsy or blood samples from venipuncture (Taberlet et al. 1999). As such, a range of sample types including feathers are now frequently being used for pathogen detection (Davidson & Skoda 2005; Yamamoto et al. 2008; Dhinakar Raj et al. 2013), particularly when investigations involve species that are difficult to trap (feathers may be collected when they drop from the bird, without the need for capture), are endangered (feather collection results in shorter handling times), or involve a large sampling effort, such as avian influenza virus detection in waterfowl (Stallknecht et al. 2012). Muscle tissue from dead birds (e.g. birds culled as pests, road kill or during natural mass death events) may provide a sample type that could be compared to blood in pathogen prevalence and load
studies. This could be the case because muscle is likely to contain blood, which otherwise might be difficult to obtain from dead birds. Muscle tissue would also be easier to collect and would require less training than the sampling of other tissues. However, there is a need for more studies that offer a direct comparison between common sample types (e.g. blood, tissue from dead animals, feathers) from the same individual at the same time, particularly with respect to the results from quantitative PCR for pathogen detection. This is particularly the case in light of recent concerns that have been raised about the lower quality and quantity of DNA obtained from feathers compared to more invasive methods (Taberlet et al. 1999; McDonald & Griffith 2011), and the risk of false positives in feather samples in some cases (Shearer et al. 2009).

In this study, we considered these issues following development of a quantitative real-time PCR (qPCR) method for the detection and relative quantitation of beak and feather disease virus (BFDV) in crimson rosellas (Platycercus elegans). BFDV provides a valuable case study because it is a significant conservation threat to parrots globally; it is listed as a ‘Key Threatening Process’ to biodiversity by the Australian Government and has been implicated in wild parrot declines in Australia and Mauritius (Heath et al. 2004; Australian Department of the Environment and Heritage 2005; Kundu et al. 2012). Furthermore, parrots are one of the world’s most threatened orders of birds, with approximately 37% of species at risk of extinction (Baillie et al. 2004). Extensive detection of wild parrot populations and more detailed studies of the fitness effects of infection are considered key priorities in the conservation management of BFDV (Australian Department of the Environment and Heritage 2005). The virus is thought to be capable of infecting all members of the order Psittaciformes (parrots, lorikeets and cockatoos) and is highly prevalent in wild and captive populations...
BFDV is a single-stranded DNA circovirus that is approximately 2000 nucleotides in length and has two primary coding regions, the replication associated protein and capsid protein (Bassami et al. 2001). BFDV is thought to have originated in Australia and has spread globally through the pet trade (Varsani et al. 2011; Harkins et al. 2014). Phylogenetic inference of BFDV suggests that different variants are associated with geographic location (country) and are considered also to be species specific (Eastwood et al. 2014). However, variants have been found to cross species boundaries within captive breeding centres (Varsani et al. 2011; Harkins et al. 2014), and has recently been found to occur in a wild and critically endangered population of Orange Bellied Parrots (Neophema chrysogaster) (Peters et al. 2014).

BFDV has been found to cause death without clear clinical signs (Latimer et al. 1991), and so PCR methods that target viral DNA in host samples are an excellent means to detect BFDV infected birds (Katoh et al. 2008; Shearer et al. 2009). Conventional (non-quantitative) and nested PCR methods have previously been developed and are commonly used for detecting BFDV DNA in host samples for diagnosis and screening studies (e.g. Ypelaar et al. 1999; Kiatipattanasakul-Banlunara et al. 2002). Although these methods are accurate and provide an estimate of prevalence, conventional PCR methods do not allow a quantitative estimate of viral load. Viral load estimates are useful, because higher pathogen burdens are typically associated with lower host fitness (Medzhitov et al. 2012). qPCR is a reliable and
sensitive method for detecting viral DNA and is used extensively in virology to measure viral load (Mackay et al. 2002). Currently, dye-based quantitative PCR assays have been reported for the quantification of BFDV load in blood, feather and cloacal samples from parrots (Raue et al. 2004; Katoh et al. 2008; Shearer et al. 2009).

Molecular studies of BFDV could benefit from feather sampling, particularly when a non-invasive means of sampling endangered or difficult to capture species is required. However, studies using both conventional PCR (Hess et al. 2004; Khaledi et al. 2005) and quantitative PCR (Katoh et al. 2008; Shearer et al. 2009) have reported conflicting outcomes in relation to whether feathers or blood are more reliable samples (Khaledi et al. 2005; But see; Raidal et al. 2008), while muscle tissue from dead birds has not been assessed in previous studies but could provide an alternative means of detecting viraemia in dead birds. This had led to significant uncertainty in the suitability and comparability of different common sample types. Further, the use of relative quantification, which can offer throughput and repeatability benefits, has not been investigated in qPCR assays for BFDV (Shearer et al. 2009).

In this study, we first developed a probe-based qPCR test that would allow reliable, high-throughput detection of BFDV and relative quantification of BFDV load in crimson rosella samples, and analysed the statistical repeatability of the results. Then, we used this test to compare qualitative (viral prevalence) and quantitative (viral load) results from blood, muscle tissue and feathers sampled at the same time from the same individuals to improve knowledge of the appropriate sample types for avian virus detection. In particular, we sought to determine whether feathers and muscle may be an adequate sampling method alone or in combination with blood for future virus prevalence and load studies. Finally, we
compared our assay with an established conventional PCR test which is widely used for BFDV screening and diagnosis (Ypelaar et al. 1999). Overall, this study provides valuable new data on the prevalence of BFDV in free-living crimson rosellas in Australia, a widespread and abundant species which may act as a reservoir for this pathogen (Eastwood et al. 2014). This study also highlights the importance of appropriate avian sample collection for pathogen detection using PCR.
2.3 Materials and methods

2.3.1 Sample collection

The crimson rosella (*Platycercus elegans*) is a widely distributed parrot species in south eastern Australia (Joseph *et al.* 2008). Sixty-one adult birds were caught using a variety of methods, including walk-in traps and mist nets; we also used nest box traps as described in Berg and Ribot (2008). On capture, approximately 100 μl of blood was collected from the basilic vein and stored immediately in ethanol. An additional 23 birds were collected from permit holding individuals who cull crimson rosellas as orchard pests or sacrifice them for other research purposes (Carvalho *et al.* 2010; Knott *et al.* 2010; Knott *et al.* 2013). These carcasses were stored in a plastic bag at -20°C immediately after death. Thirteen of these had blood collected either just before or just after death as described above. Feather and muscle tissue was collected from the frozen carcasses later in the laboratory. A single feather from the throat was plucked and finely chopped to be used immediately in DNA extraction. All 23 culled birds had Pectoralis Major muscle tissue collected from a position either side of the sternums centre which was immediately used for DNA extraction. To avoid contamination when sampling muscle tissue we first removed feathers from the area where tissue was to be collected. To avoid contamination in the lab while collecting feathers and muscle tissue all lab equipment was disposable and the work area cleaned after each bird was processed. Feather contamination may arise in the field prior to capturing the bird or due to the uncontrolled conditions of fieldwork. One of the sacrificed birds was used as a BFDV positive control, which was determined using a conventional PCR (described below) and sequence verified, GenBank accession no. KJ953858. No birds displayed clear signs of the psittacine beak and feather disease (PBFD; feather malformation/loss or beak
deformity). However, no pathological examination was undertaken of any of the birds used in this study.

2.3.2 DNA extraction and quantitation

An ammonium acetate DNA extraction method was used to extract bird and BFDV DNA (Bruford et al. 1998). In brief, blood, muscle tissue or finely chopped feather samples were added to 250 μl of a cell lysis buffer (20mM EDTA, 120mM NaCl, 50mM Tris-HCl, 20% SDS), with 200 units of proteinase K. A no template control was added to each extraction to control for potential contamination during the extraction process. After incubating for 15 hours at 37°C, 4M ammonium acetate was added for protein removal, and after centrifugation (18,000 g, for 15 minutes), ethanol was then added to the supernatant for DNA precipitation. DNA was stored in low Tris-EDTA buffer (10mM Tris.HCl, 0.1 mM EDTA; pH 7.5-8.0). DNA quality and quantity was determined using a DU 640B spectrophotometer (Beckman Coulter, CA, U.S.A) according to the manufacturer’s instructions, with a 1: 200 dilution. To verify spectrophotometer DNA quantitation we used a fluorometer (Qubit Fluorometer, Life Technologies) to re-quantify N = 39 samples from blood, muscle tissue and feathers. PCR quality DNA was further confirmed for each sample by sexing each sample following the PCR method of Griffiths et al. (1998) (results not shown).

2.3.3 Probe-based quantitative real-time PCR

Beacon Designer (Version 7, Premier Biosoft International) and the published sequence AF071878 (Niagro et al. 1998) were used to design primers in the highly conserved replication associated protein open reading frame of the BFDV genome (Bassami et al. 2001; Heath et al. 2004). A forward primer (5’ -GAC GCG GAT AAC GAG AAG TAT TG- 3’) and a
reverse primer (5’-GCA ACA GCT CCA TCG AAA GC-3’), were selected that amplified a 100 nucleotide region between nucleotide positions 90 and 190. An oligonucleotide probe containing the fluorophore FAM and quencher TAMRA was designed to bind to the amplicon produced (5’-FAM CCG TCT CTC GCC ACA ATG CCC AGG TAMRA-3’). Both primer and probe concentrations were optimized using a standard SyBr green method. To perform the PCR, Brilliant Multiplex qPCR master mix solution (Agilent Technologies, U.S.A.) with 900 nM forward primer, 300 nM reverse primer and 100 nM probe was added to 400 ng of sample DNA. For samples where the DNA extraction did not yield a sufficient quantity of DNA for the qPCR reaction we added a standardised volume of stock DNA (7 μl). We then adjusted for the lower concentration after calculating the relative estimation of viral load (see below).

Quantitative PCR was performed in a Stratagene Mx3005P (Agilent Technologies, CA, U.S.A.) with conditions as follows: Initial denaturation of ten minutes at 95°C; followed by 40 cycles of: 30 seconds at 95°C, 60 seconds at 60°C and 30 seconds at 72°C; followed by final extension of 5 minutes at 72°C.

Quantitative PCR plates contained positive and negative controls, and all samples were run in duplicate on each plate. Duplicate samples with Ct (Cycle at which probe fluorescence crosses the arbitrarily set detection baseline) values differing by more than one cycle were repeated in another PCR assay. The comparative Ct method was used to calculate relative gene expression (Schmittgen & Livak 2008). First, the data between plates was normalized using the positive control (Average Ct between sample duplicates – average Ct of positive control duplicates = ΔCt). Then a relative estimation of viral load was calculated for each sample using the equation $2^{(-\Delta Ct)}$. Ct values greater than 39 were considered negative (viral load = 0). Samples from all 84 birds were tested in this way, including the 13 individuals
with blood, muscle tissue and feather samples. Also a subsample of 22 blood samples were re-extracted twice and tested in separate PCR assays for the purpose of calculating repeatability in the detection of BFDV and the estimation of viral load (relative gene expression). Viral load was adjusted (relative gene expression multiplied by the division of expected DNA concentration by actual DNA concentration), if there was an insufficient quantity of DNA for the qPCR reaction. This method was also used to adjust viral load estimates for the DNA concentration values measured using a fluorometer.

2.3.4 Comparison with an established PCR method

To determine whether our method was consistent with an established, conventional PCR method for detecting BFDV, 39 DNA samples from blood sampled live-caught birds and 10 DNA samples from tissue samples from culled birds were tested with a slightly modified conventional PCR method developed by Ypelaar et al. (1999), which is presently a ‘gold standard’ in BFDV detection (Katoh et al. 2008; Sarker et al. 2014b). Briefly, 100-400 ng of DNA template was added to 6 μl of AmpliTaq Gold 360 Master Mix (Applied Biosystems, California U.S.A.). The forward and reverse primer sequences 5’-AAC CCT ACA GAC GGC GAG-3’ and 5’-GTC ACA GTC CTC CTT GTA CC-3’, respectively, that amplified a 717 nucleotide region of the replication associated protein, were added at a concentration of 10 μM. Reactions were made up to 12 μl using nuclease free water. PCR cycling was conducted in a GeneAmp 9700 PCR system (Applied Biosystems, California U.S.A.), with initial denaturation at 96°C for 10 minutes, and 40 cycles of 96°C for 30sec, 60°C for 30sec, 72°C for 90sec, then a final extension at 72°C for 7min. PCR products were separated using a 1.2% agarose gel.
2.3.5 Statistical analyses

Statistics were carried out using SPSS 21 (IBM, Armonk NY). Relative gene expression data was log transformed to improve normality before analyses. Statistical repeatability (r) of relative viral load estimates was calculated using one-way ANOVA following the method of Lessells and Boag (1987). We calculated r for two comparisons: 1) inter-assay variance (‘reproducibility’) which compares the viral loads of positive samples across repeated DNA extractions from the same blood samples and tested in different PCR assays (n = 8), and 2) intra-assay variance (‘repeatability’) which compares the duplicates of each positive DNA sample assayed on each plate (n = 5 blood and n = 10 tissue). Additionally, C_{T} values and viral load estimates were compared pairwise between the three sample types (blood, tissue, and feather) collected from 13 birds using non-parametric Wilcoxon signed ranks tests. To determine the sensitivity of our method we used the Kappa statistic (Viera & Garrett 2005).

2.4 Results

2.4.1 Repeatability and reproducibility

BFDV was detected in samples from 29 out of 84 Platycercus elegans individuals (34.5%). In order to test reproducibility (inter-assay variance) of BFDV detection and relative quantification of viral load in positive samples, blood samples from 22 birds were re-extracted and re-tested in a second PCR assay. Of these, 13 were negative both times, 8 were positive both times, and 1 sample was positive in only one of the two assays (95.5% concordance). Reproducibility of relative quantification of viral loads in these eight consistently positive samples was r = 0.85. As expected, estimates of viral load were highly repeatable amongst sample duplicates in each PCR assay (intra-assay variance; r = 0.99, n =
15 positive samples). This repeatability estimate was unchanged when considering blood and tissue samples separately, or when also including negative samples (i.e. gene expression = 0, n = 49).

2.4.2 Comparison between sample types

We compared the rate of detection of BFDV between blood, muscle tissue and feather samples collected at the same time from 13 birds. We found that the detection of BFDV in blood and tissue samples was largely consistent (Figure 2.1a), with ten individuals providing similar results from both blood and tissue. Feather concordance with blood was 7 of 13, while feather concordance with muscle tissue was 4 of 13. Thus, feather samples were less consistent in the detection of BFDV compared to blood or tissue than blood and tissue were with each other. In particular feathers had a higher chance of returning a positive result for an individual that was negative for BFDV in blood and/or tissue (Figure 2.1a). Pairwise Wilcoxon signed ranks tests revealed no significant differences in CT values between blood, tissue and feather samples (Table 2.1). However, the relative viral load estimate was significantly higher in feathers compared to blood (Table 2.1). There was also a tendency for tissue samples to show higher and more variable viral loads than blood samples (Figure 2.1b). To test for any inaccuracies in spectrophotometer DNA quantification we re-quantified all 39 samples (three sample types from 13 individuals) using a fluorometer, and found that the two DNA quantification methods were highly correlated (Spearman’s rank correlation rs = 0.867, P < 0.001). Conclusions regarding differences between blood, muscle tissue and feather sample types were qualitatively the same after adjusting viral load to reflect the fluorometer DNA concentration rather than spectrophotometer-determined DNA concentration (Table 2.1).
Figure 2.1. (a) Line plot showing differences in viral load between all three sample types for each individual (N = 11 individuals); samples in which no virus was detected are not shown (N = 2 individuals). Individuals with one positive sample type are denoted using a single dot; two sample types positive were denoted using a dotted line; and when all three sample types were positive a solid line was used. (2) indicates the presence of two overlapping points. (b) Boxplots representing differences in viral load between blood, tissue and feather samples collected from *P. elegans* individuals (n = 13).
Table 2.1 Results of Wilcoxon signed ranks tests of pairwise differences in quantitative real-time PCR CT values and viral load estimates between blood, tissue and feather samples collected at the same time from the same individuals (n = 13). The difference in viral load between sample types was also tested using fluorometer DNA concentration adjusted values.

<table>
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<tr>
<th>Measure of infection</th>
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<th>Test statistic</th>
<th>Standard error</th>
<th>p-value</th>
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<tr>
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<td>(Fluorometer adjusted DNA concentrations)</td>
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<td>8.441</td>
<td>0.038</td>
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<td></td>
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<td>-1.245</td>
<td>11.247</td>
<td>0.213</td>
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2.4.3 Comparison between PCR methods

For samples from 49 individual *P. elegans* (39 from blood, 10 from tissue), we compared the results from our qPCR assay with an established, conventional PCR assay used in detection
and diagnosis (Ypelaar et al. 1999). We found 11 samples that were positive for BFDV using both PCR methods (22.4% prevalence), and 34 samples that were negative according to both methods (69.4%). The remaining four samples (8.2%), including three of the blood samples and one of the tissue samples, were identified as positive using our new qPCR assay but negative using the conventional PCR assay. This represented high agreement between the two diagnostic tests (Kappa = 0.79, standard error 0.097), with evidence that our qPCR is more sensitive. Five randomly selected samples that tested positive, using the method described here, were confirmed as true positives by sequence verification [GenBank accession no. KJ953851, KJ953865, KJ953868, KJ953877, KJ953879].

2.5 Discussion

The study and monitoring of avian diseases in general has vital economic, conservation and public health benefits (Daszak et al. 2000). As with most avian diseases, research and detection of BFDV in wild populations can benefit from a high-throughput, cost effective, sensitive and reliable molecular screening and quantification tool. Here, we reported a probe-based qPCR method for detecting and estimating relative viral load of BFDV, a global threat to endangered Psittaciformes (Australian Department of the Environment and Heritage 2005). We tested this method in one host species, the crimson rosella (Platycercus elegans). Further work will be required to confirm whether this method detects all known genotypes, because BFDV is genetically diverse across (Varsani et al. 2011; Harkins et al. 2014) and within host species (Massaro et al. 2012; Sarker et al. 2014b), and is also susceptible to high levels of recombination (Julian et al. 2012; Julian et al. 2013). We demonstrate that our method compared favourably with previous methods with high reproducibility and repeatability (i.e. inter- and intra-assay variance) when using the same
Sample type is an important consideration for pathogen detection in wild populations, as different pathogens often reside in certain areas of the body, but not others. Feathers are increasingly being used as a convenient, readily available, non-invasive source of DNA for avian research (McDonald & Griffith 2011), including for detection studies for a range of host-pathogen systems including avian influenza virus and chicken circovirus (Davidson & Skoda 2005; Yamamoto et al. 2008; Busquets et al. 2010; Dhinakar Raj et al. 2013). However, in many such studies it is likely that comparability between sample types may be dependent on the host species, the pathogen, or the stage of infection. For example, in the case of viruses such as BFDV, feather samples could be contaminated with virus from the environment, the individual from which it was sampled (e.g. body fluids from preening
or skin follicles) or by coming into contact with infected individuals (Ritchie et al. 1991a; Shearer et al. 2009). Alternatively, feathers may represent historic infection status, because feathers grown when the bird was infected may contain viral DNA, even though the individual may have subsequently mounted an immune response and cleared the virus from the blood. Conversely, individuals that have recently become infected may not have viruses in feathers which grew when the individual was not infected. We do not presently know which of these scenarios is most likely to explain the discordant results between feathers and blood/muscle tissue found in this study. The best solution for most studies would seem to be validation of the comparability of different sample types for each research population or species before intensive screening is undertaken, preferably in light of knowledge about the progression of infection with the body.

Hitherto, the most appropriate sample type for BFDV detection has not been determined, although feathers have previously been promoted as suitable (Hess et al. 2004; but see Khalesi et al. 2005; Katoh et al. 2008). Our results suggest that there were no significant differences in BFDV load between all three sample types, although this outcome should be interpreted cautiously as this test was based on a low sample size. Following experimental challenge with BFDV in eastern long-billed corellas (Cacatua tenuiostris), Shearer et al. (2009) found increased probability of detecting the virus in feather compared with blood samples over the following six weeks, and decreased correlation between viral load estimates from feathers and blood. These results were attributed to environmental contamination.

Real-time PCR is well regarded for its accuracy, sensitivity and reproducible results (Mackay et al. 2002), making it a useful tool for the detection of wildlife pathogens, including
BFDV (Shearer et al. 2009). We considered viral load to be zero when $C_T$ values were greater than 39; such high $C_T$ values indicate very low viral load which were rarely reproducible, and are therefore are likely to be below the reliable detection limit of this assay. Further, fluorescence artefacts may appear after a greater number of PCR cycles (Erlich et al. 1991). This approach is therefore recommended for this method, to minimise false positives and achieve relative viral load estimates that are highly repeatable within sample types. Furthermore, comparison with a conventional PCR method (Ypelaar et al. 1999) which is widely used for BFDV detection (positives are determined by visual inspection of a single band within an agarose gel), provided largely concordant results. However, our qPCR method detected four positive ($C_T < 39$) BFDV samples (out of 49) that were considered negative using the conventional PCR, which is consistent with the higher sensitivity reported for qPCR methods (Katoh et al. 2008; Shearer et al. 2009).

BFDV is considered a serious potential threat to wild parrot populations globally, as mortality induced by the virus is often high (Ritchie et al. 1989; Ortiz-Catedral et al. 2009). However, studies to date that investigate BFDV in wild populations are scarce and only consider prevalence, and many gaps remain in our knowledge of the transmission and fitness effects of BFDV in wild populations (Ortiz-Catedral et al. 2009; Kundu et al. 2012). Our study provides information about BFDV in a sample ($n = 84$) of wild crimson rosellas. Our findings suggest that BFDV infection in this host species is widespread and persistent, because we detected BFDV in samples collected across multiple years, locations, and from three subspecies ($P. e. elegans$, $P. e. flaveolus$ and $P. e. adelaidae$). This highly coloured and phenotypically variable species, represents an emerging model system in the study of speciation (Joseph et al. 2008; Ribot et al. 2012; Eastwood et al. 2014), avian communication
(Berg & Bennett 2010; Ribot et al. 2011; Ribot et al. 2013) and vision (Ribot et al. 2009; Carvalho et al. 2010; Knott et al. 2010), and now has great potential to enable us to further elucidate the evolution and ecology of BFDV infection (Eastwood et al. 2014). The finding that BFDV exists in such a common and widespread species is a serious concern for conservation managers that are attempting to recover threatened psittacine species as crimson rosellas may act as reservoirs and transmit infection to threatened species.

In summary, this study describes a probe-based qPCR which demonstrates high-throughput and repeatability/reproducibility in the detection and relative quantitation of BFDV in crimson rosellas. We provide evidence showing that the results are comparable in blood and muscle tissue samples from wild crimson rosellas, but that feathers from the same birds may provide inconsistent results. This suggests that muscle may be an easy and comparable sample type to collect from dead birds. Our study demonstrates the potential for this qPCR to accurately assess the prevalence of a widespread but little understood virus which is a significant threat to many species in one of the world’s most threatened order of birds (Baillie et al. 2004). However, our results urge increased caution in the quantitative comparison of different sample types, and support recent calls for the careful consideration of the validity of feathers as a DNA source for avian studies, in this case in the context of pathogen detection (Khalesi et al. 2005; McDonald & Griffith 2011). Finally, this study provides evidence that BFDV is present and widespread in wild crimson rosellas in Australia.
CHAPTER 3: Phylogenetic analysis of beak and feather disease virus across a host ring-species complex

Photo: a common scene in the Western Slopes hybrid zones, where the *Platycercus elegans elegans* and *P. e. flaveolus* distributions overlap. A Western Slopes hybrid rosella pair feed on grass seeds.

Courtesy of Dr Mathew L. Berg
AUTHORSHIP STATEMENT

1. Details of publication and executive author

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<td>The ecology and evolution of beak and feather disease virus in Platypterygus elegans</td>
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If there are multiple authors, give a full description of HDR thesis author’s contribution to the publication (for example, how much did you contribute to the conception of the project, the design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)

J. Eastwood made a significant contribution to all aspects of the project. Including: conception of the project, the design of methodology, data collection, analysis, interpretation of the data, drafting the manuscript, and manuscript revision.

I declare that the above is an accurate description of my contribution to this paper, and the contributions of other authors are as described below.

Signature and date: [Signature]

4. Description of all author contributions

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<td>Conception of the project, the design of methodology, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.</td>
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<td>Mathew L. Berg</td>
<td>Conception of the project, the design of methodology, data collection, analysis, interpretation of the data, drafting the manuscript, and manuscript revision.</td>
</tr>
<tr>
<td>Raoul F.H. Ribot</td>
<td>The design of methodology, data collection, interpretation of the data, drafting the manuscript, and manuscript revision.</td>
</tr>
<tr>
<td>Shane R. Raidal</td>
<td>Methodology (lab reagents and analytical tools), interpretation of the data, and manuscript revision.</td>
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<tr>
<td>Katherine L. Buchanan</td>
<td>Conception of the project, the design of methodology, data collection, interpretation of the data, drafting the manuscript, and manuscript revision.</td>
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<tr>
<td>Ken R. Walker</td>
<td>Conception of the project, the design of methodology (lab reagents and analytical tools), interpretation of the data, drafting the manuscript, and manuscript revision.</td>
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3.1 Significance statement

The roles of disease and species hybridisation in maintaining biodiversity are of wide interest, yet rarely studied simultaneously in wild populations. Using genomic analysis of beak and feather disease virus in an avian ring-species complex, *Platycercus elegans*, to our knowledge we find viral phylogenetic structure analogous to Mayr’s ring-species hypothesis, for the first time in any pathogen. Across the 8 years, the host’s viral prevalence and infection load was lower in hybrid birds and in phenotypically intermediate subspecies. Viral genetic variation did not explain host prevalence or infection load, supporting conclusions that the evolved host response is more important. We show how host-species complexes and viral genomic analyses can provide insight into maintenance of biodiversity.
3.2 Abstract

Pathogens have been hypothesised to play a major role in host diversity and speciation. Susceptibility of hybrid hosts to pathogens is thought to be a common phenomenon that could promote host population divergence and subsequently speciation. However, few studies have tested for pathogen infection across animal hybrid zones, whilst testing for co-divergence of the pathogens in the hybridizing host complex. Over 8 years, we studied natural infection by a rapidly evolving single-stranded DNA virus, beak and feather diseases virus (BFDV) which infects parrots, exploiting a host ring-species complex (Platycercus elegans) in Australia. We found that host subspecies and their hybrids varied strikingly in both BFDV prevalence and load: both hybrid and phenotypically intermediate subspecies had lower prevalence and load compared with parental subspecies, whilst controlling for host age, sex, longitude and latitude, as well as temporal effects. We sequenced viral isolates throughout the range, which revealed patterns of genomic variation analogous to Mayr’s ring-species hypothesis, to our knowledge for the first time in any host-pathogen system. Viral phylogeny, geographic location, intraspecific host density, and parrot community diversity and composition did not explain the differences in BFDV prevalence or load between subpopulations. Overall, our analyses suggest that functional host responses to infection, or force of infection, differ between subspecies and hybrids. Our findings highlight the role of host hybridisation and clines in altering host-pathogen interactions, dynamics which can have important implications for models of speciation with gene flow, and offer insights into how pathogens may adapt to diverging host populations.
3.3 Introduction

A long-standing puzzle in evolutionary ecology concerns the processes that promote speciation, and particularly the factors that favour or constrain genetic divergence in the absence of physical barriers to gene flow (Mayr 1942, 1963). Co-evolution between pathogens and their hosts is considered a fundamental interaction which influences micro-evolutionary changes in both the host and pathogen, and could potentially mediate gene flow between populations and consequently speciation (Haldane 1949; Schmid-Hempel 2011). Parasites have the potential to influence incipient speciation of their hosts by differentially influencing the fitness of diverging and/or intermediate host lineages, and thus the genetic exchange between host populations (Sage et al. 1986; Fritz 1999; Schmid-Hempel 2011). Conversely, differing selection pressures exerted by host populations may lead to specialization and subsequent speciation of their parasites, especially if transmission between host populations is limited (Schmid-Hempel 2011). Excellent opportunities to study such phenomena are provided by clinal and hybridising populations, which offer natural laboratories in which to investigate population divergence and the early stages of speciation (Mayr 1963; Barton & Hewitt 1989). Parasitism may either promote or penalize hybridisation, depending on a range of host, parasite or environmental factors (Wolinska et al. 2008). Currently, our understanding of how host-parasite co-evolution proceeds in diverging or hybridising populations and its role in speciation is limited, due in large part to the small number of studies that examine variation in both hosts and parasites over sufficient spatial or temporal scales, or in hybridising communities (Wolinska et al. 2008; Moulia & Joly 2009).
To date, studies of host-parasite interactions in hybridising species have been overwhelmingly focussed on plants (Fritz et al. 1999; Wolinska et al. 2008; Moulia & Joly 2009). Moulia and Joly (2009) identified only eight animal hybridisation models where parasitism has been studied under natural conditions. Overall, both plant and animal studies suggest that higher parasite loads in hybrids compared with parental forms is the norm (Wolinska et al. 2008; Moulia & Joly 2009), suggesting that hybrids are typically more susceptible to parasites compared to their parental species, and may therefore restrict gene flow between parental populations. For example, a hybrid population between two subspecies of house mice (Mus domesticus) were found to have higher helminth loads (Sage et al. 1986; Moulia et al. 1991; Moulia & Joly 2009), suggesting that parasites could be selecting against hybridisation. A similar but more complex pattern was found in hybridogenetic water frogs (Rana lessonae and R. esculenta). Joly et al. (2008) reported a higher load of lung flukes in hybrids, but this pattern varied depending on the particular parasite being tested, as this study also demonstrated that parental frogs had higher loads of lung roundworms. A separate study supporting this claim on the same system reported no differences in prevalence in loads of a nematode or two trematode species between hybrid and parental frogs (Planade et al. 2009). Baird et al. (2012) recently found, in the same house mouse system mentioned above, that hybrids between two subspecies have lower helminth loads, the opposite pattern to what was previously found. This finding questions whether parasitic selection against hybrids in this system is consistent enough to prevent gene flow between the parental subspecies. Furthermore, doubt has been raised over whether helminth parasites have a fitness cost on hybrid mice (Gouy de Bellocq et al. 2012). These studies are indicative of a dynamic interaction between hosts and parasites. Most studies have attempted to explain differences in infection levels across diverging host
populations in terms of host genes or environmental variation (Wolinska et al. 2008). However, in general exogenous selection from environmental variation and differences in host architecture arising from hybridisation have not provided satisfactory explanations for the infection scenarios observed (Wolinska et al. 2008). Recent explanations for discrepancies between studies have invoked the possibility of Red Queen dynamics leading to dynamic infection scenarios in hybridising communities over space or time (Wolinska et al. 2008; Planade et al. 2009; Gouy de Bellocq et al. 2012), although the empirical data required to fully test this has been inadequate both in spatial and temporal terms (Wolinska et al. 2008). Notably, few field or laboratory studies of hybrid parasitism have examined genetic variation in the parasite (Wolinska et al. 2008; Gouy de Bellocq et al. 2012); but see Jackson and Tinsley (2003). Experimental infections employing different house mouse strains have demonstrated that host genotype affects host/protozoa interactions, but these experiments only used a single parasite strain. This is potentially a significant shortcoming, because parasites can evolve faster than their hosts (Duffy et al. 2008) and host populations may be subject to specific parasite variants early in the process of divergence, potentially leading to variation in virulence when transmitted to a different population. Thus, parasite divergence may play a crucial role in host divergence and incipient speciation of their hosts.

We studied geographic variation in the prevalence, infection load and genetic variation of a virus (beak and feather disease virus, BFDV) infecting a parrot species complex (crimson rosella, Platycercus elegans). The P. elegans complex is a long-postulated example of a ‘circular overlap’ or ‘ring-species’, of which only about 25 have been proposed worldwide (Cain 1955; Mayr 1963; Irwin et al. 2001b; Alcaide et al. 2014), because it features clinally diverging populations with ongoing gene flow (Joseph et al. 2008; Ribot et al. 2012), in an
approximate horse shoe-shaped distribution, which culminate in a zone of overlap between the most divergent taxa (terminal forms). Such species complexes offer powerful and unique insights into co-evolution of traits, population divergence and speciation (e.g. Irwin 2000; Irwin et al. 2001b; Irwin et al. 2005), but surprisingly, the opportunity presented by such systems has not yet been used to understand host-parasite interactions (Irwin et al. 2001b). BFDV occurs in many wild and captive parrot populations worldwide, with the potential to cause high mortality (Raidal et al. 1993b; Rahaus & Wolff 2003). Accordingly, it is considered a significant conservation threat and has been implicated in parrot declines in Australia and globally (Heath et al. 2004; Kundu et al. 2012; Peters et al. 2014; Sarker et al. 2014a). BFDV possesses a single-stranded DNA (ssDNA) genome of approximately 2000 nucleotides (Bassami et al. 2001). Like most small ssDNA and RNA viruses, BFDV shows high levels of genetic variation and recombination (Heath et al. 2004; Varsani et al. 2011; Julian et al. 2013), and evolves rapidly in novel conditions (Domingo-Calap & Sanjuán 2011), with multiple variant infections present in individual animals (Sarker et al. 2014a). This parrot-virus system is thus an excellent candidate to study how pathogens interact with diverging and hybridising hosts.

We investigated the prevalence and infection load of BFDV over eight years across a 1200-km-wide study area, which included the three main host subspecies (P. e. elegans, P. e. flaveolus, P. e. adelaidae), and a zone of hybridisation (Western Slopes or WS hybrid) where the most phenotypically distinct host subspecies overlap (Figure 3.1). In this way we could determine the role of host factors (subspecies, sex, age) and ecology (host population density, host community diversity and composition, temporal and spatial location) on both viral prevalence and viral load. We also sequenced the virus throughout the host range to
determine how it differs in response to host divergence and hybridisation, and how it may differ phylogenetically from BFDV virus in other host species. We used these data to test whether BFDV phylogeography supports the hypothesis that *P. elegans* is a ring-species.

**Figure 3.1.** Map of *Platycercus elegans* geographic distribution in south eastern Australia. Colours indicate the approximate range of each subspecies based on observational data from Birdlife Australia (Barrett et al. 2003); *P. e. melanoptera* was not used in this study.
3.4 Materials and methods

3.4.1 Study species and sampling

We studied the crimson rosella (*Platycercus elegans*) species complex, which consists of several geographically continuous but phenotypically divergent subspecies encircling unsuitable habitat (Figure 3.1). This species complex contains three distinct subspecies (*Adelaide, P. e. adelaidae*; crimson, *P. e. elegans*; and Yellow *P. e. flaveolus*; the latter two subspecies culminating in three zones of overlap, Western slopes hybrid) (Figure 3.1) (Joseph *et al.* 2008). To sample BFDV, we collected whole blood or muscle tissue samples between the years 2004 and 2011. Birds were caught at the nest or in walk-in traps. For detailed sampling locations (Table 3.S5), primers (Table 3.S6), and techniques, see SI Materials and Methods.

3.4.2 BFDV seroprevalence, PCR detection, and sequencing

DNA was extracted using a standard ammonium acetate method and birds were sexed following (Griffiths *et al.* 1998). Samples from 406 individuals were screened for BFDV using a probe based quantitative real-time polymerase chain reaction (qRT-PCR) developed and tested in this species. Seroprevalence was assayed using Haemagglutination Inhibition (HI) as described in Khalesi *et al.* (2005). See SI Materials and Methods for a detailed description of these techniques.

3.4.3 Phylogenetic inference, recombination and selection

Maximum-likelihood phylogenies of each sequence subset [Capsid ORF, non-coding region, partial replication-associated protein ORF, and all regions concatenated (Partial BFDV genome)] were inferred using MEGA (Tamura *et al.* 2011). The program BaTS (Bayesian Tip-
association Significance testing) was used to test for a host species association among the phylogenetic tips using all known BFDV sequences from endemic Australian host species but also among *P. elegans* subspecies in a separate analysis (Parker et al. 2008). MrBayes was used to produce a posterior set of trees to be used in BaTS (Ronquist et al. 2012). Recombination was tested using RDP 4.16 (Martin et al. 2010). DnaSP version 5 was used to test for selection (Librado & Rozas 2009). We used the Mantel test function in the Genalex 6.5 add-in for Microsoft Excel 2007 (Peakall & Smouse 2012) to test for IBD (See SI Materials and Methods).

3.4.4 Statistical analyses

Using the statistics package SPSS 22 (IBM, Armonk NY) we created a series of Generalized Linear Mixed Models (GLMM) (all subset approach) to analyse two response variables: (i) individual infection status (infected or not infected), and (ii) BFDV infection load. Fixed predictors included: (i) subspecies, (ii) age, (iii) Julian date (iv) Julian date$^2$ and (v) host sex. DNA source (blood/tissue), longitude and latitude were controlled for in all models as fixed effects. The corrected Akaike Information Criterion ($\text{AIC}_c$) was used to select the best fitting models and calculate the weights ($w_i$) for each model and predictor (Symonds & Moussalli 2011). In separate analyses we tested whether geographic location (0.5 × 0.5 decimal degree grid squares), *P. elegans* population density, or the community diversity (species richness) and composition ($\beta$-diversity) of potential hosts (Psittaciformes species), were predictors of prevalence and load (See SI Materials and Methods).
3.5 Results

3.5.1 BFDV prevalence, infection load and seroprevalence

Although we detected BFDV in all subspecies of *P. elegans*, both prevalence ($\chi^2 = 63.1$, d. f. = 3, $P < 0.001$) and load (Kruskal-Wallis: $H = 28.13$, d. f. = 3, $P < 0.001$) varied significantly between the different host subspecies. The phenotypically most distinct subspecies, *P. e. elegans* (crimson rosella) and *P. e. flaveolus* (yellow rosella), had the highest BFDV prevalence and load, whereas the phenotypically and geographically intermediate forms (*P. e. adelaidae* and WS hybrids) had a lower prevalence and load (Figure 3.2). Model selection revealed a single best model ($w_i > 0.9$) predicting prevalence ($k = 17$, $\text{AIC}_c = 1890.205$, $w_i = 0.999$), which comprised the terms subspecies, date, host sex and an interaction between subspecies and date. The null model, containing DNA source (fixed effect), year (random effect), longitude and latitude (fixed effects) received much less support ($\Delta\text{AIC}_c = 107.73$, $w_i = < 0.0001$, evidence ratio = $2.48 \times 10^{23}$) than the most plausible model, and was ranked 12 of 64 models compared.
Figure 3.2. (a) Model predicted prevalence (%) of BFDV infection between the two terminal subspecies *P. e. elegans* (crimson rosella) and *P. e. flaveolus* (yellow rosella) and the significantly less infected, *P. e. adelaidae* and WS hybrid. (b) Model predicted mean BFDV infection load (Log10 gene expression) of infected subspecies and age classes (full: sub-adult, shaded: young adult, dotted: old adult). Bars represent one standard error. Sample sizes of each subspecies (*n*) in both Figures 3.2a and 3.2b are included above the bars.
We identified a single best model that predicts BFDV load \((k = 27, \text{AIC}_c = 579.077, w_i = 0.970)\). This model included the predictors subspecies, host age, host sex and an interaction between subspecies*age. In line with the results for prevalence, \(P. \ e. \ elegans\) and \(P. \ e. \ flaveolus\) had higher BFDV load than \(P. \ e. \ adelaidae\) and WS hybrids (Figure 3.2b). Sub-adults (1-2 years of age) showed a higher BFDV load than adults (young adults 2-5 years and old adults >5 years) in both \(P. \ e. \ elegans\) and \(P. \ e. \ flaveolus\) (Figure 3.2b). Females had lower BFDV load than males. The null model for BFDV load (as above) was less likely than the most plausible models \((\Delta \text{AIC}_c = 109.16, w_i = <0.0001, \text{evidence ratio} = 5.06 \times 10^{-23})\). When considering BFDV load of blood and tissue samples separately, we found no difference in the overall results (most plausible model: subspecies + host age + host sex + subspecies*host age) (see SI results; Table 3.S1).

Difference in BFDV prevalence and load at sampling locations was not correlated with the geographic distance between sampling locations (prevalence: Mantel \(R = -0.125, \ P = 0.301\), load: Mantel \(R = -0.177, \ P = 0.211\)). To further exclude the possibility that BFDV varies according to sampling location, we partitioned our data to include only a 90-km transect encompassing two separate WS hybrid populations (Moyhu/Edi and Bonegilla/Tangambalanga) and two nearby \(P. \ e. \ elegans\) sampling sites (Stanley, 35km from Bonegilla/Tangambalanga, and Myrrhee, 15km from Moyhu/Edi). WS hybrids had a significantly lower prevalence \((\chi^2 = 35.82, \ d. \ f. =3, \ P < 0.001)\) and load \((\text{Kruskal-Wallis: } H = 15.57, \ d. \ f. = 3, \ P = 0.001)\) despite the close proximity of the subpopulations in these areas. BFDV infection was also not correlated with community diversity (Prevalence: Spearman’ \(r = -0.449, \ P = 0.264\), load: Spearman’s \(r = -0.289, \ P = 0.485\) ), or two measures of community composition: Sørensen’s similarity index (Prevalence: Mantel \(R = -0.027, \ P = 0.188\), load:
Mantel $R = -0.027, P = 0.372$) and Beta diversity (Prevalence: Spearman’s $r = 0.449, P = 0.264$, load: Spearman’s $r = 0.289, P = 0.488$). Prevalence at sampling locations was not correlated with *P. elegans* density (Spearman’s $r = -0.419, P = 0.301$) but was negatively correlated with load (Spearman’s $r = -0.714, P = 0.047$). However, this was not significant when using a measure of density that took account of subspecies limits (Spearman’s $r = -0.488, P = 0.153$).

One individual from *P. e. elegans* had antibodies for BFDV, although it was a weak signal (antibody titre 1:20). This individual was a sub-adult and was PCR positive for BFDV. All other individuals were negative for antibodies or had levels below the detection limit of this test (antibody titre <1:20).

### 3.5.2 Phylogenetic inference and recombination

Bayesian phylogenetic inference of all known endemic BFDV sequences shows clear structuring consistent with both host species and host subfamily [association index (AI) and parsimony score (PS) statistics; see Table 3.S2 and for Bayesian phylogenetic tree see Figure 3.S1]. Figure 3.S1 shows that *P. elegans* BFDV isolates branch out from all other Australian endemic species and that they show a common ancestor with BFDV isolates from *Platycercus eximius* that were introduced into New Zealand (Massaro *et al.* 2012). One exception is the presence of a BFDV isolated from *Calyptorhynchus lathami*, an endangered species that was in captivity. Significant association of subspecies was also found when analysing BFDV sequences isolated from *P. elegans* (Table 3.S2). Phylogenetic inference of the 1629 nucleotide (nt) partial BFDV region showed five groups (Figure 3.3), with the largest representing all *P. e. elegans*, some *P. e. adelaidae*, *P. e. flaveolus*, and WS populations and two representing different populations of *P. e. flaveolus* with some isolates from *P. e.*
adelaidea in each. Within the first and larger group some structure exists with hybrids generally clustering together within the distal group. *P. e. adelaidea and P. e. nigrescens* formed minor groups within the phylogeny. The capsid ORF (Figure 3.52a) was similar to the partial BFDV tree, with only minor changes in topology. There is little support for phylogenetic separation within the partial replication-associated protein ORF sequences, although some topological structure exists, depicting four main groups (Figure 3.52b). Non-coding region sequences again show clustering of subspecies, but weaker support for phylogenetic separation (Figure 3.52c).

We found evidence of recombination in 12 sequences (30%) in our dataset. These recombination events occurred in all subspecies except the WS hybrid (Figure 3.3 and Figure 3.52); see Table 3.53 for recombination breakpoints and detection methods. Recombination events did not qualitatively alter phylogenetic inference. This observation was based on comparing trees with and without sequences containing evidence of recombination (Figure 3.3 and Figure 3.53 respectively).
Figure 3.3. Maximum likelihood phylogenetic tree based on a ~1629 nucleotide region of the beak and feather disease virus genome (support values represent a percentage from 1000 bootstrap replicates). Colours indicate *P. elegans* subspecies (see Figure 3.1, blue represents *P. e. nigrescens* located in far North Queensland), and shapes indicate evidence of recombination (square = recombination detected, circle = no recombination detected).
We found evidence for weak but significant isolation-by-distance (IBD) in all coding regions of BFDV (Figure 3.S4). Using the shortest geographic distance between virus sampling locations within the host range, the capsid ORF showed a clear pattern of IBD (Mantel $R = 0.407$, $P = 0.001$). Similarly, the partial genome (Mantel $R = 0.267$, $P = 0.001$) and replication-associated protein ORF (Mantel $R = 0.099$, $P = 0.001$) both showed significant IBD as well, although the effect sizes were smaller. When using the geographic distances corrected to assume no viral gene flow across the WS hybrid zone, these patterns were the same for the capsid ORF (Mantel $R = 0.394$, $P = 0.001$), the partial genome sequences (Mantel $R = 0.247$, $P = 0.001$), and the replication-associated protein ORF (Mantel $R = 0.088$, $P = 0.001$), with similar effect sizes.

Tajima’s neutrality test supported neutral selection in the capsid ORF (Tajima’s $D = -0.87$, $P = 0.2$). Splitting subspecies into separate populations supported the same conclusion (Table 3.S4). The $d_{ns}/d_s$ ratio for the capsid ORF within $P. e. adelaidae$ and $P. e. elegans$ had evidence for positive selection $d_{ns}/d_s > 1$ (Table 3.S4). In contrast, the $d_{ns}/d_s$ ratio for the capsid ORF in all subspecies, $P. e. flaveolus$ and WS hybrid populations separately was < 1, indicating negative selection. Neutrality was rejected for replication-associated protein ORF (Tajima’s $D = -1.5$, $P = 0.05$), implying positive selection. Splitting subspecies into separate populations supported neutrality (Table 3.S4). The $d_{ns}/d_s$ ratios for the replication-associated protein ORF indicated slight purifying selection in all subspecies, except the $P. e. elegans$ population, where $d_{ns}/d_s$ was > 1, indicating positive selection (Table 3.S4).
3.6 Discussion

We tested the role of host factors in viral prevalence and load in a hypothesised ring-species host. Our data showed that subspecies was the most important predictor of prevalence and load, with WS hybrids and the phenotypically intermediate subspecies *P. e. adelaidae* having much lower prevalence and load than the terminal subspecies (*P. e. elegans* and *P. e. flaveolus*). Phylogenetic analyses of the virus, geographic location, intraspecific host density, or host community diversity and composition, did not explain these striking differences. Our results therefore provide support for differences in susceptibility, mortality or force of infection between subpopulations. Host-parasite associations are considered responsible for much of the genetic diversity present in wild populations (Anderson & May 1982), and we propose that BFDV may contribute to the maintenance of diversity in the *P. elegans* species complex, which has been a long standing puzzle (Mayr 1942, 1963).

3.6.1 Why is hybridisation associated with lower infection?

Perhaps the most parsimonious hypothesis explaining low BFDV prevalence and load in WS hybrids is that they are more resistant to BFDV infection. Our data suggests that sub-adult birds have a significantly higher BFDV load than the two adult classes, but this occurs to a lesser degree in WS hybrids and *P. e. adelaidae*. Higher resistance in the latter two populations could explain the reduced load and faster rate to baseline load levels that appear consistent across all subspecies in the old adult age class. Reduced pathogen infection among hybrid populations compared to parental species has rarely been identified in both plant and animal systems, but is particularly rare in animal models (Fritz *et al.* 1999; Wolinska *et al.* 2008; Moulia & Joly 2009). Investigations of animal hybrid systems that have found hybrid resistance patterns (reduced parasite prevalence and load) (Joly *et al.* 2008;
Mouli & Joly 2009; Baird et al. 2012) have typically been contradicted in subsequent studies where hybrids were found to have a higher prevalence than the parental species (Wolinska et al. 2008). This inconsistency has been hypothesised to result from frequency-dependant selection (Wolinska et al. 2008; Baird et al. 2012). However, our data suggest that the lower BFDV prevalence and load in WS hybrids and the subspecies P. e. adelaidae are temporally consistent, at least over the eight years of this study. This hybrid resistance scenario is dependent on the assumption that lower prevalence and load represents higher resistance, however other explanations are possible. The lower prevalence and load in WS hybrid and P. e. adelaidae could alternatively be explained by a lower susceptibility, which does not necessarily depend on factors that influence resistance. The host community diversity or composition, or the density of P. elegans populations, can be hypothesised to contribute to variation in exposure and the force of infection. However, analyses of these factors indicated that they did not account for our findings concerning prevalence and load. Our assessment of BFDV seroprevalence, using the most sensitive assay available, to determine if there was variation in BFDV exposure (i.e. higher seroprevalence would suggest higher exposure) revealed surprisingly that all except one individual were negative for antibodies. However, this result is consistent with equal exposure among all subspecies and hybrid populations. Antibody levels that are absent or below the detection limit may also arise from high tolerance to BFDV in P. elegans. Higher resistance and subsequently lower susceptibility in hybrids that is constant, could indicate a lower selective pressure and could favour hybridisation between P. e. elegans and P. e. flaveolus. This hybrid advantage could potentially explain the maintenance of the WS hybrid population and therefore the maintenance of a genetic bridge, preventing complete speciation between P. e. elegans and P. e. flaveolus.
An alternate hypothesis to explain our results is that WS hybrids and *P. e. adelaïdae* are more susceptible to BFDV. Many studies conclude that a hybrid susceptibility model is appropriate based on the observation that hybrids have a higher parasite prevalence and load (reviewed in Wolinska et al. 2008). However, lower parasite prevalence and load within a population may also indicate susceptibility by representing a higher mortality of infected individuals in that population. Therefore, it is possible that lower BFDV prevalence/load indicates higher mortality in WS hybrids and *P. e. adelaïdae* and that a higher BFDV prevalence/load represents tolerance in *P. e. elegans* and *P. e. flaveolus*. In this scenario, BFDV could potentially be promoting speciation between *P. e. elegans* and *P. e. flaveolus*. One mechanism that could explain hybrid susceptibility is through genetic admixture between *P. e. elegans* and *P. e. flaveolus*, which could lead to a breakdown of co-adapted gene complexes. This situation arises when parental populations diverge sufficiently enough that, during secondary contact, alleles that have evolved with one genome are less functional with another (Moulia & Joly 2009; Baird et al. 2012). Alternatively, genetic admixture between *P. e. elegans* and *P. e. flaveolus* is likely to produce rare host variants that are less susceptible to pathogens, which are adapted to common host genotypes, as a Red Queen model might predict (Wolinska et al. 2008). This situation could also result in more genetically diverse populations with a greater number of heterozygous individuals. It is generally accepted that more genetically diverse individuals and populations have a lower prevalence and load than more inbred populations (reviewed in Schmid-Hempel 2011). This genetic mechanism could explain a hybrid resistant model, as suggested above and in studies that have measured intermediate or higher immunity in avian hybrid systems (Tompkins et al. 2006; Wiley et al. 2009).
3.6.2 Viral phylogenetic structure

Hosts exert selective pressure on their pathogens and in some cases this can lead to pathogen specialization and perhaps even speciation of the pathogen (Schmid-Hempel 2011). There is substantial evidence in our data to suggest that BFDV is genotypically associated with host species and that BFDV sequences isolated within *P. elegans* are specific to this species, although there does appear to be evidence for isolate spill-over. The most notable example is *C. lathami*, a captive endangered cockatoo species that was likely exposed to wild *P. elegans*. Similar BFDV spill-over and host-switch events have been shown recently for the endangered orange-bellied parrot (*Neophema chrysogaster*) (Peters *et al.* 2014; Sarker *et al.* 2014a). Our results showed that the partial BFDV genome had phylogenetic structure consistent with the host from which it was sampled with five groups, two of which represent different *P. e. flaveolus* sampling locations (Figure 3.3). The larger group in Figure 3.3 contains all BFDV sequences from *P. e. elegans* but also BFDV samples from the WS hybrids. This finding was surprising, as we might expect that BFDV in a host hybrid zone would be intermediate between *P. e. elegans* and *P. e. flaveolus*, particularly as the host mitochondrial data suggests that WS hybrids cluster with both parental species (Joseph *et al.* 2008). However, this finding is consistent with the host microsatellite data, which shows phenotypic WS hybrids clustering with *P. e. elegans* (Joseph *et al.* 2008). *P. e. adelaidae* subspecies were found to have BFDV sequences that occurred in all groups in Figure 3.3. This pattern is consistent with an intermediate population separating the terminal host subspecies, but is also consistent with the host’s mitochondrial data (Joseph *et al.* 2008). However, the host’s microsatellite data indicate that there is a genetic discontinuity between *P. e. adelaidae* and *P. e. flaveolus* birds. Therefore, the microsatellite data is
inconsistent with *P. e. adelaidae* BFDV data that suggest gene flow between *P. e. flaveolus* and *P. e. adelaidae* BFDV sequences. The nucleotide region that encodes the capsid protein had a similar phylogeography as the partial genome mentioned above (Figure 3.S2a). However, there is little support for all groups to be considered separate clades. BFDV capsid sequences from *P. e. elegans* seem to be closely related to one BFDV population from *P. e. flaveolus* (Figure 3.S2a), a result likely to be explained by evidence for recombination in those BFDV sequences from *P. e. flaveolus*. This evidence for gene flow between isolates and the relatively conserved nature of the replication-associated protein ORF may explain the lack of phylogenetic separation in this coding region. Overall, BFDV in this system appears to have co-evolved with the terminal subspecies, but only limited specialization has occurred, allowing the intermediate subspecies (*P. e. adelaidae*) to be infected. Across hybrid zones parasite isolates have been found to specialize on parental species, leaving F1 hybrids unaffected (Jackson & Tinsley 2003). However, our results are more consistent with one other study that found that only one parental parasite species was able to infect the hybrid population (Heaney & Timm 1985).

### 3.6.3 Evidence for the ring-species concept within *P. elegans*

As highlighted in recent studies (Irwin et al. 2005; Joseph et al. 2008; Alcaide et al. 2014), ring-species provide powerful insights into speciation, because we may infer from spatial variation in populations how divergence can proceed over time, and because they show how divergence and reproductive isolation can occur despite gene flow. Ring-species feature two distinct and non-recombining ‘terminal’ populations that meet upon secondary contact, yet are connected through a series of intermediate populations with ongoing gene flow encircling unsuitable habitat (Mayr 1963; Alcaide et al. 2014). As the ancestral populations
expand around the unsuitable habitat, divergence around the ring is thought to arise, at least in part, because of IBD (Irwin et al. 2005). However, ring-species are exceptionally rare, and – moreover to our knowledge – have not been studied with respect to their pathogens. The ring-species model has formerly only been applied to animals and plants, and its study has presented numerous challenges (Alcaide et al. 2014). P. elegans has long been held to be a possible example of a ring-species, but conclusive evidence has remained elusive (Cain 1955; Mayr 1963; Joseph et al. 2008). Genomic analyses of highly diverse and rapidly evolving pathogens, such as viruses, can offer unique inferences about host population history (Sugimoto et al. 1997; Falush et al. 2003; Biek et al. 2006). Our findings show how this can offer a novel perspective on the long-standing evolutionary puzzle of ring-species.

The ring-species model invokes three key predictions (Mayr 1963; Joseph et al. 2008; Alcaide et al. 2014). First, a genetic discontinuity is predicted where the terminal forms meet. In line with this prediction, we did not find any evidence for recombination within the BFDV sequences from the WS host populations at the east of the ring, although the sample size was small. This lack of recombination and the fact that BFDV sequences from WS hybrid hosts are amongst the most genetically dissimilar to P. e. flaveolus BFDV sequences (Figure 3.3, Figure 3.S1, and Figure 3.S4) suggests that there is BFDV genetic discontinuity across the host hybrid zone. Second, the model predicts that the distinct terminal forms are connected by a series of intermediate populations, with gene flow encircling unsuitable habitat. Our BFDV phylogenies, in conjunction with the tip association test, revealed that BFDV isolates from P. e. flaveolus and P. e. elegans form separate clades. In geographically and phenotypically intermediate P. e. adelaidae hosts in the western end of the ring, virus variants representing all main clades were observed (Figure 3.3). Third, a pattern of IBD
around the ring, from one terminal form to the other, is predicted. We found significant IBD within the BFDV genome. However, IBD did not account for the majority of genetic divergence in BFDV, suggesting other processes, such as selection, may also contribute substantially to BFDV genetic variation. Although tests of selection suggested that much of the genetic variation in BFDV is neutral, some evidence for positive selection was found in the replication-associated ORF; this region also showed the weakest IBD (Mantel $R = 0.099$). Weak patterns of IBD may be caused by recent range expansion (Slatkin 1993; Irwin et al. 2005), which could arise if ancestral BFDV originated in species other than $P. elegans$ (as supported by our phylogeny; Figure 3.51) and crossed to $P. elegans$ relatively recently.

Overall, our data suggests that gradual evolutionary changes around a large geographic barrier may have accumulated and resulted in genetic discontinuity of BFDV at the terminal ends of the distribution. As such, these patterns in BFDV are analogous to the key predictions outlined under the ring-species concept, and thus support the notion that divergence in the face of gene flow, to the extent that recombination may be reduced or eliminated, is possible in such viruses. We suggest that our data represent a worthy first step in identifying whether quasi-species-like variation in BFDV in $P. elegans$ offers, to our knowledge, the first known example of a pathogen analogue of a ring-species. Further work will be required to test predictions arising from this conclusion, and could offer important advances in our understanding of the role of host-pathogen interactions in speciation.

3.6.4 Conclusions

We show that a phenotypically intermediate subspecies ($P. e. adelaidae$) and hybrid populations (WS) have much lower BFDV prevalence and load than their parental subspecies ($P. e. elegans$, and $P. e. flaveolus$). We found no evidence the results were explained by
potential geographic confounds, *P. elegans* population density, or host community diversity and composition. Our phylogenetic analyses of BFDV sequence variation provides empirical support for the ring-species concept, and thereby demonstrates a novel approach to testing ring-species predictions in hosts and pathogens. Hybrid zones and ring-species complexes are natural laboratories that have greatly enhanced our understanding of speciation processes. We hypothesise that BFDV infection in *P. elegans* gives rise to differential selective pressures with implications for the maintenance of host diversity, and potentially host speciation. Further study of such systems should provide powerful new insights into how hosts and parasites diverge and co-evolve.
3.7 Supporting information

3.7.1 SI materials and methods

Study species and sampling

The host that we studied is the crimson rosella (*Platycercus elegans*) species complex, a highly phenotypically variable parrot which inhabits forests and woodlands in eastern Australia (Figure 3.1). In south eastern mainland Australia, *P. elegans* forms a species complex, with a series of clinal and parapatric populations (collectively the Adelaide rosella, *P. e. adelaidae*), which culminate in two highly divergent subspecies (crimson, *P. e. elegans*, and Yellow rosellas, *P. e. flaveolus*), which hybridise only in three narrow contact zones, hereafter collectively called the Western Slopes (WS) hybrid (Forshaw & Cooper 2002; Joseph *et al.* 2008). Together, these populations occupy a near-continuous ring-shaped distribution. Consequently, the species has long been regarded as a possible rare example of a ring-species (Cain 1955; Irwin *et al.* 2001b), although population genetic analysis suggests a ring-species model oversimplifies the distribution pattern (Joseph *et al.* 2008). This host is subject to infection by Beak and Feather Disease Virus (BFDV), a single-stranded DNA circovirus that infects parrots. The pathogenic changes caused by this virus are potentially lethal to Psittaciformes species, the virus is highly contagious and transmitted directly by close contact between infected individuals (Ritchie *et al.* 1991b; Rahaus & Wolff 2003). It is considered endemic in Australia, but has spread throughout the world in captive populations of Psittaciformes (Raidal *et al.* 1993a; Varsani *et al.* 2011; Ogawa *et al.* 2013).

From 2004 – 2011, whole blood or muscle tissue samples were collected from birds at 23 locations (Table 3.S5), encompassing the distribution of *P. elegans* in south eastern
mainland Australia (Figure 3.1; *P. e. elegans*: 12 locations, *P. e. adelaidae*: 12 locations, *P. e. flaveolus*: 3 locations, WS hybrids: 7 locations). Birds were caught at the nest or in walk-in traps, and bled from the brachial vein (approx. 100 μl), or muscle tissue was sampled from dead birds. Birds were aged based on plumage characteristics, including the presence of green sub-adult (1-2 years) plumage and the underwing stripe of young adults (2-3 years). Individuals are classed as old adults (> 4 years) if there is no underwing stripe (Forshaw & Cooper 2002).

**BFDV seroprevalence, PCR detection and sequencing**

DNA was extracted using an ammonium acetate method adapted from (Bruford et al. 1998). A Beckman DU 640B spectrophotometer (Beckman Coulter, CA, U.S.A) was used to quantify DNA for downstream applications. Birds were sexed using DNA following the method of Griffiths et al. (1998). Samples from 403 individuals were screened for BFDV using a linear oligonucleotide probe based quantitative real-time polymerase chain reaction (qRT-PCR) developed and tested in this species (Eastwood et al. 2015). In short, primers were designed using the reported sequence AF071878 (Niagro et al. 1998), and the program Beacon Designer (Version 7, Premier Biosoft, International). The final concentrations in the reaction included 900nM forward primer (5’-GACGCGGATAACGAGAAGTATTG- 3’) and 300nM reverse primer (5’-GCAACAGCTCCATCGAAAGC- 3’). To measure amplification an oligonucleotide probe with fluorophore and quencher was designed (5’-FAM CCGTCTCTCGCCACAAAAT GCCCAGG TAMRA- 3’), and a concentration of 100nM was added to each reaction. Brilliant Multiplex quantitative PCR (qPCR) master mix solution (Agilent Technologies, U.S.A.) was used with 400ng of sample DNA. Sample dilutions were made using low TE (TE$_{10}$ E$_{0.1}$) (pH 7.5-8.0) buffer and were run in duplicate. qPCR was performed
using a Stratagene MX3005P thermocycler (Agilent Technologies, CA, U.S.A) with conditions as follows: Initial denaturation of ten minutes at 95°C; followed by 40 cycles of: 30 seconds at 95°C, 60 seconds at 60°C and 30 seconds at 72°C; followed by final extension of 5 minutes at 72°C. Each sample was run in random order, in duplicate, and with positive and negative controls on each plate. Samples were repeated if the duplicates showed a difference in Ct > 1.0 to limit false positives. To calculate BFDV gene expression (hereafter ‘BFDV infection load’), which we define as a relative measure of viral load across host samples, we used the comparative Ct method, $2^{(-\Delta Ct)}$, as previously described (Schmittgen & Livak 2008). ΔCt equals the difference between the positive control duplicate average and the unknown sample duplicate average.

To estimate exposure to BFDV and to compare differences in immune response we used a Haemagglutination Inhibition (HI) assay to test for BFDV specific antibodies (Khalesi et al. 2005). Dried blood on filter paper was used for serum extraction. A total of N = 49 samples that were PCR negative for BFDV were assayed corresponding to N = 18 P. e. elegans, N = 2 P. e. flaveolus, N = 13 P. e. adelaidae and N = 16 WS hybrids. A total of N = 11 samples were assayed from PCR positive birds, corresponding to N = 6 P. e. elegans, N = 2 P. e. flaveolus, N = 1 P. e. adelaidae and N = 2 WS hybrids.

A randomly selected subsample of 40 DNA samples that were positive for BFDV were sent to the Australian Genome Research Facility (AGRF; Brisbane, Australia) for sequencing using five primer sets based on a previously reported sequence AF071878 (Niagro et al. 1998) (Table 3.56). This subsample covered the three host subspecies described above, the WS hybrid populations, and one sample from an allopatric host subspecies in far north
Queensland (P. e. nigrescens). The resulting sequences were submitted to GenBank nucleotide sequence database (Table 3.S5).

*Phylogenetic inference, recombination and selection*

Sequences were constructed using the program Chromas Pro (Technelysium, Brisbane, Australia) and aligned using the Muscle function in MEGA 5.05 (Tamura *et al.* 2011). Published whole genome sequences from NCBI were used to root the phylogenies. These were sequences from wild caught birds and included two sulphur-crested cockatoos (*Cacatua galerita*, AF311301, AF311302), a galah (*Eolophus roseicapillus*, AF311298), (Bassami *et al.* 2001) and an unknown species AF071878 (Niagro *et al.* 1998). Model selection and phylogenetic inference were completed using MEGA and tested four different sequence subsets: (1) partial replication-associated protein ORF (substitution model K2+G), (2) capsid ORF (substitution model K2+G), (3) a non-coding region separating the replication-associated protein ORF and capsid ORF (substitution model T92+G), and (4) a phylogeny with all regions concatenated, hereafter termed ‘partial BFDV genome’ (substitution model GTR+G+I). Maximum likelihood phylogenies of each sequence subset were inferred using MEGA. Settings were based on the model selection results (above), with 1000 bootstrap replicates (partial deletion of gaps). To investigate host specificity we phylogenetically analysed all known BFDV sequences from endemic Australian species (see GenBank accession numbers: Figure 3.S1). Sequences were cropped and aligned to our ‘partial BFDV sequences’ from *P. elegans* subspecies and analysed using MrBayes version 3.2 (Ronquist *et al.* 2012). MrBayes default priors were used with two million MCMC generations, sampling every 500 generations. The substitution model was GTR+G+I as determined using the model selection function in MEGA. Figtree version 1.3 (Rambaut 2007) was used to produce a
consensus tree (discarded 25% burn-in) using the MrBayes output. To test whether viral isolate host species is a phylogenetically associated trait we applied the association index (AI) and the Fitch parsimony score (PS) as implemented in the program BaTS (Bayesian Tip-association Significance testing) (Parker et al. 2008). BaTS uses the posterior sets of trees output from MrBayes (excluding burn-in) to account for phylogenetic uncertainty when testing for a correlation between phenotypic traits on the phylogenetic tips. To test for an association between species we ran the analysis using 21 states corresponding to each species or subspecies with 500 replications. This analysis was also run testing for sub-family correlation using eight states and 500 replications. BFDV sequences obtained from *P. elegans* were analysed separately under a Bayesian MCMC framework using the same substitution model and priors as above. BaTS was again used to test for subspecies trait association with four character states and 500 replications.

Recombination was tested using RDP 4.16 (Martin et al. 2010) using the default settings. The methods used within the RDP program to test for recombination were Chimaera, RDP, Bootscan, 3Seq, GENECONV, MaxChi, and SiScan. Recombination was accepted if more than two methods were significant (*P* < 0.05) and phylogenetic evidence was supportive.

To indicate potential selective pressures on the two main coding regions of BFDV (capsid and replication-associated protein ORF), the ratio between non-synonymous (*d*$_{n}$) and synonymous (*d*$_{s}$) mutations was calculated using DnaSP version 5 (Librado & Rozas 2009). Tajima’s D statistic (Tajima 1989) and its confidence intervals were calculated by coalescent simulation (1000 replications) as implemented by DnaSP. We first considered all subspecies
as a single population then partitioned our data to treat subspecies as individual populations.

**Statistical analyses**

The data were analysed using the statistics package SPSS 22 (IBM, Armonk NY). We used Generalized Linear Mixed Models (GLMM) with restricted maximum likelihood estimation. We created models to analyse two response variables: (i) individual infection status (infected or not infected), which was modelled using a binomial distribution and logit link function to test for effects on infection prevalence, and (ii) BFDV infection load, which was modelled using linear regression following log_{10} transformation to fulfil the assumption of normality.

To determine the best predictors of the prevalence and infection load of BFDV infection in *P. elegans*, we used information theoretic based model selection to compare a candidate set of models, which contained the following fixed predictors: (i) subspecies, (ii) age, (iii) Julian date (the beginning of the breeding season, September 1st, was set as day 1; hereafter ‘date’), (iv) Julian date^2 (to account for a non-linear effect of date), and (v) host sex. The set of 63 competing models were derived using an all subset approach, including all combinations of main effects, as well as all two-way interactions involving the term subspecies (Symonds & Moussalli 2011). In all models, DNA source (tissue or blood sample) was controlled as a fixed predictor, longitude and latitude as fixed continuous effects and sampling year was modelled as a random intercept. All candidate models were compared to the null model containing only sample source, longitude and latitude plus year. The corrected Akaike Information Criterion (AIC_c) was used to select the best fitting models and calculate the weights (\( w_i \)) for each model and predictor (Burnham & Anderson 2002; Grueber *et al.* 2011; Symonds & Moussalli 2011). Models were considered plausible if \( w_i \) was
> 0.1. A single best model was accepted if \( w_i \) was greater than 0.9 (Symonds & Moussalli 2011). To fully eliminate the possibility that DNA source could be a confounding variable we split our dataset accordingly (blood and muscle tissue) are re-ran the candidate set of models and used the same model selection methods above.

To further exclude the possibility that BFDV varies according to sampling location we partitioned our data to include only a 90 km transect encompassing two separate WS hybrid populations (Moyhu/Edi and Bonegilla/Tangambalanga) and two nearby \textit{P. e. elegans} sampling sites (Stanley, 35km from Bonegilla/Tangambalanga, and Myrrhee, 15km from Moyhu/Edi). In addition, we address whether geographic location alone predicted prevalence and viral load, we divided the \textit{P. elegans} distribution by longitude and latitude (0.5 × 0.5 decimal degree grid squares; hereby ‘location’) and calculated BFDV prevalence and load (if \( N \geq 10 \) individuals) within each location regardless of the subspecies present (\( N = 8 \) grid squares in total). A Mantel test was then used to test for a correlation between geographic distance between the centre of the two locations squares and also the difference in prevalence and load between them. Similarly we used the same approach to calculate \textit{P. elegans} population density (number of rosellas per square metre) using a typical distance based method (Buckland et al. 2008), BFDV host diversity (total number of Psittaciforme species) and two measures of host species composition. (i) Beta diversity was measured using Whittaker’s original concept that divides the total number of Psittaciforme species (i.e. Gamma diversity) by the number of species at a particular location (i.e. Alpha diversity)(Jurasinski et al. 2009); (ii) To account for differences in species composition we calculated Sørensen’s similarity index (Wolda 1981). Density, species diversity and composition data were collected by members of Birdlife Australia using standardised field
observation techniques between the years 2004-2011 (Barrett et al. 2003). Non-parametric correlations were then tested between location prevalence/viral load and either P. elegans density, host species diversity or host species composition (Beta diversity). We also tested for a correlation between P. elegans density and prevalence using the same approach but respecting the subspecies/hybrid boundary limits. A Mantel test implemented in the Genalex 6.5 add-in for Microsoft Excel 2007 (Peakall & Smouse 2012) was used to test for a correlation between Sørensen’s similarity index and the difference in prevalence and viral load between locations.

For testing isolation-by-distance (IBD) we used a Mantel test to compare pairwise genetic distances between virus sequences with geographic distances between their sampling locations. Pairwise genetic distance was calculated in MEGA using Kimura-2 model parameters, following a Gamma shaped distribution (Kimura 1980). We used two different calculations of geographic distance. First, we calculated distance as the shortest distance between locations within the boundaries of the host range. Second, we calculated ‘corrected’ distance in the same way but assuming a barrier to gene flow between P. e. elegans and P. e. flaveolus samples across the WS hybrid area, as would be predicted by the ring-species hypothesis (Irwin et al. 2005).
3.7.2 SI results

BFDV infection load models in blood and tissue samples separately

Although we controlled for DNA source (blood or tissue) in all models, we repeated the analyses of infection load with blood and tissue samples separated to account for the possibility that infection load results may differ between these samples due to differences in viral replication. However, viral DNA would still be expected to be present in both of these sample types (Katoh et al. 2008). The most plausible blood only and tissue only models showed the same combination of fixed factors as each other (Subspecies + age + host sex), except for an interaction in each model. Blood and tissue only models disagreed only in regards to an interaction, subspecies*host sex (blood only) or subspecies*age (tissue only) (Table 3.S1).
3.7.3 SI tables

**Table 3.S1.** Results of model selection (based on corrected Akaike information Criterion; AICc) for general linear mixed models used to predict the infection load of beak and feather disease virus infection blood and muscle samples collected from the *P. elegans* species complex. Julian date was set at the beginning of the breeding season, September 1st, and is referred as ‘date’. The results show the change in AICc, the model weights and evidence ratios for models within a 95% confidence interval. Year was considered a random variable in all models with blood samples only but was included as a fixed effect when considering tissue samples only.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Model rank</th>
<th>Fixed parameters</th>
<th>k</th>
<th>AICc</th>
<th>wi</th>
<th>Evidence ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>1</td>
<td>Subspecies + host age + host sex + subspecies*host age</td>
<td>24</td>
<td>256.43</td>
<td>0.905</td>
<td>1</td>
</tr>
<tr>
<td>Tissue</td>
<td>1</td>
<td>Subspecies + host age + host sex + subspecies*host age</td>
<td>28</td>
<td>290.76</td>
<td>0.856</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Subspecies + year + host age + date + host sex + subspecies*age</td>
<td>29</td>
<td>295.39</td>
<td>0.084</td>
<td>10.11</td>
</tr>
</tbody>
</table>
Table 3.52. Association index (AI) and parsimony score (PS) statistics testing for an association between Bayesian phylogenetic tips and either host species or host subfamily (Figure 3.51). AI and PS statistics are also presented that test for a subspecies association within *P. elegans*.

<table>
<thead>
<tr>
<th>Tip association</th>
<th>Statistic</th>
<th>Observed mean (95% confidence interval)</th>
<th>Expected mean (95% confidence interval)</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host species</td>
<td>AI</td>
<td>3.96 (3.47-4.38)</td>
<td>12.34 (11.56-12.99)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>39.96 (39-40)</td>
<td>88.34 (84.45-91.78)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Host subfamily</td>
<td>AI</td>
<td>1.85 (1.64-2.01)</td>
<td>8.75 (7.72-9.87)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>16.0 (16.0-16.0)</td>
<td>53.21 (49.07-57.31)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><em>P. elegans</em> subspecies</td>
<td>AI</td>
<td>1.14 (0.72-1.49)</td>
<td>2.72 (2.11-3.31)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>10.55 (10.0-11.0)</td>
<td>20.17 (17.84-22.49)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
**Table 3.53.** Recombination events identified using the recombination detection program RDP4.16 (Martin *et al.* 2010). * indicates possible false positive recombination event.

<table>
<thead>
<tr>
<th>Recombination event</th>
<th>Subspecies</th>
<th>Recombinant (accession no.)</th>
<th>Begin-end in recombinant sequence</th>
<th>Significance detection methods (P&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>P. e. flaveolus</em> KJ953863</td>
<td>543-978</td>
<td></td>
<td>Bootscan, Maxchi, Chimaera, SiScan, 3Seq</td>
</tr>
<tr>
<td>2</td>
<td><em>P. e. elegans</em> KJ953883</td>
<td>993-1426</td>
<td></td>
<td>RDP, Bootscan, Maxchi, Chimaera, SiScan, 3Seq</td>
</tr>
<tr>
<td></td>
<td><em>P. e. elegans</em> KJ953881</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>P. e. elegans</em> KJ953860</td>
<td>1586-770</td>
<td></td>
<td>Maxchi, Chimaera, SiScan, 3Seq</td>
</tr>
<tr>
<td></td>
<td><em>P. e. elegans</em> KJ953853</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>P. e. adelaidae</em> KJ953857</td>
<td>428-728</td>
<td></td>
<td>Maxchi, Chimaera, SiScan</td>
</tr>
<tr>
<td></td>
<td><em>P. e. adelaidae</em> KJ953869</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5*</td>
<td><em>P. e. flaveolus</em> KJ953877</td>
<td>507-859</td>
<td></td>
<td>RDP, Bootscan, Maxchi, SiScan, 3Seq</td>
</tr>
<tr>
<td></td>
<td><em>P. e. flaveolus</em> KJ953878</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. e. flaveolus</em> KJ953876</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. e. flaveolus</em> KJ953874</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. e. flaveolus</em> KJ953866</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3S4. Tests of selection pressure in the capsid (CAP ORF) and replication-associated protein (REP ORF) of the beak and feather disease virus in *P. elegans*. Tajima’s Test of Neutrality with confidence intervals as implemented in DnaSP (Librado & Rozas 2009), non-synonymous \(d_n\) and synonymous \(d_s\) ratios were also calculated using this program. In these analyses, host subspecies are considered separate populations. An analysis where all subspecies are considered one population is also shown.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Subspecies</th>
<th>n</th>
<th>No. of sites</th>
<th>Nucleotide diversity</th>
<th>Polymorphic sites</th>
<th>(d_n/d_s)</th>
<th>Tajima’s D</th>
<th>95% confidence interval</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. elegans</em></td>
<td>33</td>
<td>605</td>
<td>0.029</td>
<td>87</td>
<td>0.05</td>
<td>-0.87</td>
<td>(-1.65, 1.79)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td><em>P. e. elegans</em></td>
<td>12</td>
<td>649</td>
<td>0.011</td>
<td>29</td>
<td>1.64</td>
<td>-1.24</td>
<td>(-1.79, 1.65)</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td><em>P. e. flaveolus</em></td>
<td>10</td>
<td>672</td>
<td>0.036</td>
<td>55</td>
<td>0.59</td>
<td>1.06</td>
<td>(-1.69, 1.59)</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td><em>P. e. adelaidae</em></td>
<td>6</td>
<td>649</td>
<td>0.041</td>
<td>57</td>
<td>2.18</td>
<td>0.34</td>
<td>(-1.42, 1.61)</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>WS hybrid</td>
<td>4</td>
<td>693</td>
<td>0.008</td>
<td>11</td>
<td>0.86</td>
<td>-0.56</td>
<td>(-0.85, 2.01)</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td><em>P. elegans</em></td>
<td>22</td>
<td>647</td>
<td>0.016</td>
<td>55</td>
<td>0.67</td>
<td>-1.50</td>
<td>(-1.74, 1.80)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td><em>P. e. elegans</em></td>
<td>9</td>
<td>670</td>
<td>0.011</td>
<td>22</td>
<td>2.28</td>
<td>-0.84</td>
<td>(-1.80, 1.78)</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td><em>P. e. flaveolus</em></td>
<td>6</td>
<td>697</td>
<td>0.015</td>
<td>19</td>
<td>0.99</td>
<td>0.50</td>
<td>(-1.44, 1.63)</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td><em>P. e. adelaidae</em></td>
<td>6</td>
<td>438</td>
<td>0.023</td>
<td>20</td>
<td>1.18</td>
<td>0.68</td>
<td>(-1.44, 1.52)</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>WS hybrid</td>
<td>4</td>
<td>455</td>
<td>0.011</td>
<td>10</td>
<td>0.86</td>
<td>-0.83</td>
<td>(-0.85, 2.11)</td>
<td>0.11</td>
</tr>
</tbody>
</table>
Table 3.S5. The locations and subspecies of all sampling sites, and the GenBank accession numbers for the viral nucleotide sequences obtained in this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Subspecies</th>
<th>Coordinates</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bellbrae, VIC</td>
<td>P. e. elegans</td>
<td>S38°19' E144°11'</td>
<td>KJ953858.</td>
</tr>
<tr>
<td>Deans Marsh, VIC</td>
<td>P. e. elegans</td>
<td>S38°26' E143°51'</td>
<td>KJ953865, KJ953881, KJ953885.</td>
</tr>
<tr>
<td>Port Campbell, VIC</td>
<td>P. e. elegans</td>
<td>S38°38' E143°03'</td>
<td></td>
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<tr>
<td>Lower Glenelg, VIC</td>
<td>P. e. elegans</td>
<td>S38°3' E141°16'</td>
<td>KJ953856.</td>
</tr>
<tr>
<td>Pomonal, VIC</td>
<td>P. e. elegans</td>
<td>S37°11' E142°36'</td>
<td>KJ953854, KJ953855.</td>
</tr>
<tr>
<td>Ballan-Daylesford Rd, VIC</td>
<td>P. e. elegans</td>
<td>S37°28' E144°08'</td>
<td></td>
</tr>
<tr>
<td>Steiglitz, VIC</td>
<td>P. e. elegans</td>
<td>S37°52' E144°11'</td>
<td>KJ953882, KJ953884.</td>
</tr>
<tr>
<td>Stanley, VIC</td>
<td>P. e. elegans</td>
<td>S36°23' E146°44'</td>
<td>KJ953863, KJ953868, KJ953872, KJ953873</td>
</tr>
<tr>
<td>Myrriere, VIC</td>
<td>P. e. elegans</td>
<td>S36°43' E146°20'</td>
<td>KJ953851, KJ953860.</td>
</tr>
<tr>
<td>Neerim Junction, VIC</td>
<td>P. e. elegans</td>
<td>S37°56' E145°57'</td>
<td></td>
</tr>
<tr>
<td>Bunbartha, VIC</td>
<td>P. e. flaveolus</td>
<td>S36°16' E145°21'</td>
<td></td>
</tr>
<tr>
<td>Cadell, SA</td>
<td>P. e. flaveolus</td>
<td>S34°4' E139°46'</td>
<td>KJ953847, KJ953848.</td>
</tr>
<tr>
<td>Berri, SA</td>
<td>P. e. flaveolus</td>
<td>S34°20' E140°35'</td>
<td></td>
</tr>
<tr>
<td>Bonegilla, VIC</td>
<td>WS hybrid</td>
<td>S36°9' E146°59'</td>
<td></td>
</tr>
<tr>
<td>Brungle, NSW</td>
<td>WS hybrid</td>
<td>S35°11' E148°12'</td>
<td></td>
</tr>
<tr>
<td>Tangambalanga, VIC</td>
<td>WS hybrid</td>
<td>S36°11 E147°0'</td>
<td>KJ953850, KJ953870.</td>
</tr>
<tr>
<td>Edi, VIC</td>
<td>WS hybrid</td>
<td>S36°39' E146°25'</td>
<td></td>
</tr>
<tr>
<td>Barnawartha North, VIC</td>
<td>WS hybrid</td>
<td>S36°3' E146°45'</td>
<td></td>
</tr>
<tr>
<td>Moyhu, VIC</td>
<td>WS hybrid</td>
<td>S36°34' E146°23'</td>
<td>KJ953849.</td>
</tr>
<tr>
<td>Mundarla, NSW</td>
<td>WS hybrid</td>
<td>S35°5' E147°50'</td>
<td></td>
</tr>
<tr>
<td>Carey Gully, SA</td>
<td>P. e. adelaidae</td>
<td>S34°58' E138°45'</td>
<td>KJ953857, KJ953869.</td>
</tr>
<tr>
<td>Craifers, SA</td>
<td>P. e. adelaidae</td>
<td>S35°0' E138°42'</td>
<td></td>
</tr>
<tr>
<td>Marne River, SA</td>
<td>P. e. adelaidae</td>
<td>S34°39' E139°23'</td>
<td></td>
</tr>
<tr>
<td>Quorn, SA</td>
<td>P. e. adelaidae</td>
<td>S32°17' E138°0'</td>
<td></td>
</tr>
<tr>
<td>Sevenhill, SA</td>
<td>P. e. adelaidae</td>
<td>S33°54' E138°36'</td>
<td></td>
</tr>
<tr>
<td>Stirling, SA</td>
<td>P. e. adelaidae</td>
<td>S35°00' E138°43'</td>
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</tr>
<tr>
<td>Oakbank, SA</td>
<td>P. e. adelaidae</td>
<td>S34°57' E138°49'</td>
<td>KJ953867, KJ953871, KJ953875.</td>
</tr>
<tr>
<td>Murray Town, SA</td>
<td>P. e. adelaidae</td>
<td>S32°56' E138°14'</td>
<td></td>
</tr>
<tr>
<td>Mylor-Echunga Rd, SA</td>
<td>P. e. adelaidae</td>
<td>S35°5' E138°47'</td>
<td></td>
</tr>
<tr>
<td>Melrose-Wilmington, SA</td>
<td>P. e. adelaidae</td>
<td>S32°45' E138°10'</td>
<td></td>
</tr>
<tr>
<td>Currency Creek, SA</td>
<td>P. e. adelaidae</td>
<td>S35°25' E138°45'</td>
<td>KJ953862.</td>
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<tr>
<td>Queensland</td>
<td>P. e. nigrescens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yulti Wirra, SA</td>
<td>P. e. adelaidae</td>
<td>S35°37' E138°11'</td>
<td>KJ953859, KJ953861, KJ953864.</td>
</tr>
</tbody>
</table>
**Table 3.56.** Primers used to amplify beak and feather disease virus for partial nucleotide sequencing of the viral genome. The amplicon sizes and the nucleotide regions of beak and feather disease virus amplified are noted for each primer pair.

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
<th>Range</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACTCCCCATTGCAAGG</td>
<td>TATCCATCCACCATTCA</td>
<td>380 - 680</td>
<td>438</td>
</tr>
<tr>
<td>GCATAGCCTTCGTATTGG</td>
<td>ACGCCTAACTTTATTAACACCC</td>
<td>680 - 980</td>
<td>477</td>
</tr>
<tr>
<td>CTGTTCCGGAGATTCCTTG</td>
<td>ACTACAAGGAGGACCC</td>
<td>980 - 1280</td>
<td>479</td>
</tr>
<tr>
<td>TGTTAGGGGCAACTGACG</td>
<td>CCCCAAAACACTGAATTTCG</td>
<td>1280 - 1580</td>
<td>461</td>
</tr>
<tr>
<td>GGGTCTTGTGTGGTTCTCG</td>
<td>ATGGGGCACCTCTA</td>
<td>1580 - 1880</td>
<td>438</td>
</tr>
</tbody>
</table>
3.7.4 SI figures
Figure 3.51. Bayesian phylogenetic inference of all known BFDV sequences from endemic Australian species including *P. elegans* BFDV isolates sequenced in this study (highlighted yellow). All sequences were aligned to our partial BFDV genome (1629 nucleotides) and cropped. Tree node values represent posterior probabilities and branch colours denote subfamily (see key). GenBank accession numbers of each BFDV isolate are presented on the tree tips.
**Figure 3.52.** Maximum likelihood phylogenetic tree (support values represent a percentage from 1000 bootstrap replicates) based on (a) the capsid ORF (n = 39), (b) the partial replication-associated protein ORF (n = 29), and (c) a 173 putatively non-coding nucleotide sequence from beak and feather disease virus (n = 39). The non-coding sequence is between the capsid ORF and replication-associated protein ORF, approximately between nucleotide positions 1000 and 1200. Colours indicate subspecies: yellow (*P. e. flaveolus*), red (*P. e. elegans*), orange (*P. e. adelaidae*), black (WS hybrid), white (BFDV sequences from NCBI). Shapes indicate evidence of recombination (square = recombination detected, circle = no recombination detected).
Figure 3.53. Maximum likelihood phylogenetic inference (support values represent a percentage from 1000 bootstrap replicates) based on a ~1629 nucleotide region of the BFDV genome. Samples with evidence of recombination were excluded from this analysis. Colours of circles indicate subspecies: yellow (P. e. flaveolus), red (P. e. elegans), orange (P. e. adelaidae), black (WS hybrid), white (BFDV sequences from GenBank).
Figure 3.54. The top row represents genetic distances (pairwise) based on the beak and feather disease virus (BFDV) capsid ORF (a, b), the middle row the BFDV replication-associated protein ORF (c, d) and the bottom row the BFDV partial genome (e, f). The first column (a, c, e) represents geographic distances calculated using the shortest distance
between sampling location within the boundaries of the host distribution. The second column (b, d, and f) the shortest distance within the host distribution assuming no gene flow between *P. e. elegans* and *P. e. flaveolus* across the WS hybrid area (as predicted by the ring-species hypothesis). The inner/outer circle colours indicate which host subspecies BFDV sequences were sampled from: yellow (*P. e. flaveolus*), red (*P. e. elegans*), orange (*P. e. adelaidae*), black (WS hybrid). Geographic distance was calculated between paired sequences. Lines of best fit (ordinary least squares regression), represent a visual aid only; tests of isolation-by-distance reported in the text are based on Mantel tests.
CHAPTER 4: Heterozygosity and genotype rarity affect viral infection dynamics in wild bird populations

Photo: the Adelaide rosella (*Platycercus elegans adelaidae*).

Courtesy of Dr Raoul F. H. Ribot. This image also appears on the cover of the Journal of Experimental Biology, Volume 216.
4.1 Abstract

Genetic diversity has been a central focus in ecological and evolutionary studies because of its importance in population adaptation to novel and changing environments (Forcada & Hoffman 2014). Pathogens are a crucial selective force against susceptible host individuals (Anderson & May 1982; Karlsson et al. 2014), and are thought to contribute to determining host population genetic diversity (Haldane 1949; Coltman et al. 1999; Bérénos et al. 2011; King et al. 2011; Schmid-Hempel 2011). Additionally, host genetic diversity may also reduce an individual’s susceptibility to pathogen infections (Lively 2010; Schmid-Hempel 2011). Recently, in the putative ring-species, crimson rosella (*Platycercus elegans*), it was shown that beak and feather disease virus (BFDV) varies in prevalence and viral load between each subspecies (Eastwood et al. 2014). Eastwood et al. (2014), suggested that these differences in pathogen selection could contribute to the maintenance of genetic variation within the *P. elegans* species complex. As associations between pathogen infection and genetic diversity have been identified (King et al. 2011; Hoffman et al. 2014), it has been proposed that both heterozygosity and genotype rarity could explain this variability in virus susceptibility (Eastwood et al. 2014). Here, we show for the first time that individual heterozygosity and genotype rarity predict virus prevalence and load respectively. Our results demonstrate that susceptibility is driven by heterozygosity, whilst the severity of infection is potentially subject to Red Queen dynamics acting against common host genotypes. Hence, our results show how pathogens may play a fundamental role in the evolution of animal species. Additionally, these findings have important implications for avian conservation and mate choice.
4.2 Introduction

Pathogens by definition have negative consequences for their host and often reduce their likelihood of survival or reproductive success (King & Lively 2012). Therefore, pathogens have long been considered a strong selective force in nature against susceptible host individuals (Haldane 1949). Genetic diversity is hypothesised to directly influence the susceptibility of hosts to infection, and it is generally accepted that lower genetic diversity predicts susceptibility (King & Lively 2012). In natural populations, inbred and less genetically diverse (more homozygous) individuals tend to display a higher prevalence (Acevedo-Whitehouse et al. 2003; MacDougall-Shackleton et al. 2005; Ortego et al. 2007a; Townsend et al. 2009; Isomursu et al. 2012) and load of pathogens (MacDougall-Shackleton et al. 2005; Luikart et al. 2008; Isomursu et al. 2012; Hoffman et al. 2014). The mechanism explaining this phenomenon relates to the reduced capability of individuals that are less diverse at loci involved in immunity (e.g. Major Histocompatibility Complex or Toll-like receptors) to defend against pathogens (Tschirren et al. 2013; Kamath et al. 2014). In addition, susceptibility may also be related to individuals with common genotypes to which a pathogen is co-adapted, giving rare individuals a selective advantage (Lively et al. 1990; Lively & Dybdahl 2000; Decaestecker et al. 2007; Schmid-Hempel 2011). Whilst selection against homozygotes results in greater overall population heterozygosity (Coltman et al. 1999), rare genotype advantage due to selection against common genotypes results in negative frequency-dependant selection on genotypes (Jokela et al. 2009; Wolinska & Spaak 2009). Hence, the capacity for pathogens to explain the maintenance of genetic diversity and subsequently influence host divergence between wild populations is expansive (Bérénos et al. 2011; King et al. 2011; Eastwood et al. 2014). However, no study has set out to assess the relative
importance of heterozygosity and genotype rarity. Furthermore, while there is evidence for pathogen selection against common alleles involved in immunity (Trachtenberg et al. 2003; Kamath et al. 2014) and against common clonal variants (Lively et al. 1990; Lively & Dybdahl 2000; Decaestecker et al. 2007; Schmid-Hempel 2011), no study has investigated the effect of genome wide rarity within a sexually reproducing vertebrate in the wild. The use of highly variable and hybridising species complexes offers a unique opportunity to investigate the host-pathogen interactions and determine how diversity influences the spread of pathogen infection (Eastwood et al. 2014).

In wild P. elegans we tested whether genetic diversity influences BFDV susceptibility. P. elegans is a common parrot species of south eastern Australia, which consists of several phenotypically variable (i.e. plumage colouration, vocalizations) subspecies and hybrid populations (Joseph et al. 2008; Ribot et al. 2009) (Figure 4.1). Recently, we found that the highly pathogenic BFDV varied in both prevalence and infection load between the P. elegans subspecies (Eastwood et al. 2014). BFDV is a single-stranded DNA circovirus which consists of two primary open reading frames (ORF), the capsid ORF (CAP), and the replication-associated protein ORF (REP) (Sarker et al. 2014a). Signs of the disease vary between species but include: feather malformation and/or loss, beak and claw deformity and immune suppression (Pass & Perry 1984; Todd 2000). The latter complication often results in death, thus explaining the high mortality rates and the worldwide status of BFDV as a key conservation concern (Kundu et al. 2012; Peters et al. 2014). Previously, we found that BFDV prevalence and load was notably lower in phenotypically intermediate and hybrid forms (Western Slopes and P. e. adelaïdae), than the two most phenotypically distinct subspecies (P. e. elegans and P. e. flaveolus) (Eastwood et al. 2014). These data are consistent with the
interpretation that the subspecies differ in their susceptibility to the disease. It seems possible, therefore, that higher individual and population level genetic diversity within the intermediate and introgressed populations may explain this variation in BFDV infection (Eastwood et al. 2014). We tested this hypothesis by measuring genetic diversity at both individual [homozygosity-by-loci (HL), distant relatedness ($d^2$), and genotype rarity (probability of assignment and average pairwise relatedness)] and subspecies (HL, $d^2$, genotype rarity and allelic richness) levels, and whether these measures are differentially associated with an increased risk of BFDV infection and viral load. In addition, we investigated whether host genetic distance is correlated with BFDV genetic distance, as would be predicted in an antagonistically co-evolving host-pathogen relationship (Schmid-Hempel 2011).
Figure 4.1. Distribution map of *Platycercus elegans* across south eastern Australia (Eastwood et al. 2014). Approximate *P. elegans* subspecies and WS hybrid distributions are colour coded (See key).
4.3 Materials and methods

4.3.1 Fieldwork

During 2004 – 2011, *P. elegans* samples were caught throughout the species range encompassing four subspecies (*P. e. elegans*, *P. e. flaveolus*, *P. e. adelaidae*, and *P. e. melanoptera*) and the WS hybrid populations (Figure 4.1). This sampling included n = 282 individuals from a previous study in this system (Joseph et al. 2008), but here, we only included birds within the main section of the species complex (samples were excluded above latitude S 32°56' within the *P. e. elegans* distribution because they represent a separate population i.e. north of Hunter river, NSW) (Joseph et al. 2008). An additional 90 birds from five new localities were included in this study (Bellbrae S 38°19', E 144°11', Steiglitz S 37°52', E 144°11', Pomonal S 37°11' E 142°36', Moyhu S36°35', E146°24', Bunbartha S 36°16' E 145°21'). Our primary capture method was to use nest box traps (Berg & Ribot 2008). Alternative methods included: mist netting, baited walk in traps, collection of road kill, and the collection of birds culled as orchard pests (Joseph et al. 2008; Ribot et al. 2011). Live birds had approximately 100 μl of blood taken by brachial vein puncture which was stored in ethanol. A small tissue (muscle) sample was collected from dead birds to be used for DNA instead of blood (Eastwood et al. 2015). The presence or absence of BFDV clinical signs (beak deformity, and feather malformation or loss) was noted and the approximate age of each individual was observed based on distinct plumage characteristics. Green plumage denoted sub-adult birds (1-2 years). Young adults (3-4 years) were identified by a white stripe underneath the wing, which is lost after five years of age. Subsequently, birds without a wing-stripe are classified as old adults (>5 years) (Eastwood et al. 2015).
4.3.2 Molecular techniques

An ammonium acetate DNA extraction method was used for all samples (Eastwood et al. 2015). To ensure high quality DNA was extracted and to quantify the concentration of DNA, we used a Beckman DU spectrophotometer (Beckman Coulter, CA, USA). To determine the sex of each individual we used a common molecular sexing method following Griffiths et al. (1998). To detect BFDV prevalence and load we used a probe based quantitative real-time PCR technique, which has previously been used in P. elegans (Eastwood et al. 2015). Microsatellite genotyping was conducted by the Australian Genome Research Facility (AGRF: Brisbane, Australia) using nine previously published microsatellite loci (AgGT07, AgGT21, CP03E01, CP52A09, Ero03, Ero08, Cl2, Cl3, and Cfor2627) (Joseph et al. 2008).

4.3.3 Microsatellite genotyping and analysis

We used a full dataset of n = 372 for our preliminary microsatellite data analysis. Using IRmacroN3 (W. Amos, Cambridge University) implemented in Microsoft Excel 2010, we determined the number of alleles and the allele size ranges per locus, and estimated the proportion of null alleles. For a priori reasons we then partitioned our data according to the five primary subspecies including P. e. elegans, P. e. flaveolus, P. e. adelaidae, P. e. melanoptera, and the WS hybrids. Deviations from Hardy-Weinberg equilibrium or linkage disequilibrium were then tested using Arlequin version 3.5 (Excoffier & Lischer 2010). In this study, we calculated genetic diversity at two different levels:

(a) Individual genetic diversity

Two measures of individual genetic diversity were measured using the program IRmacroN3. Homozygosity by loci (HL) as described in (Aparicio et al. 2006), is a measure designed to
index the level of inbreeding (homozygosity) within an individual by weighting the allelic
correspondence of each loci. The HL index results in more homozygous individuals having a
value closer to 1 and more heterozygous individuals a value closer to 0. Using the same
program as above we calculated $d^2$ which estimates the long term mutational divergence
between alleles. $d^2$ may therefore be better for populations that are highly admixed (Amos
et al. 2001; Aparicio et al. 2006), which is expected to the case in P. elegans.

To measure the rarity of an individual’s genotype within the population, we used a
genetic assignment method implemented in the software package Geneclass 2 (Piry et al.
2004). Typically used for identifying dispersers, Geneclass 2 calculates a likelihood estimate
(Likelihood computation used = L_home) based on Paetkau et al. (2004). Then using Monte
Carlo re-sampling methods the program simulates a random sample of expected genotypes
(n = 10,000). This allows the calculation of a probability that an individual’s genotype is likely
to be found within a given population (Paetkau et al. 2004), and therefore it can be used as
an estimate of how rare an individual’s genotype is within a given population. We also
attempted to measure how rare an individual’s genotype was by calculating the average
pairwise relatedness to all the other individuals sampled within the population. This idea is
based on the assumption that a common genotype will have a higher average pairwise
relatedness compared to a rare genotype in the same population. To calculate pairwise
relatedness to all individuals, we used the method described by Queller and Goodnight
(1989) implemented in the program CoAncestry (Wang 2011). In all measures of individual
genetic diversity (HL, $d^2$, probability of assignment and average pairwise relatedness) we
considered subspecies as a population. P. e. Melanoptera and P. e. flaveolus birds that occur
within a genetic cline (Joseph et al. 2008) were not included in the calculation of these
measures because we do not have any BFDV data in these populations. All four measures of individual genetic diversity had a large value range (HL: 0 – 0.896, $d^2$: 0.007- 0.373, probability of assignment index: 0.001 - 0.999 and average pairwise relatedness: -0.179 – 0.140). HL was significantly correlated with $d^2$ (Pearson’s r = -0.399, P < 0.001) and average pairwise relatedness (Pearson’s r = 0.285, P < 0.001). $d^2$ was correlated with the probability of assignment index (Pearson’s r = -0.286, P = <0.001) and average pairwise relatedness (Pearson’s r = -0.385, P < 0.001). The probability of assignment index was highly correlated with average pairwise relatedness (Pearson’s r = 0.757, P < 0.001). See Extended Data Figure 4.S2 for genetic diversity measure distributions.

(b) Population genetic diversity

Populations in this study are represented by subspecies classification (P. e. elegans: n = 100; P. e. flaveolus: n = 52; WS hybrid: n = 99; P. e. adelaidae: n = 112). To determine the genetic diversity of each subspecies we used several simple measures. First, using the program HP-rare version 1 (Kalinowski 2005) we calculated allelic richness (average number of alleles within a population) using the rarefaction method (Kalinowski 2004). Second, using the same method, we calculated private allele richness (average number of unique alleles within a population).

4.3.4 Host distance versus BFDV distance

A partial mantel test was used to test for a correlation between P. elegans and BFDV genetic distance whilst controlling for geographic distance (as implemented in XLSTAT, version 2014.02.03, Addinsoft). P. elegans genetic distance was calculated by transforming the Queller and Goodnight (1989) relatedness coefficient for each dyad by subtracting this value
from one. BFDV data was sequenced as per Eastwood et al. (2014), and are available from GenBank (accession numbers KJ953847 - KJ953885). BFDV genetic distance, recombination detection, and geographic distance was calculated using the same approach as Eastwood et al. (2014). We used three different BFDV genome segments for analysis, which correspond to different coding regions that may be under different selective pressures. Including, a 726 nucleotide segment of the REP, a 744 nucleotide segment of the CAP, and lastly, these regions concatenated with a non-coding region, hereby termed partial BFDV genome (1629 nucleotides). Sequences with evidence for recombination were removed in separate Mantel tests.

4.3.5 Statistical analysis

All statistical analyses were completed using the program SPSS version 22 (IBM, Armonk NY). HL, $d^2$, average relatedness and rarity were considered dependent variables in each linear mixed model (LMM) with restricted maximum likelihood estimation (REML). BFDV infection (infected or not infected) was a fixed effect while controlling for subspecies ($P. e. elegans$, $P. e. flaveolus$, $P. e. adelaidae$ and WS hybrid), age (sub-adult, young adult and old adult), sex (male and female), centred Julian date (September 1st was set as day one to mark the beginning of the breeding season; hereafter ‘date’) and centred Julian date squared (to account for non-linearity). BFDV viral load was modelled as a dependent variable with one of the genetic diversity measures (HL, $d^2$, average relatedness and rarity) as a fixed effect. Subspecies, age, sex, date and date squared were controlled for in all LMMs. The final model was achieved by removing fixed control variables that were clearly non-significant ($P > 0.1$) and re-running the model with the remaining variables (excluding the variables of primary interest: subspecies and either HL, $d^2$, rarity, or average pairwise relatedness).
4.4 Results and discussion

Our dataset consisted of $n = 226$ individuals with known age, sex, date of capture, subspecies, BFDV infection data. Individuals were genotyped using nine microsatellite markers that were not found to be out of linkage disequilibrium (Extended Data Table 4.S1) or Hardy-Weinberg equilibrium (Extended Data Table 4.S2). Using a linear mixed modelling (LMM) approach (see methods), we found that HL was predicted by individual infection status (LMM, $F = 4.81$, $P = 0.029$), indicating that more inbred individuals were more likely to be infected with this virus (Figure 4.2). Subspecies (LMM, $F = 4.449$, $P = 0.005$) and sex (LMM, $F = 5.370$, $P = 0.021$) also significantly predicted HL in our final LMM model (see Extended Data Table 4.S3). We also tested whether on an individual level $d^2$, rarity (probability of assignment and average pairwise relatedness) were predicted by BFDV infection, but no association was detected (All indices $P > 0.05$; Extended Data Table 4.S4). In all models with a genetic diversity dependent variable (HL, $d^2$, probability of assignment and average pairwise relatedness) we considered an interaction between BFDV and subspecies, however, none were found to be significant and were therefore omitted from our final models. The finding that less heterozygous individuals are more likely to be infected with BFDV is consistent with the interpretation that genetic diversity offers an advantage in terms of reducing susceptibility to infection, similar to other studies (Coltman et al. 1999; Hawley et al. 2005; MacDougall-Shackleton et al. 2005; Fossøy et al. 2009; Gompper et al. 2011). Individuals that have higher levels of heterozygosity may be able to resist a wider range of pathogens or in this instance greater BFDV subtype variation. Although a single individual can host several different genetic strains, BFDV is relatively conserved antigenetically (Sarker et al. 2014a). Alternatively, higher levels of heterozygosity may reflect an immunogenic
advantage due to overdominance or because homozygous individuals are more likely to display deleterious alleles resulting in increased pathogen susceptibility (Fossøy et al. 2009).

Figure 4.2. Homozygosity-by-loci index (HL) is predicted by BFDV infection status. Results of the final linear mixed models showing the mean model predicted HL of BFDV infected (striped bars) and non-infected (solid bars) individuals across all subspecies and WS hybrid populations (n = 226). Error bars represent the 95% confidence intervals.

At the subspecies level, if pathogens select against high levels of homozygosity, a reasonable hypothesis would be that populations under greater pathogen selective pressure would be more heterozygous (Coltman et al. 1999). We found that prevalence did vary...
between the subspecies ($\chi^2 = 61.924$, d.f. = 3, $P < 0.001$) as our larger dataset shows (Eastwood et al. 2014). WS hybrids and *P. e. adelaidae* had a lower BFDV prevalence, but a higher average HL (Extended Data Table 4.S3). Hence, this could potentially reflect a reduced selective pressure of BFDV on these populations compared to *P. e. elegans*, which was found to be the most heterozygous population (Extended Data Table 4.S3), with a higher BFDV prevalence. In a contradiction to this hypothesis, *P. e. flaveolus* had a high prevalence but similar HL levels to less infected populations (WS hybrid and *P. e. adelaidae*). Similar patterns were present when investigating genetic diversity in terms of allelic richness and private allelic richness (Extended Data Table 4.S5, Extended Data Figure 4.S1). There was no qualitative pattern when comparing BFDV to $d^2$, probability of assignment and average pairwise relatedness measures (Extended Data Table 4.S4) and there was no clear variation in the distribution shape of all genetic diversity measures (Extended Data Figure 4.S2). An alternative explanation for the variability in genetic diversity between subspecies may be a consequence of a broader range of selective forces or differences in population size (Frankham 1996).

When investigating population BFDV load and genetic diversity (all indices of genetic diversity) there was also no clear qualitative pattern (see both Extended Data Table 4.S3 and Table 4.S6). In addition, our results also showed that individual HL, $d^2$, and probability of assignment did not predict BFDV load (All indices $P > 0.05$; Extended Data Table 4.S6). However, we found that average pairwise relatedness was a significant predictor of BFDV load in the final model (LMM, $F = 4.519$, $P = 0.036$; Extended Data Table 4.S3), whilst controlling for subspecies (LMM, $F = 5.042$, $P = 0.003$), age (LMM, $F = 7.847$, $P = 0.001$), sex (LMM, $F = 9.804$, $P = 0.002$), date (LMM, $F = 6.715$, $P = 0.011$), and date$^2$ (LMM, $F = 6.125$, $P =$
Average pairwise relatedness was positively associated with BFDV load in all subspecies and hybrid populations (Figure 4.3a and b). In other words, the more related an individual was to others within its population, the more likely it was to harbour a higher infection load than less related, infected individuals. We speculate that this finding is the result of viral tracking of common host genotypes, whereby viral genotypes coevolve with host susceptibility. BFDV isolates that have coevolved to common host genotypes may be more compatible with, and more able to replicate in, closely related or common host genotypes (Lively et al. 1990; Lively & Dybdahl 2000; Decaestecker et al. 2007). This may give rare and less related genotypes within a population a selective advantage, potentially leading to a negative frequency-dependent scenario (i.e. ‘Red Queen’ dynamics) (Schmid-Hempel 2011). However, when comparing host and virus genetic distance between individuals whilst controlling for geographic distance, we found no strong evidence to suggest that paired BFDV genetic distance is correlated with paired host genetic distance (Partial BFDV genome: Mantel r = 0.008, n = 36, P = 0.847; REP: Mantel r = 0.003, n = 26, P = 0.938; CAP: Mantel r = -0.087, n = 31, P = 0.052). Although, the CAP region warrants further investigation as this correlation is only mildly non-significant. Removing sequences that showed evidence of recombination did not change these results (Partial BFDV genome: Mantel r = 0.073, n = 25, P = 0.202; REP: Mantel r = 0.108, n = 16, P = 0.249; CAP: Mantel r = 0.008, n = 22, P = 0.837). This negative result may appear to reject a BFDV/host coevolution hypothesis, however, there are multiple alternative explanations. The most likely is that our sampling spatial and temporal scale was too large to detect any correlation (Gandon et al. 2008). The finding that individuals which had more common genotypes had a higher BFDV load could also explain the evolution of host dispersal. Individuals that possess a common genotype are under more intense selective pressure to disperse in order to escape co-
adapted pathogens (Townsend et al. 2009). We identified dispersers using the probability of assignment index ($P < 0.01$) and found 11 individuals which likely originate from a different population to which they were found. Interestingly, we did find that dispersal may be sex biased with six out of seven individuals with data on gender found to be male. To test whether dispersers were more or less susceptible to BFDV infection we needed to relax our disperser criteria to increase our sample size (probability of assignment index $P < 0.05$). Classification as disperser or non-disperser was not associated with BFDV prevalence ($\chi^2 = 0.113$, d.f. = 1, $P = 0.737$) or BFDV load (T-test; $t = 1.245$, d.f. = 106, $P = 0.216$). Additionally, we cannot fully exclude a disease escape by dispersal hypothesis because one interpretation of the average pairwise relatedness measure is that it could also reflect dispersal (Rollins et al. 2012).
Figure 4.3. BFDV infection load ($\log_{10}$ transformed) is predicted by individual average pairwise relatedness. This positive relationship is consistent across all subspecies including: (a) subspecies with a high infection load (Red = *P. e. elegans*; yellow = *P. e. flaveolus*) and, (b) subspecies and hybrids with a low infection load (black = WS hybrid, orange = *P. e. adelaidae*). Trend lines were calculated using least squares regression, using predicted values from the final model of $\log_{10}$ BFDV infection load. Data points represent raw BFDV infection load data from $n = 108$ infected individuals.
The study of how genetic diversity influences the spread of infection among individuals and populations is crucial for our understanding of host-pathogen dynamics and coevolution. Here, using multiple distinct measures of host genetic diversity, we show that whilst relatively heterozygous individuals are less likely to be infected, it is the individual’s genotype rarity that predicts viral load. These data imply that an individual’s susceptibility and severity may be under differing selection regimes. Homozygous hosts may be selected against due to increased pathogen susceptibility and therefore under diversifying selection (Coltman et al. 1999). However, host and pathogen genotypes may be under negative frequency-dependent selection, with common genotypes suffering a higher infection severity (Lively et al. 1990; Wolinska & Spaak 2009). Hence, for the first time, both pathogen mediated mechanisms for explaining the maintenance of host genetic diversity are found to be important concurrently. This offers novel evidence that pathogens are an important factor in host divergence (Eastwood et al. 2014). Furthermore, mate choice benefits could be affected as choosing a heterozygous partner does not guarantee genotypic rarity (Roberts et al. 2006). Hence, there are possible mate-choice trade-offs between choosing a partner who is susceptible to becoming infected or susceptible to a higher infection severity. Finally, these results provide insight into the genetic determinants of the spread of infection in natural populations and highlight a practical conundrum for genetic conservation.
4.5 Supporting information

4.5.1 Extended data

Table 4.51. Summary statistics of each loci used in this study assuming all subspecies are one continuous population. Each locus was tested for linkage disequilibrium within each subspecies.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Number of individuals</th>
<th>Number of alleles per locus</th>
<th>Allele size range</th>
<th>Estimated proportion of null alleles</th>
<th>Evidence of linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgGT07</td>
<td>361</td>
<td>27</td>
<td>251-286</td>
<td>0.1</td>
<td>No</td>
</tr>
<tr>
<td>Cp03E01</td>
<td>361</td>
<td>23</td>
<td>184-234</td>
<td>0.1</td>
<td>No</td>
</tr>
<tr>
<td>AgGT21</td>
<td>363</td>
<td>4</td>
<td>315-328</td>
<td>0.02</td>
<td>No</td>
</tr>
<tr>
<td>Cfor2627</td>
<td>364</td>
<td>10</td>
<td>132-159</td>
<td>0.05</td>
<td>No</td>
</tr>
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<td>C12</td>
<td>364</td>
<td>6</td>
<td>61-78</td>
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<td>No</td>
</tr>
<tr>
<td>C13</td>
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<td>189-209</td>
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<td>CP52A09</td>
<td>361</td>
<td>20</td>
<td>361-401</td>
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<td>No</td>
</tr>
<tr>
<td>Ero03</td>
<td>360</td>
<td>12</td>
<td>192-218</td>
<td>0.06</td>
<td>No</td>
</tr>
<tr>
<td>Ero08</td>
<td>364</td>
<td>17</td>
<td>119-155</td>
<td>0.01</td>
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</table>
Table 4.52. These data provide no convincing evidence for any locus being out of Hardy-Weinberg equilibrium. Arlequin results for Hardy-Weinberg equilibrium for each subspecies (n = 372 individuals) and each loci (n = 9). “Y” denotes significance after Bonferroni correction (P < 0.001). Empty cells denote a non-significant result.

<table>
<thead>
<tr>
<th></th>
<th>AgGT07</th>
<th>CPO3E01</th>
<th>AgGT21</th>
<th>Cfor2627</th>
<th>Cl2</th>
<th>Cl3</th>
<th>CP52A09</th>
<th>Ero03</th>
<th>Ero08</th>
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<tr>
<td>P. e. elegans</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WS hybrid</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. e. flaveolus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>P. e. adelaidae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>P. e. melanoptera</td>
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<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Table 4.53. Final linear mixed models predicting homozygosity-by-loci (HL) and BFDV infection load ($\log_{10}$ transformed). Models were considered final following a stepwise removal of non-significant fixed effects (see methods). Julian date was set on September 1$^{st}$ at the beginning of the breeding season (referred to as “date”) and squared to account for non-linearity.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Fixed effects</th>
<th>Mean ± s.e.</th>
<th>Estimate ± s.e.</th>
<th>d.f.</th>
<th>$F$</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL (n = 226)</td>
<td>BFDV infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td></td>
<td>0.367 ± 0.015</td>
<td>219</td>
<td>4.810</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>Non-infected</td>
<td></td>
<td>0.313 ± 0.018</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subspecies</td>
<td>P. e. elegans</td>
<td>0.281 ± 0.022</td>
<td>219</td>
<td>4.449</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WS hybrid</td>
<td>0.395 ± 0.020</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. e. flaveolus</td>
<td>0.341 ± 0.030</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. e. adelaidae</td>
<td>0.342 ± 0.018</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>0.364 ± 0.014</td>
<td>219</td>
<td>5.370</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0.316 ± 0.016</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFDV load (n = 108)</td>
<td>Average relatedness</td>
<td>6.196 ± 2.915</td>
<td>95</td>
<td>4.519</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Subspecies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. e. elegans</td>
<td>-2.096 ± 0.504</td>
<td>95</td>
<td>5.042</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WS hybrid</td>
<td>-1.646 ± 0.651</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. e. flaveolus</td>
<td>-0.232 ± 0.371</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. e. adelaidae</td>
<td>-1.680 ± 0.454</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Subadult</td>
<td>-0.519 ± 0.265</td>
<td>95</td>
<td>7.847</td>
<td>0.001</td>
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<tr>
<td></td>
<td>Young adult</td>
<td>-1.638 ± 0.390</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Old adult</td>
<td>-2.083 ± 0.358</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>-0.904 ± 0.265</td>
<td>95</td>
<td>9.804</td>
<td>0.002</td>
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<tr>
<td></td>
<td>Female</td>
<td>-1.923 ± 0.282</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td></td>
<td>0.015 ± 0.006</td>
<td>95</td>
<td>6.715</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>Date$^2$</td>
<td></td>
<td>2.05 ×10$^{-4}$ ± 8.29 ×10$^{-5}$</td>
<td>95</td>
<td>6.125</td>
<td>0.015</td>
<td></td>
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</table>
Table 4.54. Final linear mixed models predicting individual (n = 226) genetic diversity (d²), probability of assignment and average pairwise relatedness). Models were considered final following a stepwise removal of non-significant fixed effects (see methods). Julian date was set on September 1st at the beginning of the breeding season (referred to as “date”) and squared to account for non-linearity.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Fixed effects</th>
<th>Mean ± s.e.</th>
<th>Estimate ± s.e.</th>
<th>d.f.</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>d²</td>
<td>BFDV infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>0.130 ± 0.008</td>
<td>0.130 ± 0.008</td>
<td>219</td>
<td>0.002</td>
<td>0.963</td>
</tr>
<tr>
<td></td>
<td>Non-infected</td>
<td>0.131 ± 0.009</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Subspecies</td>
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</tr>
<tr>
<td></td>
<td>P. e. elegans</td>
<td>0.120 ± 0.11</td>
<td>0.120 ± 0.11</td>
<td>219</td>
<td>4.596</td>
<td>0.004</td>
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<tr>
<td></td>
<td>WS hybrid</td>
<td>0.104 ± 0.010</td>
<td></td>
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<tr>
<td></td>
<td>P. e. flaveolus</td>
<td>0.168 ± 0.015</td>
<td>0.168 ± 0.015</td>
<td></td>
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<tr>
<td></td>
<td>P. e. adelaidae</td>
<td>0.130 ± 0.009</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.122 ± 0.007</td>
<td>0.122 ± 0.007</td>
<td>219</td>
<td>3.011</td>
<td>0.084</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0.139 ± 0.008</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Rarity</td>
<td>BFDV infection</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Infected</td>
<td>0.452 ± 0.028</td>
<td>0.452 ± 0.028</td>
<td>224</td>
<td>0.086</td>
<td>0.769</td>
</tr>
<tr>
<td></td>
<td>Non-infected</td>
<td>0.463 ± 0.027</td>
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</tr>
<tr>
<td>Average relatedness</td>
<td>BFDV infection</td>
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</tr>
<tr>
<td></td>
<td>Infected</td>
<td>0.000 ± 0.006</td>
<td>0.000 ± 0.006</td>
<td>218</td>
<td>2.379</td>
<td>0.124</td>
</tr>
<tr>
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<td>Non-infected</td>
<td>-0.015 ± 0.007</td>
<td>-0.015 ± 0.007</td>
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</tr>
<tr>
<td></td>
<td>Subspecies</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. e. elegans</td>
<td>0.003 ± 0.010</td>
<td>0.003 ± 0.010</td>
<td>218</td>
<td>2.829</td>
<td>0.039</td>
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<tr>
<td></td>
<td>WS hybrid</td>
<td>0.003 ± 0.008</td>
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</tr>
<tr>
<td></td>
<td>P. e. flaveolus</td>
<td>-0.036 ± 0.012</td>
<td>-0.036 ± 0.012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. e. adelaidae</td>
<td>0.000 ± 0.007</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Date²</td>
<td>-4.850 ×10⁻⁶ ±</td>
<td>-4.850 ×10⁻⁶ ±</td>
<td>218</td>
<td>5.351</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.097×10⁻⁶</td>
<td>2.097×10⁻⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 4.55.** Wilcoxon signed ranked test comparing pairwise population allelic richness (shaded) and population private allelic richness (un-shaded). Standardised test statistic is denoted by W. Significance ($P < 0.05$) is highlighted in bold ($P. e. elegans$: $n = 100$; $P. e. flaveolus$: $n = 52$; WS hybrid: $n = 99$; $P. e. adelaidae$: $n = 112$).

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>$P. e. elegans$</th>
<th>WS hybrid</th>
<th>$P. e. flaveolus$</th>
<th>$P. e. adelaidae$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P. e. elegans$</td>
<td>(W = -1.72, $P = 0.09$)</td>
<td>(W = -2.55, $P = 0.01$)</td>
<td>(W = -1.84, $P = 0.07$)</td>
<td></td>
</tr>
<tr>
<td>WS hybrid</td>
<td>(W = -1.72, $P = 0.09$)</td>
<td>(W = -0.42, $P = 0.68$)</td>
<td>(W = -0.56, $P = 0.58$)</td>
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</tr>
<tr>
<td>$P. e. flaveolus$</td>
<td>(W = -1.72, $P = 0.09$)</td>
<td>(W = -1.48, $P = 0.14$)</td>
<td>(W = -1.96, $P = 0.05$)</td>
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</tr>
<tr>
<td>$P. e. adelaidae$</td>
<td>(W = -2.66, $P &lt; 0.01$)</td>
<td>(W = -0.06, $P = 0.95$)</td>
<td>(W = -1.24, $P = 0.21$)</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.56. Final linear mixed models with BFDV load as the dependent variable (n = 108). Including each measure of individual genetic diversity tested separately as a fixed effect: Homozygosity-by-loci (HL), distant relatedness ($d^2$), and probability of assignment. Models were considered final following a stepwise removal of non-significant fixed effects (see methods). Julian date was set on September 1st at the beginning of the breeding season (referred to as “date”) and squared to account for non-linearity.
<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Fixed effects</th>
<th>Mean ± s.e.</th>
<th>Estimate ± s.e.</th>
<th>d.f.</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFDV Load</td>
<td>HL</td>
<td>0.919 ± 1.023</td>
<td>95 0.807 0.371</td>
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<tr>
<td></td>
<td><em>P. e. elegans</em></td>
<td>-2.113 ± 0.514</td>
<td>95 3.994 0.010</td>
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<tr>
<td></td>
<td>WS hybrid</td>
<td>-1.484 ± 0.662</td>
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<tr>
<td></td>
<td><em>P. e. flaveolus</em></td>
<td>-0.376 ± 0.371</td>
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<tr>
<td></td>
<td><em>P. e. adelaidae</em></td>
<td>-1.647 ± 0.464</td>
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<tr>
<td></td>
<td>Age</td>
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<tr>
<td></td>
<td>Sub-adult</td>
<td>-0.459 ± 0.268</td>
<td>95 8.357 &lt;0.001</td>
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<td></td>
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<td>-1.688 ± 0.397</td>
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<tr>
<td></td>
<td>Old adult</td>
<td>-2.068 ± 0.367</td>
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<tr>
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<td>-0.938 ± 0.270</td>
<td>95 7.523 0.007</td>
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<td>-1.872 ± 0.294</td>
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<td>Date</td>
<td>0.016 ± 0.006</td>
<td>95 7.366 0.008</td>
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<td>Date^2</td>
<td>1.86 ×10^-4 ± 8.41 ×10^-5</td>
<td>95 4.866 0.030</td>
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<tr>
<td>BFDV Load</td>
<td><em>d^2</em></td>
<td>-1.346 ± 2.060</td>
<td>95 0.427 0.515</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Subspecies</td>
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<td><em>P. e. elegans</em></td>
<td>-2.105 ± 0.515</td>
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<td>WS hybrid</td>
<td>-1.572 ± 0.667</td>
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<tr>
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<td><em>P. e. adelaidae</em></td>
<td>-1.695 ± 0.465</td>
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<tr>
<td></td>
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<td></td>
<td>Sub-adult</td>
<td>-0.468 ± 0.270</td>
<td>95 8.683 &lt;0.001</td>
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<td>-1.699 ± 0.397</td>
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<td>-2.115 ± 0.366</td>
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<tr>
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<tr>
<td></td>
<td>Male</td>
<td>-0.927 ± 0.270</td>
<td>95 9.065 0.003</td>
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<tr>
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<td>Female</td>
<td>-1.928 ± 0.288</td>
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<td></td>
<td>Date</td>
<td>0.015 ± 0.006</td>
<td>95 6.552 0.012</td>
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<td>1.81 ×10^-4 ± 8.46 ×10^-5</td>
<td>95 4.596 0.035</td>
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<td>Rarity</td>
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<td><em>P. e. elegans</em></td>
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<td>95 4.035 0.010</td>
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<tr>
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<td>-1.598 ± 0.664</td>
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<td></td>
<td><em>P. e. flaveolus</em></td>
<td>-0.373 ± 0.370</td>
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<td></td>
<td><em>P. e. adelaidae</em></td>
<td>-1.653 ± 0.463</td>
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<td></td>
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<td>-0.492 ± 0.270</td>
<td>95 8.109 0.001</td>
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<td>-1.677 ± 0.396</td>
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<td></td>
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<td>-2.098 ± 0.364</td>
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<td>Sex</td>
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<td>-0.937 ± 0.269</td>
<td>95 8.525 0.004</td>
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<td>-1.908 ± 0.288</td>
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<td></td>
<td>Date</td>
<td>0.015 ± 0.006</td>
<td>95 6.485 0.012</td>
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<td>Date^2</td>
<td>1.83 ×10^-3 ± 8.40 ×10^-5</td>
<td>95 4.763 0.032</td>
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</table>
Figure 4.51. *P. e. elegans* is the most genetically diverse subspecies. According to the two population level genetic measures (a) allelic richness, and (b) private allelic richness. Both measures were calculated across all nine loci within each subspecies (*P. e. elegans*: n = 100; *P. e. flaveolus*: n = 52; WS hybrid: n = 99; *P. e. adelaidae*: n = 112).
Figure 4.S2. Frequency distribution of each individual genetic diversity measure from n = 363 individuals.
CHAPTER 5: Pair fidelity in long-lived parrots: genetic and behavioural evidence from the crimson rosella (*Platycercus elegans*)

Photo: nestling crimson rosella (*Platycercus elegans*).

Courtesy of Dr Mathew L. Berg
5.1 Abstract

Genetic analyses of parentage have revolutionised our understanding of avian mating systems. However, the majority of such studies to date have focussed on passerine species, which limits comparative analyses and the generality of our knowledge on avian breeding biology. Despite this taxonomic bias, extra pair paternity (EPP) and conspecific brood parasitism (CBP) are often assumed to be less common or absent in taxa with characteristics including longevity, socially monogamous mating systems, and bi-parental care. However, recent molecular studies in taxa such as seabirds have cast doubt on the generality of these assumptions. The order Psittaciformes, with around 350 species worldwide, is a long-lived taxon that is relatively understudied with respect to parentage and pair fidelity, although most species are assumed to be both socially and sexually monogamous. However, there is little research testing whether EPP or CBP occur in Psittaciformes using modern molecular methods. To test for genetic and social pair fidelity, we studied an Australian parrot, the crimson rosella (*Platycercus elegans*), at three sites over eight years in south eastern Australia. Using nine microsatellite markers in 43 pairs and their offspring, we found no cases of EPP as expected. However, we found one case of CBP, demonstrating that *P. elegans* females do adopt alternative breeding strategies, albeit at low levels. Furthermore, we show that over the eight years of study, 32% of re-captured individuals paired with two or more partners in different years. Our results are consistent with assumptions of low EPP in parrots, but challenge the notion that this is associated with long-term pair bonds.
5.2 Introduction

In birds, social monogamy is an extremely common mating strategy with approximately 92% of species forming single pair bonds during breeding (Wan et al. 2013). However, genetic parentage analysis of offspring in monogamous breeding pairs has shown that true sexual monogamy, i.e. no extra-pair paternity (EPP), is actually rare (Griffith et al. 2002). Comparative analyses suggest that an exception may occur in monogamous breeding systems that require a high reproductive effort from both parents, and where individuals are long lived, with these characteristics being associated with low rates of EPP and long-term pair bonds. These patterns have found support in several orders including Sphenisciformes, Procellariiformes, Strigiformes, and Falconiformes (Masello et al. 2002). However, recent evidence has cast doubt on the generality of low EPP in these orders, particularly within Procellariiformes, where some species have had up to 30 percent of nests with EPP (Quillfeldt et al. 2012). These studies highlight the need for more research to test for inter- and intra-order variability in EPP.

Psittaciformes, including parrots, lorikeets, and cockatoos, are amongst the most long lived birds (Brouwer et al. 2000; Carvalho et al. 2010), and are considered to feature social monogamy and bi-parental care for nestlings in most species (Forshaw & Cooper 2002; Berg & Bennett 2010). These characteristics give rise to expectations that pair fidelity will be high, and EPP will occur rarely in this order. However, there has been little empirical evidence testing this assumption. To our knowledge, there have been two studies that have tested for and distinguished EPP and conspecific brood parasitism (CBP) in Psittaciformes. These studies suggest that, in this order, EPP and CBP do occur but at markedly variable levels in different species. Beissinger (2008) reported that in green rumped parrotlets
(Forpus passerines), EPP was present in 14.4% of broods and 7.7% of young were found to be the result of EPP. In contrast, Masello et al. (2002) did not find any EPP in burrowing parrots (Cyanoliseus patagonus), but did find that CBP occurred, albeit at low levels (4%). Studies on Echo parakeets (Psittacula eques) (Taylor & Parkin 2009) and on blue-and-yellow macaws (Ara ararauna) (Caparroz et al. 2001), suggest that EPP and/or CBP may occur in these species. However, these latter two studies had low sample sizes and the methods used were unable to reliably distinguish EPP and CBP. More research on parentage and pair fidelity in the order Psittaciformes is necessary to determine whether the data fit predictions based on life-history in this order, and will enhance comparative analyses of avian mating systems.

Platycercus elegans is a common parrot species that occurs across south eastern Australia and can be divided into five phenotypically and genetically distinct subspecies (Joseph et al. 2008). The species complex has been extensively studied with regard to vocalizations (Ribot et al. 2009; Ribot et al. 2011; Ribot et al. 2012; Ribot et al. 2013), vision (Carvalho et al. 2010; Knott et al. 2010; Knott et al. 2013), and colouration (Berg & Bennett 2010). In this study, we focus on three subspecies/populations where we have long term breeding data (up to 8 years): P. e. elegans, P. e. adelaidae and the ‘Western Slopes’ hybrid population sensu Joseph et al (2008). As with many parrot species, P. elegans are long-lived birds with bi-parental care, and have therefore been hypothesised to have low levels of EPP and CBP and high levels of pair fidelity (Krebs 1998; Forshaw & Cooper 2002; Krebs et al. 2002a). In this study we aimed to determine whether EPP and/or CBP occurs in this species, whether social mate fidelity continues across breeding seasons, and whether breeding pairs maintain nest site fidelity across years. Overall, our study tests several common assumptions about Psittaciforme mating systems, but for which there is little published evidence, and reduces the deficiency of literature as to whether or not EPP and CBP are common in this order.
5.3 Materials and methods

5.3.1 Field methods

We studied *P. elegans* that were breeding in nest boxes during the main breeding season (October to January), by trapping breeding pairs and their offspring using the method outlined in Berg and Ribot (2008). Once captured, birds were fitted with a uniquely numbered steel leg ring for permanent identification. We collected approximately 80 μl of blood from the brachial vein of both nestlings and their putative parents, which was then stored in ethanol. The number of eggs that did not hatch was also noted. In south-eastern Australia, *P. elegans* comprises four subspecies and hybrid populations (Forshaw & Cooper 2002; Joseph *et al.* 2008). We sampled birds at 14 sites, which represented two subspecies and hybrid populations, over eight breeding seasons, as follows. From 2004-2006 birds were caught in South Australia at six sites in the distribution of the *P. e. adelaidae* subspecies: Carey Gully (S34°58', E138°45'), Marne River (S34°39', E139°23'), Sevenhill (S33°54'E138°36'), Currency Creek (S35°25', E138°45'), Quorn (S32°17', E138°0') and Yulti Wirra (S35°37', E138°11'). In 2010 and 2011 birds were caught at two study sites in the *P. e. elegans* distribution in Victoria: Bellbrae (S38°19', E144°11'), and Steiglitz (S37°52', E144°11'). From 2004 to 2011 we caught birds at six sites in Victoria and New South Wales where *P. e. elegans* and *P. e. flaveolus* hybridise (forming the ‘Western Slopes’ hybrid, *in sensu* Joseph *et al.* (2008): Barnawartha (S36°3', E146°45'), Bonegilla (S36°9', E146°59'), Brungle (S35°11', E148°12'), Tangambalanga (S36°11', E147°0'), Moyhu (S36°35', E146°24') and Mundarlo (S35°4', E147°51').
5.3.2 DNA extraction and molecular sexing

DNA was extracted using a standard ammonium acetate extraction method based on (Bruford et al. 1998). DNA quality and quantity was assessed using a Beckman DU spectrophotometer (Beckman Coulter, CA, USA). The sex of each bird was determined following the protocol described in Griffiths et al. (1998).

5.3.3 Microsatellite genotyping and parentage analysis

Microsatellite genotyping was conducted by the Australian Genome Research Facility (AGRF: Brisbane, Australia). In this study, we used nine microsatellite loci (AgGT07, AgGT21, CP03E01, CP52A09, Ero03, Ero08, CL2, CL3, and Cfor2627) which had been previously used in population genetics studies of P. elegans (Joseph et al. 2008; Ribot et al. 2012). For parentage, we compared nestling allele sizes across all nine loci to that of their parents. Offspring were considered to be the result of extra pair paternity (EPP) or conspecific brood parasitism (CBP) if there were greater than two allele mismatches with their putative parents across the nine loci used; if two or less loci contained mismatching alleles to either parent, then they were considered a result of either mutations or genotyping errors (Ferretti et al. 2011). Accordingly, offspring were considered the result of EPP if they possessed an allele that matched the mother for at least seven of the nine loci but which matched the father for seven or less loci. CBP was concluded if an offspring’s alleles mismatched both parents for at least three loci.
5.4 Results

We tested parentage in 42 families, comprising the breeding pair and 185 nestlings (1-8 nestlings per pair), and found that all but one nestling (0.5%) showed genetic similarity with both of their putative social parents. One nestling, from the *P. e. elegans* population at Bellbrae, was observed to have six mismatching alleles across five loci. At two loci, one allele mismatched the female and at another two loci one allele mismatched the male. One locus had two alleles that were not present in either parent. This would result if an unknown female had deposited an egg in the nest (i.e. CBP). We found no evidence for EPP. Out of the 42 breeding pairs, 18 nests had all eggs hatch and all offspring in these nests were genotyped. A total of 20 clutches had eggs that failed to hatch and were not genotyped, with a mean ± Standard deviation of 2.0 ± 1.3 unhatched eggs per clutch among these 20 clutches. Nine broods had missing samples from hatched nestlings, usually due to early mortality, with a mean of 1.8 ± 1.1 unsampled nestlings per brood among these nine broods.

To investigate whether social pair bonds in *P. elegans* persist across years, we compared the ring numbers of breeding adults over the 8 year study period, including those that were not genotyped for the parentage analyses. This resulted in a total of 100 complete breeding pairs and 19 individuals that were caught more than once (re-captures). Five of those 19 (26%) were paired with the same partner, for 2 or 3 breeding seasons (Table 5.1). Six out of 19 individuals (32%) were found to pair with more than one partner, within the period that we caught them (Table 5.1). One female (individual 16 in Table 5.1) was found to pair with two different males during the same breeding season. The fate of the first male is unknown. We found that most breeding individuals (84%) caught more than once had changed nest boxes between breeding seasons at least once, however, the distance they
moved to nearby nest boxes was very small (Table 5.1). The mean distance between nest box changes of all re-captured pairs was 0.17 km ± 0.03 (SE). The maximum distance a recaptured bird moved from the previous year’s nest box was 0.65 km (Table 5.1).

**Table 5.1.** List of breeding individuals that were captured in two or more years during the study period (2004-2011). M indicates male and F female. Numbers on M and F identify re-captured individuals that were paired with one another for at least one breeding season. *indicates at least one breeding attempt where a partner was not identified.
<table>
<thead>
<tr>
<th>Individual</th>
<th>Sex</th>
<th>Subspecies</th>
<th>Years when caught (number of times captured)</th>
<th>Number of years when partner known</th>
<th>Number of different social partners</th>
<th>Number of different nest boxes occupied</th>
<th>Mean (±SE) distance between nest boxes occupied (km)</th>
<th>Maximum distance between nest boxes occupied (km)</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>M¹</td>
<td>WS hybrid</td>
<td>2007, 2008, 2009, 2010 (4)</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>0.21 (±0.27)</td>
<td>0.51</td>
</tr>
<tr>
<td>2</td>
<td>F²</td>
<td>WS hybrid</td>
<td>2008, 2009, 2010 (3)</td>
<td>3</td>
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<td>2</td>
<td>0.07 (±0.07)</td>
<td>0.13</td>
</tr>
<tr>
<td>3</td>
<td>M²</td>
<td>WS hybrid</td>
<td>2007, 2008 (2)</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0.06 (±0.04)</td>
<td>0.17</td>
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<tr>
<td>4</td>
<td>F²</td>
<td>WS hybrid</td>
<td>2005, 2006, 2007, 2008, 2009 (5)</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>0.14 (±0.14)</td>
<td>0.40</td>
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<tr>
<td>5</td>
<td>M³</td>
<td>WS hybrid</td>
<td>2010, 2011 (2)</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0.18 (±0.12)</td>
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<tr>
<td>6</td>
<td>F³</td>
<td>WS hybrid</td>
<td>2008, 2009, 2010, 2011 (4)</td>
<td>3</td>
<td>2*</td>
<td>2</td>
<td>0.20 (±0.20)</td>
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<td>2008, 2009, 2010, 2011 (4)</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>0.06 (±0.04)</td>
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<td>8</td>
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<td>2</td>
<td>0.11 (±0.00)</td>
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<tr>
<td>9</td>
<td>F</td>
<td>WS hybrid</td>
<td>2009, 2011 (2)</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0.13</td>
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<td>10</td>
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<td>2009, 2010 (2)</td>
<td>1</td>
<td>1*</td>
<td>2</td>
<td>0.17</td>
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<td>2008, 2009 (2)</td>
<td>1</td>
<td>1*</td>
<td>2</td>
<td>0.07 (±0.07)</td>
<td>0.13</td>
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<tr>
<td>12</td>
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<td>WS hybrid</td>
<td>2010, 2011 (2)</td>
<td>1</td>
<td>1*</td>
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<td>0.13</td>
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<tr>
<td>13</td>
<td>F</td>
<td>Adelaide</td>
<td>2004, 2005 (2)</td>
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<td>1*</td>
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<td>0.11 (±0.00)</td>
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<td>Adelaide</td>
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<td>2</td>
<td>1</td>
<td>0.13</td>
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<tr>
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<td>F</td>
<td>Adelaide</td>
<td>2005, 2010 (2)</td>
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<td>1*</td>
<td>2</td>
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<tr>
<td>18</td>
<td>M⁵</td>
<td>Crimson</td>
<td>2010, 2011 (2)</td>
<td>2</td>
<td>1</td>
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5.5 Discussion

Species that demonstrate high level of bi-parental care, long life spans, and social monogamy with long-term pair bonds are often assumed to have low levels, if not absent, EPP (Wan et al. 2013). These expectations have found support in many species (Griffith et al. 2002), although exceptions are increasingly being found and comparative analyses are limited somewhat by taxonomic biases in studies of avian mating systems. The order Psittaciformes are widely known for the aforementioned life history characteristics including extreme longevity, but our knowledge of EPP/CBP in this order is severely restricted due to the paucity of studies. Our findings indicate that the Australian parrot species *P. elegans* is genetically monogamous, as we found no evidence for EPP. Our study of three subspecies and 14 populations over eight years suggests that these findings are consistent over time and space in this widespread species complex.

Interestingly, we found evidence for a low level of CBP. One individual in a nest of four was genetically dissimilar to both parents, indicating that an unknown female had deposited an egg in this clutch, which subsequently hatched successfully. To our knowledge, CBP has been unambiguously demonstrated in only one other genetic study on Psittaciformes. Masello et al. (2002) studied the colonial nesting burrowing parrot using DNA fingerprinting and found no evidence for EPP, but suggested that CBP does occur at low levels (4%). However, Masello et al. (2002) concede that brood mixing though connected burrows could explain at least one case of CBP. Brood mixing in *P. elegans* could occur if there was accidental egg incubation after nest take overs. Our results and those of Masello et al. (2002) are dissimilar to those summarised in Beissinger (2008), which mentioned moderate levels of EPP occur in green-rumped parrotlets. This marked difference in EPP
levels between *P. elegans* and green-rumped parrotlets is perhaps a consequence of the green rumped parrotlets complex social system, and high male competition for females (Beissinger 2008). Our results are consistent with what we understand about *P. elegans* breeding biology, i.e. social monogamy, long lived and provide bi-parental care (Krebs 1998; Krebs & Magrath 2000; Forshaw & Cooper 2002). Two other studies have investigated parentage in parrot species, but due to small sample sizes and inadequate methods they were unable distinguish between EPP and CBP (Caparroz *et al.* 2001; Taylor & Parkin 2009). These studies suggest that not all offspring are the result of sexual monogamy and that EPP and/or CBP could occur. Taylor and Parkin (2009) tested for sexual monogamy in the endangered Echo parakeet using four microsatellite loci. They suggest that the allele mismatches found are likely to be evidence for EPP, particularly as breeding pairs may be accompanied by one or more auxiliary males. Parentage has also been investigated in blue and yellow macaws (Caparroz *et al.* 2001). Caparroz *et al.* (2001) found that in 10 out of 11 broods nestlings were genetically similar to their siblings, however the parents were unable to be sampled in this study. In our study we were able to use a relatively large sample size and genotype samples from both putative parents, which permitted us to distinguish CBP from EPP. It should be noted that we did not genotype unhatched eggs in 20 out of 42 clutches (48%), which could lead to an underestimation of the rate of CBP if parasitic eggs were less likely to hatch than non-parasitic eggs due to mistiming or host rejection.

Our findings show that that genetic pair fidelity in *P. elegans* is high, with no evidence of EPP, and that pairs generally remain together throughout the breeding season. However, our data also indicates that individuals do change partners frequently, challenging the assumption that this species, and perhaps other parrot species, forms long term pair bonds.
(Forshaw & Cooper 2002; Krebs et al. 2002b). We show that pairs can remain with a single partner for at least one to three years, but may also change partners between or even within breeding seasons. For example, one female in our study was caught five times in three different nest boxes with four different partners, one of which was with a different species, an eastern rosella (*Platycercus eximius*). In our study, the reasons for separation are unclear and could be due to either ‘divorce’ or partner mortality (Beissinger 2008). We expect that adult mortality alone is unlikely to explain the high rates of mate switching in such a species, but *P. elegans* mortality in the wild is currently unknown and more re-capture data of more individuals and for longer periods is required to address this gap. Our recapture data also demonstrates that individuals changed nest locations regularly, but never far from the previous location, which is consistent with previous observations on *P. elegans* movements (Forshaw & Cooper 2002).

Our findings provide new evidence for occasional CBP and low levels of EPP in the order Psittaciformes, but challenge the notion that this is related to long-term social pair bonds. However, this only represents the second published study on parrots demonstrating no EPP and providing evidence for low levels of CBP. More research is needed across a broader range of species in this order with variable breeding habits to more reliably determine the levels of both EPP and CBP in wild populations. Doing so will provide valuable new data to aid comparative analyses of the ecological and life history correlates of social and genetic mating systems in birds.
CHAPTER 6: Non-random mating according to infection:

*Platycercus elegans* males infected with beak and feather disease virus rarely mate

Photo: juvenile (first year) crimson rosella (*Platycercus elegans*) with its distinct green plumage.

Courtesy of Dr Mathew L. Berg
6.1 Abstract

Pathogen transmission dynamics are highly complex with some pathogens able to influence the spread of infection using both direct and indirect modes of transmission. While it might be expected that both transmission modes result in non-random pathogen spread among families (through extended contact), tests in natural populations are limited. Here, we investigated beak and feather disease virus (BFDV) infection and load in family groups among three different subspecies of a south-east Australian parrot (*Platycercus elegans*). We found that it was rare to find BFDV infected males, while infected females were still common in breeding populations and they were often paired with uninfected males. In addition, we observed more non-infected breeding pairs than expected by chance, but cases where both sexes within a pair were infected were observed much less than expected. Interestingly, parental infection status did not explain nestling infection status, suggesting that other infection routes (e.g. from the nest material) are likely in this system. These results could be explained by female choice for non-infected males or alternatively that infected males fail to compete for females. These findings have implications for mitigating BFDV outbreaks in endangered species of Psittaciforme species. More generally, our results suggest that pathogen infection and transmission within families is highly complex with both host ecology and behaviour being important.
6.2 Introduction

Knowledge of pathogen transmission is critical to understanding the epidemiology of disease outbreaks (Woolhouse et al. 1997; Rohani et al. 2009), which in natural populations can have important conservation management implications (Altizer et al. 2003). Pathogens have evolved a variety of transmission modes, both direct and indirect, taking advantage of host ecology and increasing the spread of infection. Indirectly transmitted pathogens that persist in the environment or utilise vectors, will often show spatial variation in transmissibility related to environmental heterogeneity (Hudson 2002; Altizer et al. 2006). However, pathogens that are transmitted directly rely less on spatial variables and are often associated with host-host contact frequency (Bull et al. 2012; MacIntosh et al. 2012), or a particular behavioural interaction (e.g. grooming) with infected individuals within their social group (Drewe 2010; Blyton et al. 2014). Direct and indirect modes of transmission are not always exclusive and for some pathogens, both are likely to be involved in disease outbreaks (Rohani et al. 2009). However, in natural populations there is still a substantial knowledge gap in pathogen transmission dynamics (Blyton et al. 2014). Surprisingly, few studies have considered transmission within family groups, despite the probable importance of this social interaction in pathogen transmission, where both direct and indirect transmission are likely to be maximised (Martinez-Padilla et al. 2012; Blyton et al. 2014).

In many species, breeding pairs exhibit an array of social behaviours that create the opportunity for direct pathogen transmission (Hamede et al. 2009; Drewe 2010; Blyton et al. 2014), for example; copulation or allogrooming. This pair bond relationship is likely to be closer and more frequent than other social interactions with conspecifics, and hence increases pathogen transmission risk (Blyton et al. 2014). Avoiding a pair bond relationship
with infected individuals would therefore seem beneficial to escape pathogen transmission (Borgia & Collis 1990; Able 1996). Particularly, if virulence and transmissibility are high and can affect host survival and reproductive output (Able 1996). This avoidance behaviour characterised by the “contagion indicator hypothesis” can result in non-random infection patterns within pairs (Thomas et al. 1995; Able 1996). In wild animals, few studies have investigated non-random pairing relative to infection status of a directly transmitted pathogen. In Pied flycatchers (Ficedula hypoleuca) louse prevalence and load was correlated between male and female dyads (Potti & Merino 1995). Furthermore, this study and another by Møller (1990), found that the infection status of one or both parents was related to that of their nestlings. Suggesting in the before mentioned systems that avoiding infected potential partners can reduce the infection risk to their offspring.

Non-random pathogen prevalence and load patterns within family groups are still likely for indirectly transmitted pathogens. This mode of transmission relies on host contact with a contaminated environment or a vector. Particularly in monogamous species with bi-parental care of offspring, breeding pairs are likely to spend an extended period of time together in the same spatial area. So they are likely to be exposed to similar pathogens that are indirectly transmitted. Experimental investigation of indirectly transmitted nematodes (Trichostrongylus tenuis) in wild red grouse (Lagopus lagopus scoticus) suggest that male and female parasite loads are correlated, and that males which had parasites removed experienced increased parasite loads if paired with a naturally infected female (Martinez-Padilla et al. 2012). This study indicates that even when the mode of transmission is in indirect, pairing with an infected partner is potentially costly. Other studies have found similar positive associations among breeding pairs and pathogen prevalence (Thomas et al.}
1995; Thomas et al. 1996), and load (Thomas et al. 1995). Non-random breeding pair patterns in respect to infection has also been found for those transmitted by vector (Votýpka et al. 2003). For species that utilize confined microhabitats including dens, burrows or tree hollows, indirect transmission is potentially increased. Adult pairs and their offspring may become infected if pathogens are persisting in these environments (Møller 1990; Leu et al. 2010), again potentially resulting in non-random patterns of infection among family groups.

One example of a well-known but understudied pathogen in natural populations is beak and feather disease virus (BFDV). BFDV is a ssDNA circovirus that only infects the order Psittaciformes (Bassami et al. 2001). The signs of the disease vary among species and between individuals (Raidal & Cross 1995; Doneley 2003), but typically include feather malformation and loss, and beak and claw deformity (Pass & Perry 1984). The virus also replicates in the bursa of Fabricius (Todd 2000; Ortiz-Catedral 2010), an important organ involved in bird immune function (Ratcliff 2011). This suppresses the immune system, leaving infected individuals susceptible to secondary infections and often death (Todd 2000). BFDV has been implicated in several high mortality events worldwide (Heath et al. 2004; Kundu et al. 2012). Hence, it is a concern for Psittaciforme conservation globally and is classified as a key threatening process to biodiversity by the Australian government (Australian Department of the Environment and Heritage 2005).

BFDV is transmitted by contact with bodily excretions from an infected individual such as: faeces, crop secretions, and feather dust (Ritchie et al. 1991a; Rahaus et al. 2008). Furthermore, there is evidence to suggest that BFDV can be transmitted vertically, with the virus being detected within the egg (egg glair and embryo) and in male testes (Rahaus et al. 2008). Infected birds are known to shed large quantities of BFDV into their environment.
(Raidal et al. 1993c). This coupled with evidence to suggest that BFDV can persist for long periods of time (years) suggest that environmental transmission may also be an indirect source of infection (Peters et al. 2014). BFDV has been detected in nesting material previously in captivity (Raidal et al. 1993c), and in the aviary environment (Bert et al. 2005). Similar circoviruses (CynNCXV and CynNCKV) have been found in nesting hollows of wild yellow-crowned parakeets (*Cyanoramphus auriceps*) (Sikorski et al. 2013). These various modes of transmission have been elucidated using captive individuals, but no study has investigated BFDV transmission in wild birds.

*Platycercus elegans* is a common parrot species of south eastern Australia which has received considerable attention due to its reputation as a ‘ring-species’ and its extraordinary phenotypic diversity (Joseph et al. 2008; Ribot et al. 2009; Berg & Bennett 2010; Ribot et al. 2012; Knott et al. 2013). Individuals of this monogamous species (Krebs et al. 1999; Krebs et al. 2002a; but see Chapter 5) typically breed between October and January in naturally occurring tree hollows, with their clutch size ranging between four and eight nestlings (Krebs 1998). Krebs et al. (2002a), note that females but not males begin breeding after their first year, suggesting, that there is some age structure within breeding *P. elegans*. BFDV has previously been shown to be prevalent and widespread in wild populations (Eastwood et al. 2014; Eastwood et al. 2015). Eastwood et al. (2014), found that BFDV varied between the subspecies with *P. e. elegans* and *P. e. flaveolus* having a higher prevalence and viral load than the WS hybrid population and *P. e. adelaidae*. This study also found that date and sex were also found to be important predictors of BFDV prevalence whilst sex and age were found to be important in predicting viral load. First year sub-adult birds were found to have the highest viral load which decreased with age.
Here, we collected BFDV infection data (prevalence and load) of both breeding pairs and their offspring in three different subspecies in the *P. elegans* species complex (*P. e. elegans, P. e. adelaidae* and WS hybrid). We had three primary aims: (1) determine if there are non-random associations between the infection status of males and females paired together, (2) determine if there are non-random associations between the infection load of males and females paired together, and (3) determine if parental infection status and viral load influence that of their offspring. Investigating non-random associations among families may explicate transmission pathways both direct and indirect in little known pathogens in wild animals.
6.3 Materials and methods

6.3.1 Study area and sample collection

Using a method outlined in Berg and Ribot (2008), we used nest boxes to trap breeding pairs of *P. elegans* and their nestlings. During the years 2004-2006 birds were caught in South Australia at nine different sites which covered the distribution of the *P. e. adelaidae* subspecies: Carey Gully (S34°58', E138°45'), Marne River (S34°39', E139°23'), Sevenhill (S33°54' E138°36'), Currency creek (S35°25', E138°45'), Quorn (S32°17', E138°0') and Yulti Wirra (S35°37', E138°11'). At other sites including Carey Gully, Mylor (S 34°59', E 138°47'), Oakbank (S 34°59', E 138°50) and Crafers (S 35°0', E 138°42') we mist netted birds, and collected those that were culled as pests (Ribot *et al.* 2011; Eastwood *et al.* 2015). This was done before and after the breeding season to provide an estimate of prevalence in the *P. e. adelaidae* subspecies. We captured *P. e. elegans* at two nest box sites in Victoria, Bellbrae (S 38°19', E 144°11'), and Steiglitz (S 37°52', E 144°11'), during 2010-2011. Again, birds were mist netted and caught from these two sites using baited walk-in traps outside the breeding season. At the WS hybrid site, only breeding birds captured in nest boxes were used during the years 2004-2011. Sites included Barnawartha (S36°3', E146°45'), Bonegilla (S36°9', E146°59'), Brungle (S35°11', E148°12'), Tangambalanga (S36°11', E147°0'), Moyhu (S36°35', E146°24') and Mundarlo (S35°4', E147°51'). In all 510 birds, we sampled blood from the brachial vein which was stored immediately in ethanol and used for DNA extraction; when dead birds were collected a small amount of muscle tissue was used instead.

Nestlings in this study are defined as individuals remaining inside the nest box; adults are aged based on distinct plumage characteristics. Green plumage denoted sub-adults (1-2 years), while young adults (3-4 years) were identified by a white stripe underneath the wing.
which is completely lost after 5 years of age, when they are classified as old adults (Forshaw & Cooper 2002).

6.3.2 BFDV detection and sex identification

An ammonium acetate DNA extraction method for avian blood was used for all samples, adapted from a Sheffield University protocol based on (Bruford et al. 1998). To ensure high quality DNA was extracted and to quantitate the concentration of DNA, we used a Beckman DU spectrophotometer (Beckman Coulter, CA, USA), as per the instruments instructions. A further indication of DNA extraction success and to determine the sex of each individual we used a common molecular sexing method based on Griffiths et al. (1998). To detect BFDV infection and viral load we used a newly developed probe based quantitative real-time PCR technique, which has previously been used in P. elegans (Eastwood et al. 2014; Eastwood et al. 2015).

6.3.3 Statistical analysis

A chi-square test was used to investigate deviations from expected frequencies of breeding pairs and their infection status. Pairs were categorised as both infected, neither infected, male only infected, or female only infected. Using known proportions of males and females infected with BFDV in the respective populations, we calculated probabilities for each category and multiplied by the total number of pairs to generate the expected. We first pooled all breeding pairs together for analysis, but then separated P. e. adelaidae, P. e. elegans and WS hybrid birds to account for different populations. In these analyses, proportions were calculated using both birds caught during breeding, and those caught outside the breeding season, to better represent population prevalence and not just
breeding birds. We also conducted a separate analysis, calculating expected values using only prevalence from breeding birds, to account for a bias in potential seasonal variation. When expected values were less than five in any of the cells, Fisher’s Exact Test for association was used instead (Quinn & Keough 2002). To test for differences in viral load between all males and females sampled a T-test (two tailed) was used. Spearman’s Correlation Coefficient was used to test for assortative mating to viral load, or the possibility that infected individuals alter mate viral load.

To investigate parental infection status and BFDV transmission to nestlings a 4x2 contingency table was used to calculate expected values (both parents infected, neither parent infected, male only infected and female only infected x nestlings infected, nestlings not infected). All nestlings were considered positive if at least one nestling tested positive.
6.4 Results

6.4.1 Prevalence and viral load of BFDV within age groups and sexes

Within adult age classes BFDV prevalence in males and females was 28.3% (± 7.2, 95% CI) and 30.8% (± 7.2, 95% CI), respectively. Neither BFDV prevalence ($\chi^2 = 0.24, \text{ d. f.} = 1, P = 0.24$) nor BFDV viral load ($T$ test; $T = 0.71, \text{ d. f.} = 90, P = 0.48$, standard error = 0.26) was significantly different between the sexes. However, within breeding birds only, male (8.7% ± 5.4, 95% CI) and female (25% ± 7.4, 95% CI) prevalence was significantly different ($\chi^2 = 10.62, \text{ d. f.} = 1, P = 0.001$). Prevalence in nestlings (4.0% ± 1.7, 95% CI), sub-adult (66.2% ± 11.5, 95% CI), young adult (25.2% ± 7.9, 95% CI) and old adult (16.4% ± 6.3 95% CI), varied significantly ($\chi^2 = 178.02, \text{ d. f.} = 3, P < 0.001$). Prevalence between the three adult age classes also varied significantly ($\chi^2 = 37.5, \text{ d. f.} = 3, P < 0.001$), (Figure 6.1). Sub-adults had a significantly higher viral load (Mean ± SE: nestlings = 26.42 ± 26.40, sub-adults = 124.62 ± 51.57, young adult = 0.04 ± 0.02, old adult = 0.06 ± 0.02) than all other age classes (Kruskal-Wallis; $H = 23.9, \text{ d. f.} = 3, P < 0.001$).
Figure 6.1. Percentage prevalence of BFDV across age classes (white bars) and percentage of each age group breeding (black bars): both are shown separately for males (left panel) and females (right panel). BFDV prevalence was calculated using both breeding and non-breeding birds and only included breeding age classes (excluding nestlings).

6.4.2 Non-random infection within breeding pairs

Pooling data from across all subspecies and using the calculated proportions of male and female infection within this dataset (N = 93 pairs), we observed a much lower than expected frequency of infected males paired with non-infected females (χ² = 22.63, d. f. = 3, P < 0.001). In addition, breeding pairs with neither sex infected was observed more frequently than expected, but we observed that infected pairs (both sexes) were less than expected. Females were found to breed despite being infected (Figure 6.2a). These patterns are consistent when considering subspecies and WS hybrid populations separately in the
analysis (Figure 6.2b, Figure 6.2c, and Figure 6.2d). The *P. e. adelaidae* population data (*N* = 40 pairs) suggests that this pattern is significant (Fisher’s Exact Test: *P* = 0.019). Fisher’s exact test was used as some groups had expected values that were less than five (Figure 6.2b). The WS hybrid population (*N* = 38 pairs) and *P. e. elegans* (*N* = 15 pairs), despite showing the same pattern as the pooled data set and the *P. e. adelaidae* population (Figure 6.2c and Figure 6.2d respectively) show a non-significant result (Fisher’s Exact Test: *P* = 0.69) and (Fisher’s Exact Test: *P* = 0.49) respectively.

To eliminate the potential influence of age related breeding patterns (e.g. a low proportion of sub-adult males) and the higher BFDV prevalence of sub-adults, we excluded sub-adults from the analysis. Again, in all populations, the same pattern occurred with a greater than expected frequency of uninfected pairs and less than expected frequency of pairs consisting of male infected/female not infected. This association was significant in the pooled data set (Fisher’s Exact Test: *P* = 0.003, *N* = 67 pairs) and marginally non-significant in the *P. e. adelaidae* population (Fisher’s Exact Test: *P* = 0.07, *N* = 30 pairs). However, infection patterns in the WS hybrid population was again random (Fisher’s Exact Test: *P* = 0.195) but the same pattern as above was present. The sample size when excluding sub-adults from the *P. e. elegans* data set was too small (*N* = 8 pairs) to draw any conclusions.

We repeated our analysis using sex prevalence data from breeding birds only. We found no significant difference between observed and expected pair infection status groups (Fisher’s Exact Test: *P* = 0.98), suggesting infection among pairs could be random. When considering each population separately we again found no significant patterns (*P. e. elegans*: Fisher’s Exact Test *P* = 0.917, *P. e. adelaidae*: Fisher’s Exact Test *P* = 0.964, WS hybrid: Fisher’s Exact Test *P* = 0.999).
Figure 6.2. Observed and expected frequencies of BFDV infected and non-infected males and females within *P. elegans* breeding pairs. The white bars represent the expected frequency of breeding pairs within a particular group. Black bars represent the observed frequency of breeding pairs within a particular group. (A) shows data from all subspecies pooled together, (B) shows data from *P. e. adelaidae*, (C) shows data from WS hybrid population, and (D) shows data from *P. e. elegans*. 
6.4.3 BFDV viral load within breeding pairs

We found two breeding pairs where both the male and female were infected with BFDV out of a total of 93 pairs. We therefore could not test our hypothesis that male and female infection load would be similar within breeding pairs.

6.4.4 Parental and nestlings infection status

We screened a total of 74 breeding pairs with nestlings (N = 324 nestlings). No cases of both parents infected and chicks infected were observed during the breeding seasons sampled, so this group was omitted from the analysis. Groups defining infected males or females were reclassified as one parent infected, to increase the sample size. There was no difference between the observed frequencies of each defined group and what was expected ($\chi^2 = 0.2$, d. f. = 4, $P = 0.99$; Figure 6.3). However, we observed more frequently cases where neither parent was infected with nestlings uninfected (Figure 6.3). We also observed seven cases where one parent was infected but no nestlings within their brood were infected (Figure 6.3). In six cases, nestlings were also found to be infected with BFDV despite both parents testing negative (Figure 6.3). Groups with both parents infected with nestlings not infected, and one parent infected with nestlings infected, occurred rarely (Figure 6.3).
Figure 6.3. Observed and expected frequencies of parental and nestling infection status within *P. elegans*. The white bars represent the expected frequency within a particular group. Black bars represent the observed frequency within a particular group. + indicates the parents or nestlings tested positive for BFDV. - indicates the parents or nestlings tested negative for BFDV.
6.4.5 Age differences in breeding

Including data from all three populations sampled (WS hybrid, *P. e. adelaidae* and *P. e. elegans*), we found evidence that males and females vary in age related mating patterns (Figure 6.1). Females began to breed as sub-adults but the most frequent age group found breeding was young adults. Interestingly, females were never found to pair with a male younger than themselves. Old-adult males were more frequently found breeding, while sub-adult males were the least frequent (Figure 6.1).
6.5 Discussion

Our results show that the occurrence of BFDV infection within breeding pairs was non-random. We observed lower than expected frequencies of breeding pairs that consisted of an infected male and a non-infected female, which was significant in our overall dataset and in the *P. e. adelaidae* population. BFDV infected females were still found breeding and at expected levels. In addition, non-infected breeding pairs were considerably more frequent than expected, while cases where both male and female were infected were less frequent than expected. The occurrence of BFDV infection among breeding pairs was similar for *P. e. elegans* and WS hybrid populations, however, these findings were not significant (*P < 0.05*). This variation could be due to the low statistical power caused by the low sample sizes from these individual populations. However, we cannot fully exclude the possibility that the spread of infection among breeding pairs does vary between populations. BFDV load within breeding pairs could not be assessed, because we found only two cases where both the male and female were infected. Interestingly, the observed distribution of BFDV among nestling birds was not related to parental infection status as predicted for a directly transmitted pathogen. This study provides insight into the complicated transmission and perhaps behavioural dynamics explaining BFDV infection within family groups.

To account for the observed age variation in BFDV infection (both prevalence and load was higher in subadults and decreased with age) and the different breeding age patterns between males and females (females breed as subadults but breeding subadult males are rare (Krebs *et al.* 2002a)), we repeated our analysis excluding subadults. The findings were consistent, indicating that age is not a confounding variable. A further confound was due to potential seasonal variation in BFDV prevalence (Eastwood *et al.* 2014),
that may skew sex prevalence estimates during the breeding season. We repeated our analysis using prevalence estimates from breeding birds only, which resulted in the same observed group frequency distribution patterns as above. However, males during the breeding season had a much lower prevalence than females which likely contributed to the non-significance of this finding. We note that lower male prevalence during the breeding season is probably due to the finding that infected males rarely breed, in addition, previous work has shown that males could in fact be more susceptible to infection (Eastwood et al. 2014).

6.5.1 Why infected males rarely breed?

In this study, BFDV infected males did not often pair with non-infected females. This finding and the low number of pairs where both sexes were positive, strongly suggest that infected males rarely breed. Two mechanisms, which are not mutually exclusive, may explain these results. First, females could choose males that were not infected with BFDV, hence, avoiding direct transmission of the virus to themselves, and potentially their nestlings (Able 1996). Moreover, infected females may avoid infected partners to prevent further exposure to the virus (Martinez-Padilla et al. 2012). However, the rarity of finding infected pairs prevented us from testing this hypothesis. Infected males may also be less capable of performing parental care activities, which may be an indirect cost to females which pair with infected males (Ilmonen et al. 2000). Female preference for non-infected males may also benefit reproductive output through offspring inheriting resistance genes (Clayton 1991). A second hypothesis to explain why few BFDV infected males breed is that they are less capable of obtaining and/or retaining a mate (Schall & Dearing 1987; Galipaud et al. 2011). Infected males conceivably have fewer resources to dedicate to particular traits that are important
for obtaining a mate (Able 1996). Alternatively, male traits may have evolved to allow females to identify parasites and therefore able to avoid infected males (Borgia & Collis 1990). In addition, behavioural traits may also be negatively affected by pathogens, including, male-male completion (Schall & Dearing 1987; Galipaud et al. 2011), reduced willingness to pair (Bierbower & Sparkes 2007), or a reduced capacity to defend mates from other males. Females may also have an increased propensity to “divorce” infected males (Bull & Burzacott 2006), which might also contribute to our observations.

Given that BFDV is transmitted via contact it was surprising to find many cases where one partner was infected but not the other. However, this interaction is complex and other important factors such as acquired immunity may have a role in explaining pathogen occurrence within families (Woolhouse et al. 1997). Furthermore, recent evidence has suggested that close social interactions, including pair bonding, may not always explain pathogen transmission (Blyton et al. 2014). But perhaps different social encounters or indirect transmission paths may also be important (Rohani et al. 2009; Drewe 2010; MacIntosh et al. 2012; Blyton et al. 2014).

6.5.2 Indirect transmission of BFDV?

We found that the relationship between parental and nestling infection status was random. Interestingly, nestlings were found to be infected despite both parents testing negative, suggesting an indirect mode of transmission. The most likely explanation for this finding is that nestlings are becoming infected from nesting material. BFDV is thought to persist in the environment for long periods of time and has been found before in nesting material (Raidal et al. 1993c; Sikorski et al. 2013). This suggests that nest hollows, or in this case nest boxes, could be an important source of BFDV infection in wild Psittaciforme species (Peters et al.
A secondary explanation is that BFDV can be transmitted through a vector, however, there is currently no evidence to support this hypothesis. In addition, we found that nestlings can test negative for BFDV despite one or both parents testing positive. One possibility is that nestlings won’t become infected even if they are exposed by their parents. However it is also conceivable that BFDV may not be replicating until later or perhaps after the nestling period (approximately 28 days) because of a large viral latency period (Bonne et al. 2009), therefore, making it difficult to detect at low levels.

6.5.3 BFDV infection and breeding pair frequency patterns between the age classes

This study supports findings from other studies investigating age related BFDV patterns, which suggest sub-adults have a higher prevalence (Bert et al. 2005) and load (Eastwood et al. 2014), which decreases with age. The reason for this age effect is unknown, but one possibility is that infected individuals develop an acquired immune response and recover from the infection (Paré & Robert 2007). Alternatively, BFDV related mortality could explain the lower prevalence and viral load in adult age classes. Sub-adults may have a larger proportion of susceptible individuals within their class, which are subsequently removed leaving only recovered individuals and those yet to be infected. Hence, infected individuals become rarer in the population with age (Gregory et al. 1992; van Oers et al. 2010).

A qualitative comparison between the percentage of age classes found breeding and the percentage of those age groups infected showed that male sub-adults had both the highest BFDV prevalence and the lowest percentage breeding. However, male old adults had the lowest BFDV prevalence, but were paired more often with a female. This could potentially indicate that females are choosing older mates because they are less likely to be infected (Garamszegi et al. 2005). But testing the causality between age of breeding and
infection status may be difficult, particularly as other traits that older males may display and are preferred by females could confound any such study (Kokko & Lindstrom 1996; Brooks & Kemp 2001). Females were never found to mate with a male younger than themselves, with young adult females the most predominant. This may be representative of the most common age class in the population, and therefore the most likely to find in breeding populations.

6.5.4 Conclusion

Overall, our study demonstrates that BFDV infection among breeding pairs of *P. elegans* is non-random. Pairs that consisted of both male and female infected and male only infected were observed at a lower frequency than expected, even when accounting for age and seasonal variation in prevalence. Non-infected pairs were the most frequent and above expected levels. These results suggest that females still commonly breed when infected with BFDV but that infected males rarely pair. Additionally, we found that the occurrence of infection among nestlings was random according to their parent’s infection status, and that there is evidence BFDV can be indirectly transmitted via nest material. Our study indicates that in wild *P. elegans* direct transmission is not the only factor explaining the spread of infection among family groups, and that indirect modes of transmission combined with host behaviours and acquired immunity also contributes to the observed patterns.
CHAPTER 7: Discussion

Photo: Western Slopes hybrid rosella.

Courtesy of Dr Mathew L. Berg
7.1 Summary

In this thesis, the ecology and evolution of beak and feather disease virus (BFDV) was investigated within the *Platycercus elegans* species complex. Hitherto, most knowledge on BFDV had originated from the study of captive Psittaciforme species. The few studies in wild Psittaciforme populations clearly indicate the detrimental capacity this virus can have on conservation efforts (Ortiz-Catedral et al. 2009; Kundu et al. 2012; Peters et al. 2014). Given that Psittaciformes are one of the most threatened orders of birds, BFDV is outlined by the Australian Government as a “key threatening process to biodiversity”. A key priority to help mitigate the effects of BFDV on wild psittacines has been to conduct research on the ecology of BFDV in the wild (Australian Department of the Environment and Heritage 2005). The paucity of BFDV data in wild birds was addressed by using the common parrot species *P. elegans* of south eastern Australia. This study utilised samples collected across eight years in the four main subspecies: *P. e. elegans*, *P. e. flaveolus*, *P. e. adelaidae*, and the WS hybrid (*P. e. elegans* × *P. e. flaveolus*). Then, using a quantitative real-time PCR method reported in chapter 2 (Eastwood et al. 2015), BFDV viral prevalence and load was determined. The *P. elegans* species complex consists of variable populations that are phenotypically and genetically distinct, hybridise, and exist in variable environments (Forshaw & Cooper 2002; Joseph et al. 2008). So creating a ‘natural laboratory’ for elucidating factors important for determining the spread of infection, and the evolutionary implications of this host-pathogen relationship (Hewitt 1988).

In chapter 2, now published as Eastwood et al. (2015), the first aim was to develop and validate a quantitative real-time PCR method in *P. elegans* for the detection of BFDV. The second aim was to then use this method to assess the similarity between three distinct
avian sample types: blood, muscle tissue and feathers. Feather samples provided discordant results concerning virus presence when compared with muscle tissue and blood. Viral load estimates varied between different sample types, although this result was non-significant. Overall, the work reported in chapter 2 suggests that BFDV is common in wild *P. elegans*, and highlights the importance of validating sample types when generating and interpreting, qualitative and quantitative avian virus data.

In chapter 3, now published as Eastwood *et al.* (2014), the first aim was to determine what factors predict BFDV prevalence and load. Using an all subset model selection approach, prevalence was found to be predicted by a model consisting of the terms subspecies, Julian date, host sex, and an interaction between subspecies and Julian date. BFDV load was found to be predicted by a model consisting of the terms subspecies, host age, host sex, and an interaction between subspecies and age. The main finding was that WS hybrid birds and the phenotypically intermediate *P. e. adelaidae* were found to have a much lower BFDV prevalence and load compared to the ‘terminal’ subspecies (*P. e. elegans* and *P. e. flaveolus*). There are several mechanisms hypothesised to explain this pattern, first, WS hybrid and *P. e. adelaidae* birds could have been more resistant to BFDV than *P. e. elegans* and *P. e. flaveolus* (hybrid resistance hypothesis), a conclusion consistent with evidence for BFDV clearance with age. Second, WS hybrid and *P. e. adelaidae* birds could alternatively be more susceptible to infection, because these findings are also consistent with higher levels of BFDV induced mortality (hybrid susceptibility hypothesis). Third, the force of infection may vary between the subspecies and WS hybrid populations (force of infection hypothesis). Although the latter hypothesis cannot be fully rejected, there was no evidence to indicate that BFDV exposure varied between the subspecies, because BFDV was not associated with
geographic location, intraspecific host density, BFDV susceptible community diversity and composition, nor was there variation in BFDV seroprevalence. Overall, my results in chapter 3 suggest that the evolved host response and/or the force of infection are key determinants in influencing the spread of infection in wild animals. Furthermore, it appears that BFDV could have important consequences for host speciation by influencing selection across the intermediate subspecies (P. e. adelaidae) and the WS hybrid populations.

The second aim of chapter 3 was to investigate how BFDV isolates differ across a divergent and hybridising species complex and to also test the ring-species concept. BFDV isolates were found to be associated with subspecies, with isolates from P. e. flaveolus and P. e. elegans forming two clear groups. BFDV isolates from WS hybrids were found to be at the distal end of the phylogeny and these were closely related to those from P. e. elegans. P. e. adelaidae BFDV isolates were spread evenly between the P. e. elegans and P. e. flaveolus groups. This BFDV phylogeographic pattern is consistent with the ring-species hypothesis with the three main predictions supported: (i) WS hybrid BFDV isolates were the most genetically distinct from P. e. flaveolus BFDV isolates despite the likelihood of sympatry; (ii) there was no evidence of BFDV gene flow across the WS hybrid contact zone (i.e. no recombination); and (iii) BFDV isolates from P. e. adelaidae were distributed evenly between P. e. elegans and P. e. flaveolus, with evidence of isolation-by-distance throughout the ring. These results are the first to suggest that a quasi-species pathogen can have phylogeographic properties analogous to a ring-species.

In chapter 4, the role genetic diversity plays in determining the spread of infection was investigated. This study aimed to determine genetic diversity at the individual and subspecies level, and to test whether genetic diversity is associated with BFDV prevalence
and load. More broadly, this study aimed to determine the relative importance of genotype rarity and heterozygosity in predicting infection. In chapter 4, I showed for the first time that individual heterozygosity and average pairwise relatedness (genotype rarity) predict virus prevalence and load respectively. This finding was consistent within all *P. elegans* subspecies and hybrid populations. Whilst genetic diversity did vary between subspecies using a number of measures (homozygosity-by-loci, mutational divergence, rarity, and allelic diversity), there was no clear qualitative pattern with respect to BFDV prevalence at the population level. In chapter 4, I also investigated the hypothesis in which the host and pathogen are antagonistically coevolving, by testing for a correlation between host genetic distance and BFDV genetic distance. However, there appeared to be no significant correlation between host-pathogen genetic distances. Overall, my results in chapter 4 suggest that pathogens may play a fundamental role in the evolution of avian species by selecting against susceptible individuals within each population.

The findings in chapter 4 that suggest genetic diversity is important in BFDV susceptibility could potentially be relevant at the brood level. Therefore in chapter 5, the potential for infected females to increase their fitness by increasing brood genetic diversity through extra-pair paternity was explored. In this way, extra-pair paternity may be a successful breeding strategy to increase reproductive success during a disease outbreak (Johnsen *et al.* 2000; Foerster *et al.* 2003; Soper *et al.* 2014). The first aim of this study was to determine the level of extra-pair paternity or conspecific brood parasitism in *P. elegans*. Given there were no cases of extra-pair offspring within 43 broods, there was no capacity to test for an association between extra-pair paternity and infection. However, there was a single case of conspecific brood parasitism. A second aim of this study was to test whether
social mate fidelity continues across breeding seasons, and whether breeding pairs maintain nest site fidelity across years. Over a period of eight years, re-capture data showed that 32% of breeding birds paired with a different partner. In addition, 84% of breeding birds were found to change nest boxes at least once, albeit the distance between boxes was small. The finding of low or even absent extra-pair offspring is consistent with previous studies in long lived and socially monogamous psittacine species. However, these results challenge the notion that all parrot species form long term pair bonds.

In chapter 6, the spread of BFDV infection (prevalence and load) among breeding pairs and their nestlings was determined, in three *P. elegans* subspecies. Specifically, I tested if the infection status of one mate influenced that of their partner. In addition, I tested whether parental infection status influenced the infection status of their nestlings. I found few BFDV infected males breeding, whilst infected females were commonly observed paired with uninfected males. In addition, non-infected breeding pairs were observed more often than expected by chance. But it was rare to find breeding pairs where both sexes were infected. Interestingly, parental infection status did not explain nestling infection status, suggesting that other infection routes (e.g. from the nest material in the hollow) are likely to feature in this system. These findings have implications for mitigating BFDV outbreaks in endangered Psittaciforme species. More generally, they suggest that pathogen infection and transmission within families is highly complex with host ecology, immunity and behaviour all playing a role.
7.2 BFDV in the *Platycercus elegans* species complex

7.2.1 BFDV in a wild population

The most important host factor in determining both BFDV prevalence and viral load in wild *P. elegans* was subspecies. Differences in BFDV infection between Psittaciforme species has been noted previously (Bert *et al.* 2005; Shearer *et al.* 2008a; Peters *et al.* 2014), but never at a subspecies taxonomic level. Cockatoo species are noted as particularly susceptible to BFDV because they often present clear signs of disease, feather malformation and loss, beak and claw deformities (Peters *et al.* 2014), high BFDV seroprevalence and BFDV PCR prevalence (McOrist *et al.* 1984; Raidal *et al.* 1993b; Bert *et al.* 2005). In contrast, cockatiels (*Nymphicus hollandicus*), for example, seem to be relatively less susceptible to BFDV as they are only occasionally found with the virus (Shearer *et al.* 2008a). Although there were marked differences in BFDV infection between the subspecies, no birds throughout the study were found to have any signs of disease. As such, there is currently no data on the pathogenesis of this virus in wild *P. elegans*, and subsequently the mechanism behind this variation in subspecies infection dynamics is still a mystery. In finding that there were no ecological predictors of infection and no seroprevalence variation between populations, one could suggest that BFDV exposure does not vary between populations, or alternatively, differences in exposure do not influence population level BFDV prevalence and load.

Genetic diversity could play a role in explaining subspecies variation in pathogen susceptibility (reviewed in, Schmid-Hempel 2011). One hypothesis to explain the observed lower prevalence and viral load in the WS hybrid and intermediate *P. e. adelaidae* was that these populations are possibly more genetically diverse. However, I found that genetic diversity was not associated at the population level, but there were some interesting effects
at the individual level (chapter 4). Heterozygosity was found to be associated with infection risk, with more infected individuals typically having a lower heterozygosity. In contrast, BFDV viral load was associated with genotype rarity, suggesting that once infected, rare genotypes have a lower severity of infection. These findings suggest that hybridisation may reduce pathogen susceptibility, but also highlight potential implications for mitigating the effects of BFDV on endangered species.

The factors that determine infection in wild populations are often complex, but it is well known that host demography is important (Wilson et al. 2002). First year birds are typically found to display clear BFDV disease signs in captivity (Paré & Robert 2007). In chapter 3, the prevalence of BFDV was not associated with age, and older birds (> 5 years) were still sometimes infected. Although in chapter 6, age group was found to be associated with prevalence, chapter 3 is more robust because the sample sizes were much larger. BFDV viral load was found to be much higher in *P. elegans* sub-adult birds, which may be consistent with the higher proportion of younger birds displaying signs of disease in captivity. Nestlings had a low prevalence (chapter 6), but this could be due to the viruses long latency period (Raidal et al. 1993c; Bonne et al. 2009). Instead of age related effects on prevalence in chapter 3, Julian date was found to be an important predictor in the final model. This finding could be indicating that prevalence is cyclic within years. More evidence is needed to completely separate age and Julian date effects, and the possibility that this Julian date pattern differs between subspecies. Interestingly, chapter 4 suggests that BFDV load may also be predicted by date and date squared (non-linear), but a caveat here is that the sample size in this study was much smaller.
In many host-pathogen systems, host sex is important in determining the severity of infection (Poulin 1996; Zuk & McKean 1996; Wilson et al. 2002). Previously, there has been no suggestion that BFDV is sex biased (Rahaus & Wolff 2003), but in both chapter 3 and 4 I found that sex was an important predictor of both BFDV prevalence and viral load. Males are typically more susceptible to infectious disease (Poulin 1996) and my results are concordant with this pattern. The mechanism behind male biased infection patterns could be due to male specific immunosuppressive hormones such as testosterone (Peters 2000), or potentially differences in behaviour and life-history (Zuk & McKean 1996). However, in chapter 6, there was no difference in prevalence between the sexes, except when considering breeding birds alone, in which case, females had the highest prevalence. Female biased infection in this instance appears to be driven by infected males failing to pair with a mate (chapter 6). There are two hypothesised explanations for the lack of breeding infected males. First, infected males may be less capable of competing for females, and therefore breed less often (Schall & Dearing 1987; Galipaud et al. 2011). Second, females may choose non-infected males for a number of potential benefits, including good genes for increased immunity or better paternal care for offspring (Zuk et al. 1990; Ehman & Scott 2002). Chapter 6 found that BFDV transmission within breeding pairs and their nestlings was not certain. Interestingly, nestlings were found to be infected despite having uninfected parents, suggesting alternative routes for BFDV transmission including fomites or more likely, indirect transmission from the environment. Previous studies have suggested that nesting cavities could be a major BFDV transmission pathway (Raidal et al. 1993c; Sikorski et al. 2013), and my work supports that conclusion.
7.2.2 Is BFDV a key threat to biodiversity?

The order Psittaciformes represents one of the most endangered avian orders with 27% of species listed between vulnerable to critically endangered (IUCN 2014). Orange-bellied parrots (*Neophema chrysogaster*) are one example of a critically endangered species in Australia, with fewer than 50 individuals remaining in the wild (IUCN 2014; Sarker *et al.* 2014b). It is likely that severe inbreeding depression has occurred in this species reducing genetic diversity. If the findings from chapter 4 are general across species, the Orange-bellied parrot and other threatened species could be extremely susceptible to an outbreak of BFDV. Previously in several endangered species, inbreeding depression has been demonstrated to lower genetic diversity (e.g. heterozygosity), and increase the risk of infection (Cassinello *et al.* 2001; Hedrick *et al.* 2001; Spielman *et al.* 2004; Ross-Gillespie *et al.* 2007; Charpentier *et al.* 2008). An additional concern for Australian Psittaciformes was the finding that BFDV is prevalent, widespread, and persistent across years within *P. elegans*, suggesting that this species may act as a viral reservoir. Endangered species may be at risk of BFDV infection because reservoirs of infection may increase the risk of transmission and the force of infection (Jackson *et al.* 2014; Sarker *et al.* 2014b). Species that are in decline are particularly susceptible if BFDV strains are infective across different genera, which has been suggested in other studies combining captive and wild caught birds (Peters *et al.* 2014; Sarker *et al.* 2014a; Sarker *et al.* 2014b). In contrast, the data in this study suggest that BFDV isolates are somewhat associated with host species, and those found in *P. elegans* are unique, apart from an isolate obtained from a captive glossy black cockatoo (*Calyptorhynchus lathami*) (chapter 2). This infected glossy black cockatoo was a captive bird that may have been held in close proximity to *P. elegans*. Viral persistence within nesting
cavities could be an important transmission route (chapter 6). This would present an additional conservation challenge, because most Psittaciforme species including *P. elegans*, utilize tree hollows for breeding. Taken together my study indirectly supports previous conclusions that BFDV is a considerable threat to already endangered Psittaciforme species.

### 7.2.3 Possible role for BFDV in host speciation?

In the *P. elegans* circular overlapping species complex, BFDV prevalence and viral load was consistently lower in the WS hybrid and *P. e. adelaidae* populations. This finding has important implications for models of speciation and for the maintenance of phenotypic and genotypic diversity in the *P. elegans* species complex. By contrast, similar studies investigating pathogens across a hybridising species complex often find that hybrids are over-infected (Wolinska et al. 2008). The hybrid zone between *Mus musculus* and *M. domesticus* represent a classic example of hybrid susceptibility (Moulia & Joly 2009; Baird et al. 2012). Sage et al. (1986), sampled across this hybrid zone for nematodes and cestodes, and found increased infection loads within hybrids. This finding was confirmed in a separate transect across the hybrid zone (Moulia et al. 1991), and experimentally (Moulia et al. 1993). Those studies suggested that pathogens could be selecting against hybridisation between *M. musculus* and *M. domesticus*, and therefore blocking gene flow between the parental species, despite secondary contact. Here in *P. elegans*, a lower prevalence and load within hybrid and intermediate phenotypes could suggest that these populations are in fact more resistant. Subsequently, hybridisation could be advantageous against pathogen infection and could prevent speciation by mediating gene flow between the most divergent subspecies, *P. e. elegans* and *P. e. flaveolus*. Hybrid resistance is relatively rare in animals and is known to vary according to year and the particular pathogen (Jackson & Tinsley 2003; Joly et al. 2008;
Wolinska et al. 2008; Planade et al. 2009). Recently, a re-examination of the wormy mice hybrid zone found that hybrids were now under-infected when previously they were over-infected (Baird et al. 2012). This is consistent with Red Queen type dynamics where susceptibility fluctuates between populations through time. In such scenarios, pathogen selection may not have a significant role in population divergence (Baird et al. 2012). In P. elegans lower infection patterns in WS hybrid and P. e. adelaidae appear to be consistent, at least across the eight years of this study (chapter 3). However, as the pathogenesis of BFDV infection in the wild is not well understood, there is doubt as to what exactly a low prevalence and load reflects. Therefore, an alternative explanation for a lower prevalence and viral load is that WS hybrid and P. e. adelaidae are more susceptible and suffer a higher BFDV induced mortality rate, whilst in the in the terminal subspecies (P. e. elegans and P. e. flaveolus) a high prevalence and load could indicate tolerance. In a hybrid susceptibility scenario BFDV could act as a barrier to gene flow supporting divergence between the terminal subspecies. Further investigation needs to be carried out on P. elegans to separate either hybrid resistance or susceptibility hypotheses, and to detail the specific mechanisms involved.

One possible mechanism that could explain a lower prevalence and load within hybrid and intermediate subspecies is genetic diversity (Lively 2010). In chapter 4, BFDV infected individuals typically had a lower heterozygosity compared to non-infected birds. This is consistent with other studies that show similar patterns (Acevedo-Whitehouse et al. 2003; MacDougall-Shackleton et al. 2005; Ortego et al. 2007a; Townsend et al. 2009; Isomursu et al. 2012), and more broadly demonstrate that more homozygous or inbred individuals are at a disadvantage. This still appears to be the case in outbred vertebrate
species, including raccoons (Gompper et al. 2011), and is confirmed in P. elegans (chapter 4). Interestingly, genotypic rarity was positively associated with BFDV load in all P. elegans subspecies. The finding that heterozygosity and genotype rarity predict different aspects of a viral infection at the individual level, suggest contrasting evolutionary dynamics between host genetic diversity and BFDV infection. In this instance, susceptibility could be driven by heterozygosity, but the severity of an infection could be under negative frequency-dependent selection against common host genotypes. The results in chapter 4 may have implications for mate choice, with a potential conflict arising between heterozygosity and genotype rarity (Roberts et al. 2006). Overall, genetic diversity appears to be important in susceptibility to disease and therefore has important implications for host-pathogen evolution.

Given that chapter 4 demonstrated that common host genotypes typically had a higher viral load, it was hypothesised that this finding could be due to viral tracking of the most common host genotypes. Therefore, in this study it was expected that host genetic distance and BFDV genetic distance would be correlated. Such a finding was not found in chapter 4, and would suggest that there is no host-pathogen local adaptation. However, the lack of association between host-pathogen genetic distances is discordant with the findings in chapter 3, which suggest that BFDV isolates are associated with particular subspecies. A similar scenario has been found before in the Daphnia magna and Pasteuria ramose system (Ebert et al. 1998), where population variation in resistance was not associated with population local adaptation. Ebert et al. (1998) suggested that their discordant results could be because their study was over a large spatial scale (Gandon et al. 2008).
Genetic diversity at a subspecies level was not associated with *P. elegans* subspecies BFDV prevalence and viral load. But, using most measures, *P. e. elegans* was the most genetically diverse population compared to all other populations which had similar levels of genetic diversity. In this study, populations were divided into subspecies for *a priori* reasons relating to previous work done in chapter 3 and in Joseph *et al.* (2008). However, this subspecies level genetic diversity approach did not show any patterns with BFDV infection, but instead these results could reflect the broader range of selective forces or differences in population size (Frankham 1996).

### 7.2.4 Platycercus elegans and the ring-species concept

Mayr’s ring-species concept proposed that speciation by distance could occur around a geographic barrier (Mayr 1942). Therefore, complete geographic isolation was not always necessary for speciation to occur. The ring-species hypothesis has three main predictions. First, they consist of two terminal forms that encircle a geographic barrier, via a series of intermediate forms with on-going gene flow. Second, there is isolation-by-distance around the ring from one terminal population to the next. Third, there is reproductive isolation where the two terminal forms meet (Irwin *et al.* 2001a). The classic example to demonstrate the key ring-species predictions and the best studied system is the greenish warbler (*Phylloscopus trochiloides*) species complex (Irwin 2000; Irwin *et al.* 2001b; Irwin *et al.* 2005). However, a recent study using the latest genomic techniques has cast doubt on whether this system should be considered a rare example of a ring-species (Alcaide *et al.* 2014). Alcaide *et al.* (2014), found evidence indicating that over time some greenish warbler populations were separated without gene flow and that gene flow between the terminal forms has occurred, albeit limited. Similarly, Joseph *et al.* (2008), rejected a strict ring-species hypothesis in the *P.*
elegans species complex, based on the finding (and location) of three genetic discontinuities instead of one, which did not always correspond with plumage colour phenotype as predicted (Joseph et al. 2008; Ribot et al. 2009). Both studies concluded that periods of allopatry have contributed to the current genetic patterns observed as a likely response to the variability in environmental and climatic conditions.

Some pathogens, particularly RNA viruses and retroviruses, evolve faster than their hosts by having larger population sizes, faster generation times, and higher mutation rates (Duffy et al. 2008). Therefore, rapidly evolving pathogens may reflect recent host demographic and population structure (Sugimoto et al. 1997; Falush et al. 2003; Biek et al. 2006). BFDV is a rapidly evolving ssDNA virus, with comparable evolutionary rates to RNA viruses (Kundu et al. 2012). Here BFDV isolates within P. elegans were found to have patterns consistent with each of the ring-species predictions (chapter 4). First, phylogeographic analysis of BFDV revealed that P. e. elegans and P. e. flaveolus isolates formed two distinct groups, with P. e. adelaidae isolates co-occurring among the two. Second, WS hybrids clustered with P. e. elegans and formed the distal group in the phylogeny and were the most distinct from P. e. flaveolus, with no evidence of recombination or gene flow with P. e. flaveolus. Last, I found evidence within BFDV for isolation-by-distance around the ring. In addition, BFDV within P. elegans appears to be unique, with little evidence to suggest much gene flow occurs between BFDV isolates from other parrot species. Overall, my findings are consistent with the three key predictions outlined by Mayr’s ring-species concept.
The finding that BFDV has characteristics analogous to a ring-species is interesting given that genetic analysis of the host using microsatellites and mitochondrial DNA reject complete ring speciation (Joseph et al. 2008). A possible explanation is that BFDV actually reflects current host population structure, and not *P. elegans* historical evolution. This scenario is likely given the high mutation and recombination rates of BFDV (Julian et al. 2013). The suggestion that BFDV has the properties of a ring-species within *P. elegans* warrants a re-examination of the ring-species hypothesis in the host. Previous testing of this hypothesis has relied on subspecies colouration and incorporates geographical location (Joseph et al. 2008). Here, using the nine microsatellite markers used in chapter 4, the ring-species hypothesis was re-tested using the Bayesian inference program Structure (Pritchard et al. 2000). Preliminary results of this re-examination appear to show that *P. elegans* display a ring-species like population structure (Figure 7.1). The results of this new analysis differ from Joseph et al. (2008) because a single genetic discontinuity was found. However, the single discontinuity did match the one within *P. e. flaveolus* found in Joseph et al. (2008). This indicates that whilst a ring-species hypothesis could possibly still stand, it is not correlated with current subspecies classifications or with plumage colour phenotypes (Figure 7.1). The microsatellite genetic discontinuity identified in both analyses also corresponds with a sharp cline in *P. elegans* vocalisations (fundamental frequency and peak frequency pattern) (Ribot et al. 2012). It therefore seems possible that a learned signal may help promote population divergence (Ribot et al. 2012). Another signal which may prove to promote divergence is odour (Mihailova et al. 2014). Recently, a study conducted within *P. e. elegans* demonstrated that breeding females were able to discriminate between subspecies, using odour as the only signal (Mihailova et al. 2014). Other contributing factors in the maintenance of the phenotypic and genetic diversity within the *P. elegans* species complex.
may include rainfall (Joseph et al. 2008), colouration (Berg & Bennett 2010) and vision (Carvalho et al. 2010; Knott et al. 2010; Knott et al. 2012; Knott et al. 2013). Further investigation of the ring-species concept in *P. elegans* is ongoing.

**Figure 7.1.** K = 2 Structure plot from N = 372 individuals, using nine microsatellite markers from chapter 4. Model parameters included, 100 000 burn-in, 1 million MCMC reps and assumed an admixture model. The most likely number of populations based on the microsatellite data was estimated using the delta K method. Data is presented in the approximate geographic location around the ring. The colour bar on the x-axis represents *P. elegans* subspecies (yellow = *P. e. flaveolus*, orange = *P. e. adelaidae*, dark red = *P. e. melanoptera*, red = *P. e. elegans*, and black = WS hybrid). These data suggest that there is one genetic discontinuity within the *P. e. flaveolus* subspecies, and that a genetic cline through *P. e. melanoptera, P. e. elegans*, until WS hybrids exists.
7.3 Limitations of this study and future research

A clear limitation of the work presented in this thesis is that BFDV pathogenesis and its effect on host fitness in wild *P. elegans* is unknown. *P. elegans* subspecies with a high prevalence might be expected to display typical signs of the disease, however disease progression does vary between species, the age of the individual and also with individual body condition (Paré & Robert 2007). In captivity, infected birds may develop a chronic form of disease that can result in complete baldness (Paré & Robert 2007), but wild birds displaying such severe signs of the disease would likely die. Given the severe effects BFDV may enact in captive birds, one might assume similar effects in the wild, although host immune response may vary according to the environment (Buehler *et al.* 2008). The uncertainty in the effects of BFDV in wild *P. elegans* is relevant to the interpretation of findings in chapter 3, and this limitation is reflected in the numerous hypotheses that are presented to explain low prevalence and load in the WS hybrid and *P. e. adelaidae* populations. A common ‘garden type’ infection experiment controlling for BFDV strains in captivity, could potentially determine subspecies variation in resistance, tolerance, disease progression, and mortality rate. Groups of each subspecies could be housed separately and infected with viral isolates from each subspecies group, while monitoring viral replication (PCR: Eastwood *et al.* 2015), antigen excretion (Haemagglutination: Raidal *et al.* 1993c), antibody development (Haemagglutination inhibition: Raidal *et al.* 1993c) and histopathology (Shearer *et al.* 2008b). The before mentioned measures, should also be used in wild *P. elegans* to account for the different conditions in captivity. Although, as in this study, a fieldwork approach could suffer from the lack of re-capture data to properly measure disease and survival. Furthermore, wild birds may also suffer pathogen effects that
are subtle and difficult to detect (Ricklefs 1992; Soler et al. 2003; Asghar et al. 2015). To better understand the evolutionary effects of BFDV and subtle effects, the impact BFDV has on host life-history traits should be tested. In other host-pathogen systems host growth, survival and fitness can be negatively affected by pathogens (e.g. Merino & Potti 1995; Saino et al. 1998; Soler et al. 2003). Determining pathogen induced differences in fitness between hybrid and parental subspecies could indicate the role BFDV potentially plays in host population divergence.

In chapter 4, host heterozygosity and genotype rarity were associated with BFDV prevalence and viral load, respectively. However, it was surprising to find that these and other genetic diversity measures did not explain the population level variation in BFDV prevalence and load. A second unexpected finding in chapter 4, was that there was no correlation between host genetic distance and BFDV genetic distance. It is possible that a large spatial scale could explain both the lack of evidence supporting population level genetic diversity effects on BFDV infection, and the lack of a correlation between host-pathogen genetic distances. Sampling large numbers of hosts for BFDV across small spatial scales should eliminate confounding factors (Ebert et al. 1998; Gandon et al. 2008), including, asymmetries in selection pressure, differences in host population size and dynamics, and differences in environmental conditions, all of which may mask host-pathogen co-evolutionary patterns when using microsatellite markers. Additionally, the use of only nine microsatellite markers to measure genetic diversity could be another limitation of this study (Balloux et al. 2004; Westerdahl et al. 2005; Gompper et al. 2011; Forstmeier et al. 2012). Next-generation sequencing methods could be a more powerful approach to detect associations between host-pathogen genetics (Hoffman et al. 2014).
The correlational nature of chapter 4 is in itself a limitation, because I can only hypothesise that Red Queen type co-evolutionary dynamics are occurring. Measuring host-pathogen co-evolution across a large time-scale would prove a Red Queen hypothesis, however this is unlikely to be feasible given the long generation time of the host. Future work in this system could experimentally test if pathogen adaptation to genotypically similar hosts occurs, and subsequently results in higher viral loads as suggested in chapter 4. Experiments of this sort are challenging, but can be made possible by taking advantage of the fast evolutionary rate of the pathogen (i.e. high mutation rate, large population size, short generation time). A serial passage experiment, involving the trans-infection of BFDV through makeshift *P. elegans* populations (highly related or not related), could allow one to tease out whether common genotypes to which a pathogen is co-adapted can result in increased infectivity or replication rate. Similar passage experiments are common, and can demonstrate host-pathogen co-evolution (Schulte *et al.* 2010; Kubinak *et al.* 2012; Kubinak *et al.* 2015). However, this approach in a non-model species would have several technical and ethical challenges.
7.4 General conclusion

Overall, this thesis substantially contributes to our understanding of BFDV ecology and evolution, and represents the most comprehensive investigation of BFDV in a wild bird to date. My research has determined the most important predictors of infection in the *P. elegans* species complex and provided information about BFDV transmission dynamics. Given the study host, *P. elegans* has a unique and rare circular overlapping distribution, this study highlights the potential implications BFDV may have for host speciation, and vice versa. Furthermore, this study provides the first example of a pathogen displaying phylogeographic patterns analogous to a ring-species. In addition, this study is also the first to demonstrate that host heterozygosity and genotype rarity influence different components of a viral infection. The work in this thesis not only helps understand BFDV in wild birds for conservation purposes, but provides general insights into the spread of infection and host-pathogen co-evolution. To conclude, this thesis presents a plethora of interesting and novel questions for future research.
References


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