Molecular ecology of Beak and Feather Disease Virus in the Endangered Mauritius parakeet (*Psittacula eques*)

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DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any other university or tertiary institution. To the best of my knowledge and belief this work contains no material previously published or written by another person. Wherever contributions from others are involved, every effort is made to indicate this clearly, with due reference to the literature.

I also declare that all data collection, analyses and writing for the compilation of each chapter were undertaken by me. Co-author contributions were limited to corrections and comments and they have consented to their work being included in the thesis.

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CHAPTER 1: Detecting Beak and feather disease virus in Psittacines: an assessment of research trends and knowledge gaps

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ABSTRACT

The occurrence of Psittacine Beak and Feather Disease (PBFD) has been reported in both wild and captive parrot populations since the mid-1970s. PBFD has been found to be widely infectious and often fatal, affecting both Old and New World psittacine species. Consequently, determining routes of transmission and developing pathological and serological tests for screening for presence of the Beak and Feather Disease Virus (BFDV) have emerged as important priorities for biodiversity managers. Here, we consolidate information collected from 82 PBFD- and BFDV-based publications on the primary screening methods being used and identify important knowledge gaps regarding potential global disease hotspots. We present trends in research intensity in this field, and critically discuss advances in screening techniques and their applications to both aviculture and to the management of threatened wild populations. Finally we provide an overview regarding estimates of BFDV prevalence in captive and wild flocks alongside a complete list of all psittaciform species known to be susceptible. Advances in BFDV screening and diagnostic tools currently available allow for a broader application of results to conservation management strategies. Our evaluation highlights the need for standardised diagnostic tests and more emphasis on studies of wild populations, particularly when considering the intrinsic connection between global trade in companion birds and the spread of novel BFDV strains into wild populations. Increased emphasis should be placed on the screening of captive and wild parrot populations within their countries of origin across the Americas, Africa and Asia.

KEY WORDS Emerging Infectious Disease**,** PBFD, BFDV, screening, prevalence estimate, susceptible species

1.1 INTRODUCTION

Pathogens responsible for emerging infectious diseases (EIDs) have become a major concern in conservation biology owing to their potential for rapid evolution and the implications an epidemic may have on vulnerable species (Altizer, Harvell & Friedle 2003). Consequently, understanding EIDs and their management in wildlife populations has become increasingly important to conservationists (Bradley & Altizer 2007). Assessing the prevalence and impacts of disease can be challenging, particularly during the outbreak of a novel pathogen (Artois et al. 2001). Data collected and used in these circumstances often vary in sampling or assessment method, frequently with imperfect diagnostic tests providing the only available insight into infection incidence within a population (Williams & Moffitt 2010; Lachish et al. 2012). Consequently, synthesising multiple sources of information across many species can provide insight into how to improve management of EIDs, identify knowledge gaps and reveal where improvements in surveillance methods might be required.

The occurrence of Psittacine Beak and Feather Disease (PBFD) has been reported in both wild and captive parrot populations since the mid-1970s. The disease has been found to be widely infectious and often fatal, affecting both Old and New World psittacine species. PBFD is thought to have originally been documented in the late 1880s in wild Australian *Psephotus* parrots as feathering abnormalities that impaired their flight (Ashby 1907). Most commonly affecting immature and fledgling birds, classical symptoms include symmetrical loss of contour, tail and down feathers before replacement by dystrophic and necrotic feathers that fail to grow soon after emergence from the follicle (Perry 1981; Pass and Perry 1984; Ritchie et al. 1991a). Beak deformities such as fractures, abnormal elongation and palatine necrosis are also typical symptoms of PBFD but their presence and severity vary from species to species (Ritchie et al. 1989a). Other clinical symptoms include lethargy, depression, diarrhoea and immunosuppression which are individually variable, sometimes lead to death and may depend on the virulence of the viral strain or the route of viral exposure (Ritchie et al. 1989b).

BFDV was tentatively placed in the Circoviridae family (Studdert 1993), consisting of the smallest known autonomously replicating pathogenic animal viruses (Ritchie 1995; Todd 2000; Delwart & Li 2012), which was later confirmed when the first complete BFDV genome

was sequenced (Bassami et al. 1998). The structure of the Beak and Feather Disease Virus (BFDV) isolated from viral inclusion bodies was determined to be a non-enveloped, icosahedral virion between 14 and 16 nm in size and containing a single stranded DNA genome approximately 1.7 to 2.0 kilo-bases in length (Ritchie et al. 1989a).

Until the early 1990's histology and recovery of virions were the primary means of determining whether a bird was infected with BFDV. The first haemagglutination (HA) and haemagglutination inhibition (HI) assays for PBFD were then developed as a technique for both the identification and quantification of virus recovered from BFDV-positive birds (Ritchie et al. 1991b). Since the initial description of the syndrome, several attempts have been made to culture the virus in-vitro in order to provide a source of antigen for vaccinations, but as of yet these have not been successful (Pass et al. 1985; Bassami et al. 1998; Robino et al. 2014). The lack of effective vaccine has compelled researchers to develop techniques to further interrogate the molecular genetics of the virus instead; encouraging development of oligonucleotide probe based methodologies such as dot-blot DNA hybridization, DNA in-situ hybridization and a polymerase chain reaction (PCR) based assay (Latimer et al. 1992; Latimer et al. 1993). These techniques provided a means to determine whether infection was present, even if the individual being studied was asymptomatic. Due to their small size, whole-genome sequencing has now become a commonplace tool in the analysis of circoviruses, assessing their phylogenetic relationships both with other taxa within the family and between strains of the same virus occurring in different hosts or global regions (Phenix et al. 2001; Olvera, Cortey & Segales 2007; Regnard et al. 2015a).

PBFD has become a major cause for concern to conservationists and aviculturists as the disease has spread rapidly across the world, owing to BFDV's high environmental persistence and ability to shift between closely related host species (Peters et al. 2014, Sarker et al. 2014a). BFDV is easily transmitted through contact with contaminated feather dust, surfaces or objects (Ritchie, Anderson & Lambert 2003), and can also be passed directly from a female to her offspring (Ritchie et al. 1989a; Kundu et al. 2012). The management of PBFD in captivity is economically important in some countries; for example, it is estimated that aviculturists in South Africa lose up to 20% of their flock to the disease annually (Heath et al. 2004). Worryingly, many wild populations of vulnerable species are

also affected including: the kakapo (*Strigops habroptilus*) and kaka (*Nestor meridionalis*) of New Zealand (Massaro et al. 2012), the Australian orange-bellied parrot (*Neophema chrysogaster*) (Peters et al. 2014) and swift parrot (*Lathamus discolour*) (Sarker et al. 2013a), and the Mauritius (or "echo") parakeet (*Psittacula eques*) (Kundu et al. 2012). Therefore, understanding the mechanics behind the spread of BFDV and how to test for its prevalence has taken on a renewed global relevance.

Concern over the implications for conservation, aviculture and biosecurity together with methodological advances in the detection of the virus has prompted an upsurge in research effort. Methodological developments have provided the basis on which researchers are now able to model the potential routes of transmission around the world (Harkins et al. 2014), link BFDV prevalence to management related tools for endangered species recovery (Tollington et al. 2015) and determine the ways in which anthropogenic activities have changed the way in which the virus is evolving due to recombination (Julian et al. 2013). Remarkably, whilst there are many research teams worldwide working on BFDV and PBFD there is a severe lack of synthesised knowledge on the primary screening methods being used, the species affected and, consequently, potential disease hotspots that have lacked attention. Here, we aim to consolidate the most pertinent patterns and methods emerging from the literature published since the first scientific description of PBFD in 1984 to provide both a qualitative and quantitative overview of approaches and screening results. Our review provides a much-needed source of information regarding BFDV prevalence estimates in captive and wild flocks for use by conservation practitioners. Our objective was not to provide an exhaustive description of each technique but to analyse the trends in how screening has progressed over the last three decades and provide an overview of prevalence estimates for this EID alongside broader implications to biosecurity and conservation.

1.2 METHODS

1.2.1 Literature search

Searches for literature were conducted by entering English key words and terms into Google Scholar and were selected to balance search sensitivity with specificity. The terms were: "Beak and Feather Disease Virus", "Psittacine Beak and Feather Disease", "Beak and Feather Disease", "Psittacine circovirus", "BFDV screening", "PBFD screening", "BFDV

detection" and "PBFD detection". Acquisition of literature was restricted to only those articles that had been published in academic journals or as conference proceedings up to and including July 2015, thus excluding any theses and organisational reports.

1.2.2 Analysis

Information extracted from each publication included the year published, whether the birds studied originated in the wild or captivity, host species, country of origin of all specimens, tissue types and laboratory methods used in the detection of PBFD or BFDV and the outcome of diagnostic tests including detection prevalence. If a total population prevalence estimate was provided this value was also recorded.

The publications were grouped in five year intervals to examine the trend in the number of publications produced over time. If multiple species from the same country of origin were involved in the same study the country of origin was recorded once per publication. If the study was based on captive individuals, and a different country of origin for a specimen was not otherwise clearly stated in the publication, it was assumed that the country in which the study was undertaken was the country of origin. Where a study used specimens from both captive and wild individuals from the same country, the country of origin for each specimen was recorded once per category for each publication. For example: Regnard et al. (2015b) screened specimens from both captive and wild populations of *Poicephalus robustus* and this information was recorded by adding South Africa once to each category. Maps were produced using ArcGIS 10.2.1 (ESRI 2011) displaying the results of captive and wild specimens independently. Seven publications did not declare whether the specimens obtained were of wild or captive origin; comprising five incidences from Australia, one from the United States of America (USA) and one from Brazil. These incidences were all excluded from the analyses of geographical patterns. The common names of species historically recorded as positive for PBFD/BFDV were aligned to current nomenclature as per the International Union for Conservation of Nature (IUCN) Red List database, alongside additional information regarding their current IUCN status and native geographic region.

Screening methods were recorded once per publication. The annual trends in the five most frequently used screening methods were assessed, along with the overall most

commonly combined mixed-methods approaches. As with country of origin, tissues used for screening and diagnostics were divided into wild and captive specimens and, where a study used a certain tissue type from both captive and wild individuals, that type was recorded once per category for each publication.

1.3 RESULTS

1.3.1 Publication trends and affected species

There has been a linear increase in the number of publications involving testing for BFDV since the first scientific description of PBFD to present (Figure 1.1, $R^2 = 0.96$), with the total number of screening based publications reaching 82 by July 2015. The total number of publications on BFDV screening and prevalence is by far the highest between 2011 and July 2015, being 30% higher than the number of publications for the five-year period preceding it and more than three times higher than the first full categorical period from 1986-1990.

Research has been focused predominantly on captive populations, encompassing 33 different countries and with the highest number of specimens originating from the USA followed by South Africa, Australia and Japan (Figure 1.2). Conversely studies on wild populations have only been undertaken in eight nations, with 12 of the 38 studies including specimens of Australian origin and none conducted on New World parrots. Three of these 38 studies were based on screening for BFDV among exotic introduced populations of non-native species; from the United Kingdom (Sa et al. 2014), Mauritius (Kundu et al. 2012) and New Zealand (Jackson et al. 2014a). A BFDV or PBFD positive result was reported at least once in both wild and captive specimens tested from all included countries aside from Senegal (Figure 1.3).

Of the 88 species in which BFDV or PBFD has been detected in wild or captive birds (Table 1.1) approximately 65% (57 species) are categorised as Least Concern by the IUCN (2015), 10% are considered to be Near Threatened and just over a quarter are classified in Threatened categories. A declining population was observed in over 60% of BFDV affected host species. Of the 20 species in which BFDV or PBFD has been detected among wild populations, 70% (n=14) are currently categorised as Least Concern, two are classified as Near Threatened and the remaining four are classified in Threatened categories. Half (n=10) were determined to have host populations increasing in population size (IUCN 2015). In addition

wild populations of three subspecies have also tested positive for BFDV, all of which are native to the Oceania region.

The summarised captive and wild population BFDV/PBFD prevalence estimates are reported in Table 1.2. Prevalence estimates have been provided for nine national captive populations globally; comprising four from Europe (two of which were for Poland), two from Oceania, two from East Asia and one from Central America. These estimates vary in their scope from describing prevalence in a subset of species (e.g. parakeets, Ha et al. 2009) to estimating BFDV prevalence across entire national captive populations (e.g. Bert et al. 2005). Among wild populations, seven of eight publications reporting prevalence estimates are from the Oceania region, with four from New Zealand alone. *Cacatua galerita* populations in Australia were estimated to have a prevalence of between 10-20% (McOrist et al. 1984), slightly below the minimum estimate provided for populations in New Zealand two decades later (Ha et al. 2007). The lower limits of the 95% confidence interval surrounding BFDV prevalence in wild *Platycercus eximius* populations in New Zealand provided by two separate research groups, five years apart, are comparable (Ha et al. 2007; Massaro et al., 2012). However the upper limit varies from 20.4% to more than double, at 42.3%. Similarly, the two estimates for *Cyanoramphus novaezalandiae* populations differ greatly from one another (Ortiz-Catedral et al. 2009; Massaro et al., 2012), with the upper limit of the 2012 estimate approximately 12% lower than the total estimate provided in 2009. The only estimates for African populations are from Mauritius, where the endemic parakeet population was screened annually throughout the duration of the study. From 2004 to 2009 the estimated total prevalence varied from 11-41% (Kundu et al. 2012).

1.3.2 Most frequently-used laboratory methods

Of the 82 publications evaluated, 47% (n=39) of them used a single method approach for detecting BFDV; with standard PCR-based assays the most frequently applied (43%), followed by whole genome sequencing (26%) and histology (18%) respectively.

Histology using both light and scanning electron microscopy has been one of the most popular and consistently used methods from 1984 to present. Of the 14 methods available for screening and diagnostics, histology has been used at least once in combination with all but quantitative (or real-time) polymerase chain reaction (qPCR), blocking ELISA and

duplex shuttle PCR. An ELISA test was first developed for screening in the mid-1980s (Pass et al. 1985) but wasn't used in a BFDV/PBFD screening based publication until more than two decades later (Table 1.3), after which it was never used again. Similarly the duplex shuttle PCR method has been used only once. Both HA and HI were used on 12 occasions since their first application in 1991 (Table 1.3). However, HA was not used at all in the 2011 to July 2015 publication period (Figure 1.4).

The standard PCR based assay closely follows histology in terms of widespread use and is the most frequently used screening method overall, applied in 49% of reported studies (Table 1.3). Of the 35 publications that used standard PCR from 2000 onwards, 24 used the protocol and/or oligonucleotide primers developed by Ypelaar et al. (1999). The application of both PCR and whole genome sequencing is considerably higher than any other mixed method approach; used together as the only methods in nine publications and in a total of 12 mixed method studies. In the 2011 to July 2015 period, whole genome sequencing became the only method to exceed the number of applications of standard PCR for BFDV screening since it increased in popularity from 1996 to 2000 (Figure 1.4); used in 70% of publications since its first application in 2004.

1.3.3 Tissue types used for screening

A total of 13 tissue types have been used for BFDV and PBFD screening since 1984: beak, blood, bone marrow, cloacal swabs, crop samples, embryonated and non-embryonated eggs, faeces, feather dust, feathers, muscle tissue, skin and viscera. All tissue types, aside from beak, have been used for screening on at least one occasion in captive populations; with feathers used the most frequently (34%), followed by blood (32%) and skin (9%) respectively. Conversely, only six tissue types have been used in the screening of wild populations. As with captive populations, blood (41%) and feathers (37%) were the most commonly used source for samples, with viscera studied 10% of the time and beak only used on one occasion.

1.3.4 Descriptions of clinical signs

Basic visual body condition assessments were mentioned in 36 of the 82 publications and ranged from a brief statement of the presence or absence of feather disorder (Latimer et al. 1992; Ogawa, Yamaguchi & Fukushi 2005) to more in-depth observations regarding overall body condition (Sa et al. 2014; Jackson et al. 2014b). More thorough scoring systems

for the classification of clinical symptoms were applied in eight studies. The most descriptive of these systems was by Regnard et al. (2015b), consisting of six different clinical symptoms, with each broken down into five different scores of overall physical condition, which was then compared to individual viral load. Other scales, such as that applied by Ritchie et al. (1991a, b) descriptively scored only clinical feather and beak lesions.

1.3.5 Field methods used to obtain wild specimens

Only 16 of the 38 studies reporting BFDV or PBFD incidence in wild birds discussed the field methods used to obtain their specimens. The most frequently used method was mist netting, reported in 11 of the 16 publications (Massaro et al. 2012; Jackson et al. 2014a; Regnard et al. 2015b). The second most preferred method was trapping, either whilst individuals were in nests (McOrist et al. 1984; Eastwood et al. 2015) or with walk-in traps (Eastwood et al 2014, 2015). Other studies were undertaken on specimens gathered opportunistically from mortality cases and individuals brought in for health checks (Ha et al. 2009).

1.4 DISCUSSION

1.4.1 Patterns in global PBFD and BFDV research

Interest in the screening for, spread and impacts of BFDV/PBFD globally has steadily increased over the last three decades, with a particular focus on wild populations in the last five years. Over the course of this period the focus in research has shifted from basic descriptions of presence or prevalence in populations, towards question-driven studies relating to viral recombination, evolution and phylogenetics (e.g. Henriques et al. 2010; Julian et al. 2013), the drivers of outbreaks in wild populations (e.g. Jackson et al. 2014; Peters et al. 2014), and their implications for improving the management of captive and wild populations. However, despite the burgeoning interest in assessing incidence in wild populations there are some conspicuous research gaps which future research should aim to fill. Oceania is undoubtedly the most heavily researched regarding the incidence of BFDV or PBFD in both wild and captive populations globally. This finding may partly be due to evolutionary studies indicating that the virus likely originated from this region and BFDV has been listed as a “Key Threatening Process” to biodiversity in Australia (Eastwood et al.

2015). In contrast, there has been very little research on BFDV in proximate geographical regions of high parrot diversity such as South East and Southern Asia.

Given that *Cacatua* was the genus from which PBFD was first described, 11 species of which have proven to be susceptible to BFDV infection, to date there has been very little research into areas of South East Asia to which many of these species are native. The virus has been found in specimens from both wild and captive populations in Indonesia, a country that contains many psittacine breeding farms (Ogawa et al. 2013) and is heavily exploited for both the legal and illegal trapping and export of companion birds for the pet trade (IUCN 2015). Equally, with high levels of parrot endemicity in South and Central America, it is surprising that no studies have been published on BFDV or PBFD incidence in wild populations. Only two studies have been conducted on captive individuals originating from these geographical regions; one from Costa Rica (Dolz et al. 2013) and another that included specimens of Guyanese origin (Ogawa et al. 2013). Whilst one study from Brazil did not specify whether the individuals studied were of captive or wild origin and were therefore not included in Figure 1.2 (Soares, Guimaraes & Durigon 1998), this anomaly makes little difference to the overall picture. Similarly most of the African continent is data deficient, with no BFDV studies published on wild populations north of Zambia (Warburton & Perrin 2002) or from any of the Indian Ocean islands aside from Mauritius. The captive studies have been slightly more inclusive, with specimens from Cameroon and the Ivory Coast, but they were not conducted within the country of origin and therefore provided little information on the state of captive flocks locally. Also, as the specimens from captive birds originating from these nations tested positive for BFDV (Bert et al. 2005) it would be beneficial to investigate wild populations further for the occurrence of any spill over from the aviculture industry.

Notably, one species that requires further research focus is *Psittacula krameri*; the most introduced parrot globally with breeding populations in approximately 35 countries, across five continents (Tayleur 2010). No BFDV or PBFD screening has been conducted on any of the wild populations of *P. krameri* across its extensive native range. However, feral populations within its invasive range and captive individuals have both tested positive for BFDV (Kundu et al. 2012; Julian et al. 2013; Sa et al. 2014). It is therefore highly likely that the

virus is present in wild flocks which may act as a reservoir with potential spill over into other sympatric vulnerable psittacine species.

1.4.2 Advances in methods

The methods used to screen specimens for BFDV have also changed along with the questions being asked. The variety of optimised diagnostic tests and technologies available for BFDV screening have increased and improved substantially since its first scientific assessment. Whole genome sequencing has become a particularly prominent tool in recent years due to the small size of the BFDV genome, reduced costs of this technique and the availability of comparable sequence data through collective resources such as GenBank. Other methods, such as blocking ELISAs, duplex shuttle PCRs and dot-blot DNA hybridization have been used once or twice but were not as effective as other methods available or in common use at the time. Unlike the ELISA, the HI assay, currently the leading assay for anti-BFDV antibody detection, does not require a secondary antibody and is widely suitable for detection for a large proportion of psittacine species (Kahlesi et al. 2005).

With the impact of PBFD evident in a number of declining and vulnerable wild parrot populations and the wider economic impact of PBFD on aviculture it would be valuable to standardise an approach to basic viral screening, improving on both accuracy and repeatability. Standardisation would allow for more reliable modelling, extrapolation and population prevalence estimates that are comparable between countries, species or breeding facilities. Managers of captive breeding facilities used in the pet-trade may want to increase the confidence in diagnostic tests to prevent the introduction of infected individuals into healthy collections. Similarly conservation managers may require better detection accuracy in order to increase the probability of success in establishing a disease-free base population when undertaking translocations in species recovery and reintroduction programmes. Whilst steps have recently been taken to improve the standard PCR protocol by quantifying DNA extraction concentrations prior to screening (Eastwood et al. 2015), still lacking in the literature is an assessment of detection accuracy at variable DNA concentrations and how this impacts the repeatability of a result.

Quantitative (real-time) PCR techniques are now being more regularly applied to determine individual viral load (Shearer et al. 2009a; Eastwood et al. 2015; Regnard et al.

2015b) as probe-based assays are able to detect viral DNA at much lower concentrations than recorded by the naked eye when visualizing a gel. However, the reagents and equipment required for screening through standard PCR are currently substantially cheaper than those used for probe-based assays and are thus likely to have continued wide-spread use for the purpose of general BFDV screening.

1.4.3. Tissue types used for screening

Extracted DNA samples can vary greatly in yield depending on the type and amount of tissue used. For example, feathers typically produce very low genomic DNA yields, particularly when extracted from those that are cut off from the blood supply once fully grown (De Volo 2008), only representing viral incidence during the initial growth phase. Concentrations can considerably affect the sensitivity of PCR assay (Khalesi et al. 2005) as the amount of viral DNA obtained from any sample will be dependent on the infection level within the host at the time of sampling (Knowles et al. 2011; Lachish et al. 2012), making higher DNA yields preferable to increase the probability of detection. A number of studies have proven that there are inconsistencies in detection of BFDV between tissue types (Ramis et al. 1998; Hess, Scope & Heincz 2004; Khalesi et al. 2005; Eastwood et al. 2015). Feathers have been found to test positive for BFDV in the absence of clinical signs (Hess, Scope & Heincz 2004), where no HI antibody is detectable (Khalesi et al. 2005) and when an individual's blood or tissue tested negative (Eastwood et al. 2015).

Whilst samples from wild populations may be easier and require less veterinary expertise to obtain through non-invasive techniques, such as the collection of feathers, there is a higher risk of cross contamination between samples (Taberlet, Waits & Luikart 1999) and thus may increase the proportion of false-positives when screening. Also, as a primary symptom of PBFD is feather loss the collection of dropped feathers (for example from a roost site) may further bias the estimated proportion of infected individuals. Therefore, as with the variation in diagnostic methods, it would be valuable to standardise a protocol each for blood and feathers, the two most commonly screened tissue types, for widespread use between managers of both wild and captive populations. As the screening of muscle tissue and blood have been found to provide highly comparable results with standard and qPCR techniques

(Eastwood et al. 2015), a standardised blood screening protocol could therefore also be extended to use with other internal tissues such as muscle or viscera.

1.4.4 The under-reporting of body condition assessments and field techniques

Both the reporting of body conditions of screened individuals and the techniques used to capture wild birds have been highly inconsistent in the literature. As it has been shown that some individuals can remain asymptomatic despite testing positive for BFDV (Ritchie 1989a) it is difficult to determine whether body condition assessments are of value in informing management guidelines. However, overall physical condition has been found to correlate with viral load in Cape parrots (Regnard et al. 2015b) and consequently it may be of value to implement a robust and standardised scale of clinical signs as a primary means of assessment in the field. This finding will need to be tested in a number of other parrot species to determine its repeatability across the *Psittacidae* family before further reliance can be placed on this as a means of roughly estimating host prevalence without a diagnostic assay.

Mist nets and traps, the most frequently used field techniques used to catch wild birds, may facilitate the horizontal spread of infection between individuals if equipment is not adequately cleaned between uses. BFDV has been found to be highly environmentally persistent (Peters et al. 2014) and conservation managers should therefore be aware of the risks of increased transmission when a thorough cleaning regime is not implemented.

1.4.5 Applications

The application of screening and diagnostic tests for BFDV has developed from trying to understand the structure of the virus, how it is transmitted between individuals and the nature of the disease, to assessing what incidence and prevalence means for management and interrogating evolutionary relationships between strains occurring in different parts of the world. These methodological developments have proven to be particularly valuable when considering translocation and reintroduction programmes for wild populations (Cunningham 1996; Jackson et al. 2015), highlighted by a complete loss of a new founder population of Endangered *Psittacula eques* to PBFD in 2005 (Tollington et al. 2013, 2015).

Initially the virus was thought to be limited in its diversity (Ritchie et al. 1990) and some attempts were made to produce a protective vaccine (Pass et al. 1985; Raidal, Sabine

& Cross 1993; Bonne et al. 2009) but little attention has been given to this in recent years. Instead researchers and practitioners have taken a proactive stance, placing a closer focus on closer monitoring and management of the disease, trying to avoid spill over into vulnerable species (Jackson et al. 2014; Peters et al. 2014).

This review highlights the need for more focus on wild populations, particularly when taking into consideration the intrinsic connection between the trade in companion birds and the spread of novel BFDV strains into the wild. It is also clear that there are still many opportunities to study the impacts of disease in captive and wild parrot populations within their countries of origin across the Americas, Africa and Asia. Many parrot species have declining populations and exist within highly fragmented and degraded habitats (IUCN 2015) and consequently it would be of great value in the future conservation of wild populations to determine how the spread of infectious disease further impacts on survival or persistence. Only a few total prevalence estimates exist for captive and wild populations, valuable information for geographical and cross-species comparisons that, in some incidences, could be relatively easily reported with modelling existing data. The progression and refinement of the screening and diagnostic tools currently available for the study of BFDV allows for a broader application of results in management strategies and disease transmission prevention protocols. However further work still needs to be done on the standardisation of diagnostic tests to allow for more reliable extrapolation of results in the management of both captive and wild populations.

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1.6 FIGURES AND TABLES

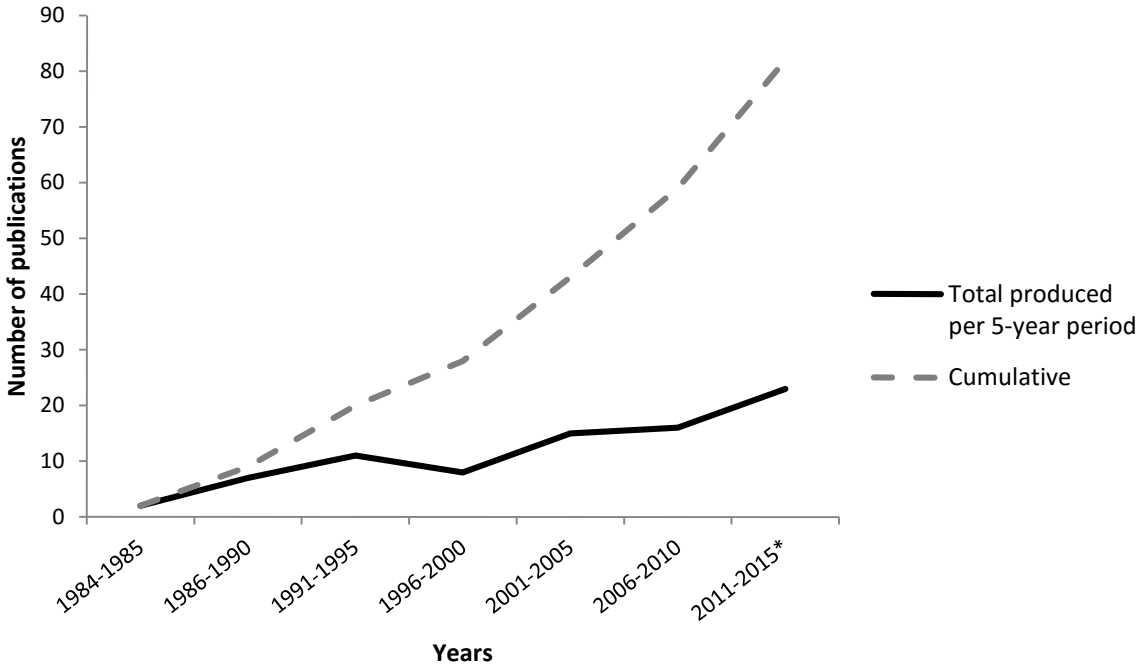

Figure 1.1 The number of PBFD and BFDV screening based publications produced between 1984 and July 2015.

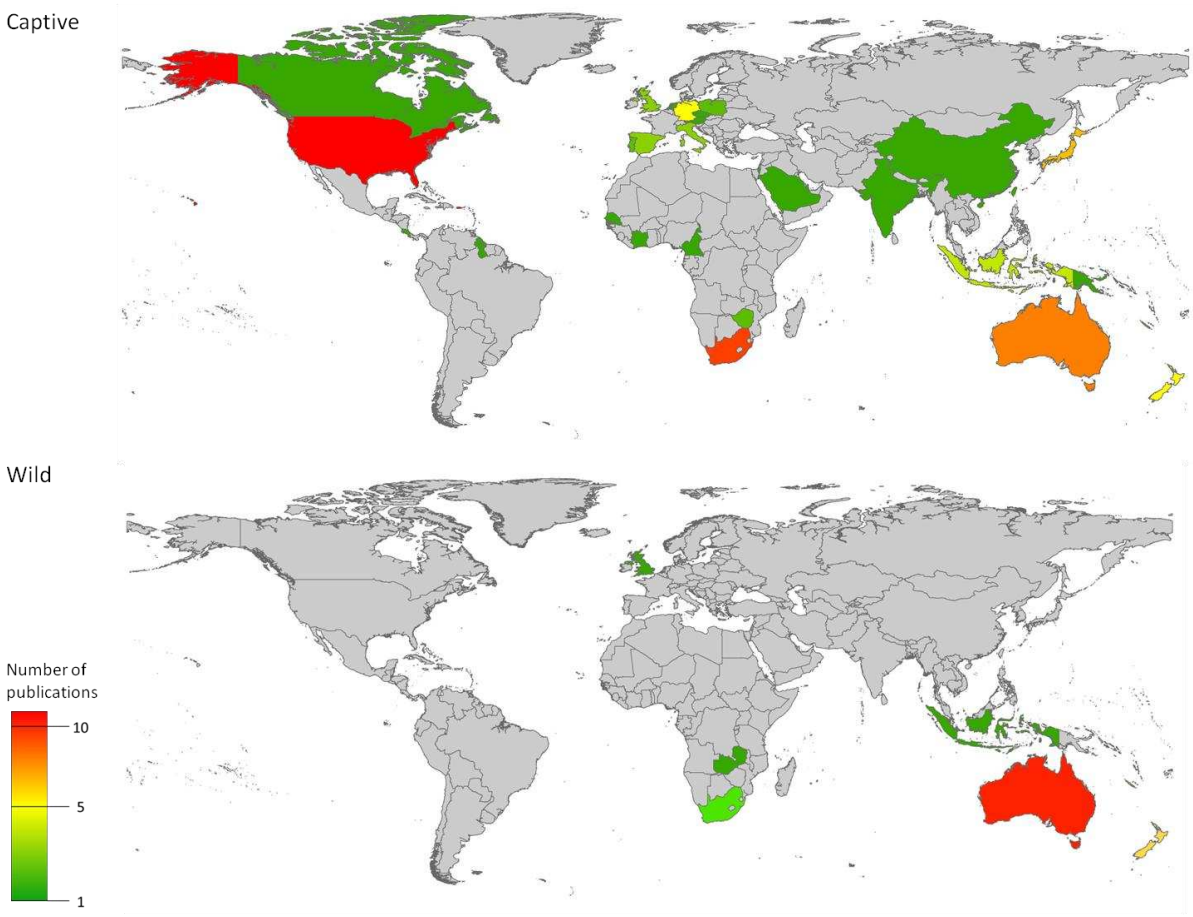

Figure 1.2 The geographical distribution of research into BFDV and PBFD during the period 1984-July 2015. Countries are coloured according to the number of publications involving specimens originating from that country.

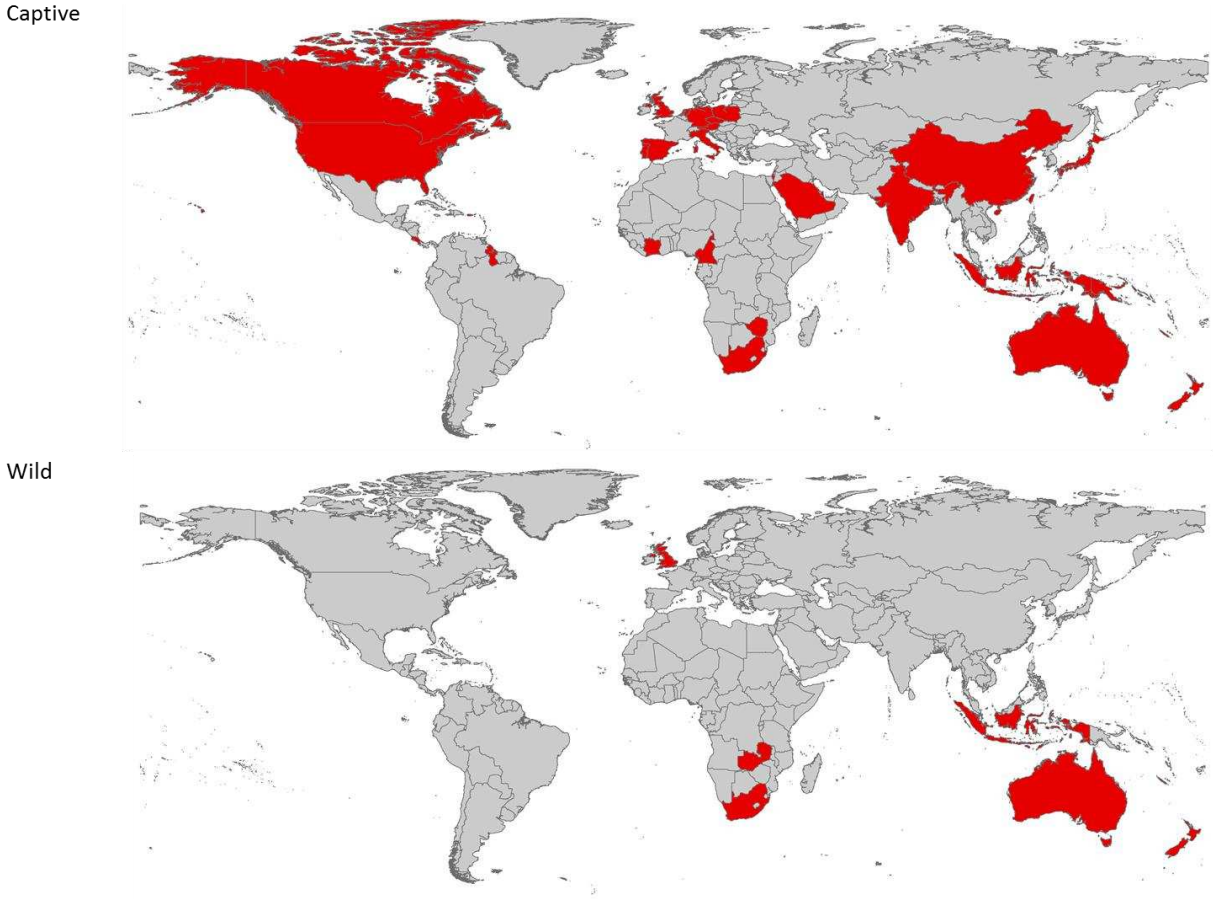

Figure 1.3 The geographical distribution of BFDV positive results (red fill) during the period 1984-July 2015.

Table 1.1 Psittacine prevalence estimates and the screening tests used in publications from 1984 to 2015 for both wild and captive psittacine populations. All recorded psittacine species that have tested positive for BFDV from 1984 to 2015. Those that have tested positive in wild populations are marked with a (*)

Common name	Scientific name	IUCN category	Population trend	Continental region
New World				
Turquoise-fronted Amazon	*Amazona aestiva*	LC	Decreasing	South America
White-fronted Amazon	*Amazona albifrons*	LC	Increasing	North and Central America
Orange-winged Amazon	*Amazona amazonica*	LC	Decreasing	South America
Yellow-naped Amazon	*Amazona auropalliata*	VU	Decreasing	Central and South America
Red-lored Amazon	*Amazona autumnalis*	LC	Decreasing	North, Central and South America
Yellow-shouldered Amazon	*Amazona barbadensis*	VU	Decreasing	South America
Yellow-crowned Amazon	*Amazona ochrocephala*	LC	Decreasing	Central and South America
Vinaceous-breasted Amazon	*Amazona vinacea*	EN	Decreasing	South America
Blue-and-yellow macaw	*Ara ararauna*	LC	Decreasing	South America
Red-and-green macaw	*Ara chloropterus*	LC	Decreasing	South America
Scarlet macaw	*Ara macao*	LC	Decreasing	South and Central America
Military macaw	*Ara militaris*	VU	Decreasing	North and South America
Red-fronted macaw	*Ara rubrogenys*	EN	Decreasing	South America
Blue-crowned parakeet	*Aratinga acuticaudatus*	LC	Decreasing	South America
Sun parakeet	*Aratinga solstitialis*	EN	Decreasing	South America
Burrowing parrot	*Cyanoliseus patagonus*	LC	Decreasing	South America
Northern red-shouldered macaw	*Diopsittaca nobilis*	LC	Stable	South America
Brown throated parakeet	*Eupsittula pertinax*	LC	Increasing	South and Central America
Pacific parrotlet	*Forpus coelestis*	LC	Stable	South America
Golden parakeet	*Guarouba guarouba*	VU	Decreasing	South America
Green-thighed parrot	*Pionites leucogaster*	EN	Decreasing	South America
Black-headed parrot	*Pionites melanocephalus*	LC	Stable	South America
Bronze-winged parrot	*Pionus chalcopterus*	LC	Decreasing	South America
Blue-winged macaw	*Primolius maracana*	NT	Decreasing	South America
Crimson-fronted parakeet	*Psittacara finschi*	LC	Increasing	Central America
Green-cheeked parakeet	*Pyrrhura molinae*	LC	Stable	South America

Old World

Nyasa lovebird	*Agapornis lilianae*	NT	Decreasing	East Africa
Black-cheeked lovebird	*Agapornis nigrigenis**	VU	Decreasing	East Africa
Peach-faced lovebird	*Agapornis roseicollis*	LC	Decreasing	Southern and Central Africa
Australian king parrot	*Alisterus scapularis*	LC	Decreasing	Oceania
Red-winged parrot	*Aprosmictus erythropterus*	LC	Increasing	Oceania and South East Asia
Australian ringneck	*Barnardius zonarius (barnardi)**	LC	Increasing	Oceania
White cockatoo	*Cacatua alba*	EN	Decreasing	South East Asia
Solomon's corella	*Cacatua ducorpsii*	LC	Stable	Oceania
Sulphur-crested cockatoo	*Cacatua galerita**	LC	Decreasing	Oceania and South East Asia
Triton cockatoo	*Cacatua galerita triton*		Not assessed	Oceania
Tanimbar corella	*Cacatua goffiniana*	NT	Decreasing	South East Asia
Philippine cockatoo	*Cacatua haematuropygia*	CE	Decreasing	South East Asia
Major Mitchell's cockatoo	*Cacatua leadbeateri*	LC	Stable	Oceania
Moluccan cockatoo	*Cacatua moluccensis*	VU	Decreasing	South East Asia
Blue-eyed cockatoo	*Cacatua ophthalmica*	VU	Decreasing	Oceania
Bare-eyed corella	*Cacatua sanguinea**	LC	Increasing	Oceania and South East Asia
Yellow-crested cockatoo	*Cacatua sulphurea*	CE	Decreasing	South East Asia
Citron-crested cockatoo	*Cacatua sulphurea citrinocristata*		Not assessed	South East Asia
Eastern long-billed corella	*Cacatua tenuirostris**	LC	Increasing	Oceania
Gang gang cockatoo	*Callocephalon fimbriatum**	LC	Increasing	Oceania
Red-tailed black cockatoo	*Calyptorhynchus banksii**	LC	Decreasing	Oceania
Glossy black cockatoo	*Calyptorhynchus lathami*	LC	Decreasing	Oceania
Vasa parrot	*Coracopsis vasa*	LC	Stable	East Africa
Yellow-fronted parakeet	*Cyanoramphus auriceps**	NT	Decreasing	Oceania
Red-fronted parakeet	*Cyanoramphus novaezelandiae (saisseti)**	NT	Decreasing	Oceania
Antipodes parakeet	*Cyanoramphus unicolor*	VU	Stable	Oceania
Eclectus parrot	*Eclectus roratus*	LC	Decreasing	Oceania and South East Asia
Galah	*Eolophus roseicapilla**	LC	Increasing	Oceania
Red lory	*Eos bornea**	LC	Decreasing	South East Asia
Horned parakeet	*Eunymphicus cornutus*	VU	Increasing	Oceania
Musk lorikeet	*Glossopsitta concinna*	LC	Stable	Oceania
Purple-crowned lorikeet	*Glossopsitta porphyrocephala*	LC	Decreasing	Oceania
Swift parrot	*Lathamus discolor**	EN	Decreasing	Oceania
Budgerigar	*Melopsittacus undulatus*	LC	Increasing	Oceania

Orange-bellied parrot	*Neophema chrysogaster**	CE	Decreasing	Oceania
Kea	*Nestor notabilis*	VU	Decreasing	Oceania
Bluebonnet	*Northiella haematogaster*	LC	Decreasing	Oceania
Cockatiel	*Nymphicus hollandicus*	LC	Stable	Oceania
Crimson rosella	*Platycercus elegans**	LC	Decreasing	Oceania
Adelaide rosella	*Platycercus elegans adelaidae**		Not assessed	Oceania
Yellow rosella	*Platycercus elegans flaveoulus**		Not assessed	Oceania
Eastern rosella	*Platycercus eximius**	LC	Increasing	Oceania
Brown-headed parrot	*Poicephalus cryptoxanthus*	LC	Stable	Southern and East Africa
Red-fronted parrot	*Poicephalus gulielmi*	LC	Decreasing	West, Central and East Africa
Cape parrot	*Poicephalus robustus**	LC	Decreasing	West, Central, East and Southern Africa
Rüpell's parrot	*Poicephalus rueppellii*	LC	Decreasing	Southern and Central Africa
Red-bellied parrot	*Poicephalus rufiventris*	LC	Stable	East Africa
Senegal parrot	*Poicephalus senegalus*	LC	Stable	West Africa
Regent parrot	*Polytelis anthopeplus**	LC	Decreasing	Oceania
Palm cockatoo	*Probosciger aterrimus*	LC	Decreasing	Oceania and South East Asia
Red-rumped parrot	*Psephotus haematonotus*	LC	Increasing	Oceania
Red-breasted parakeet	*Psittacula alexandri*	NT	Decreasing	South East and South Central Asia
Echo parakeet	*Psittacula eques**	EN	Increasing	East Africa
Plum-headed parakeet	*Psittacula cyanocephala*	LC	Decreasing	South Asia
Lord Derby’s parakeet	*Psittacula derbiana*	NT	Decreasing	East Asia
Alexandrine parakeet	*Psittacula eupatria*	NT	Decreasing	South East and South Central Asia
Rose-ringed parakeet	*Psittacula krameri**	LC	Increasing	West, Central, East Africa; South Central Asia
Edwards' fig-parrot	*Psittaculirostris edwardsii*	LC	Stable	Oceania
African grey parrot	*Psittacus erithacus*	VU	Decreasing	West, Central and East Africa
Timneh parrot	*Psittacus timneh*	VU	Decreasing	West Africa
Scaly-breasted lorikeet	*Trichoglossus chlorolepidotus*	LC	Stable	Oceania
Olive-headed lorikeet	*Trichoglossus euteles*	LC	Stable	South East Asia
Scarlet-breasted lorikeet	*Trichoglossus forsteni*	NT	Decreasing	South East Asia
Rainbow lorikeet	*Trichoglossus haematodus**	LC	Decreasing	Oceania and South East Asia
Deplanche's rainbow lorikeet	*Trichoglossus haematodus deplanchii**		Not assessed	Oceania
Red-collared lorikeet	*Trichoglossus rubritorquis*	LC	Decreasing	Oceania
Yellow-tailed black-cockatoo	*Zanda funerea*	LC	Stable	Oceania

Table 1.2 Prevalence estimates and the screening tests used in publications from 1984 to 2015 for both wild and captive psittacine populations

Population location	Test prevalence	Methods used	Reference
Captive			
Germany	39.2% from 32 captive breeding facilities	PCR	Rahaus & Wolff 2003
Australia	23% (PCR) / 66.7% (HA) of samples submitted by veterinarians	PCR, HA, HI	Khalesi et al. 2005
Italy	8.05% for entire national captive population	PCR	Bert et al. 2005
Taiwan	41.2% of birds submitted by private owners	PCR	Hsu, Ko & Tsai 2006
New Zealand	<7% cumulative parakeet species	PCR, Histology	Ha et al. 2009
Poland	25.3% for entire national captive population; 22.12% - small aviaries; 25.23% - medium aviaries; 25.99% - large aviaries	PCR	Piasecki & Wieliczko 2010
Costa Rica	19.7% for entire national captive population	PCR	Dolz et al. 2013
Japan	31.3% of imported birds for breeding	PCR	Ogawa et al. 2013
Poland	20.6% across 50 captive breeding facilities	PCR, Whole genome sequencing	Julian et al. 2013
Wild			
Australia	*Cacatua galerita* - 10 - 20% (200 - 1000 individuals) over 4 years	Histology	McOrist et al. 1984.
New Zealand	*Platycercus eximius* - 8.6-20.4%, *Cacatua galerita* - 22-33%	PCR, Histology	Ha et al. 2007
New Zealand	4–7 % across all native species	PCR, Histology	Ha et al. 2009
New Zealand	*Cyanoramphus novaezalandiae* - 28%	PCR	Ortiz-Catedral et al. 2009
New Zealand	*Cyanoramphus novaezalandiae* - 10.5 % (95 % CI: 6.1 %–16.4 %); *Cyanoramphus auriceps* - 26.7 % (95 % CI 12.3 %–45.9 %); *Platycercus eximius* - 22.9 % (95 % CI 9.9 %–42.3 %)	PCR, Whole genome sequencing	Massaro et al. 2012
Mauritius	*Psittacula eques* - 2004/05 - 38%; 2005/06 - 41%; 2006/07 - 11%; 2007/08 - 25%; 2008/09 - 17%	PCR	Kundu et al. 2012.
New Caledonia	*Trichoglossus haematodus deplanchii* - 25% (11-45%)	PCR, Whole genome sequencing	Jackson et al. 2014a
Australia	*Platycercus elegans* - 45-50%; *Platycercus elegans adelaidae* - 95-100%; *Platycercus elegans flaveoulus* - 18-22%, WS hybrids - 8-10%	qPCR, HI	Eastwood et al. 2014

Table 1.3 A summary of all methods used in screening for BFDV in wild and captive psittacine populations, a count of how many times each has been used and examples of where each has been applied.

Method	Description	Times used	Example references
Blocking ELISA	A blocking ELISA is a method used to immobilize biomolecules, primarily proteins, to a plate via passive or covalent interactions, minimising non-specific binding to the surface by saturating unoccupied binding sites with a blocking reagent.	1	Shearer et al. 2009b
DNA in-situ hybridization	DNA in-situ hybridization is a technique used in the localisation of specific nucleic acid targets within fixed tissues and cells using an oligonucleotide probe before microscopically visualizing the results.	4	Greenacre et al. 1992; Ramis et al. 1998
Dot-blot DNA hybridization	Dot blot hybridization is a technique used to determine the abundance of certain DNA in an extraction dotted onto a membrane through hybridization with universal and specific oligonucleotide probes.	2	Latimer et al. 1992; Latimer et al. 1993
Duplex shuttle PCR	Duplex PCR is a process that allows the co-amplification of separate regions of a gene in a single PCR reaction using different pairs of primers in the same reaction mixture.	1	Ogawa, Yamaguchi & Fukushi 2005
Endochrinological response	Endochrinological response is a method used to challenge the host immune system with a hormone that encourages the production and release of a stress hormone to evaluate whether any differences exist between healthy and infected individuals.	1	Jacobson et al. 1986
Haemagglutination Assay	Haemagglutination assay (HA) is a method used to quantify the amount of virus attached to molecules on the surface of host red blood cells in a series of dilutions of a viral suspension.	12	Raidal & Cross 1994; Sanada & Sanada 2000
Haemagglutination Inhibition	A modified version of the HA where a standard amount of virus and host blood cells are used with the addition of a serially diluted antiserum to determine which concentration inhibits agglutination of the cells.	12	Ritchie et al. 1991a; Khalesi et al. 2005
Haematology	Haematology is the study of the morphology and physiology of blood and, in this context, relates to the diagnosis and monitoring of diseases present in the blood stream.	3	Jacobson et al., 1986; Jergens, Brown & England 1988

Histology	Histology is the microscopal examination of stained tissues and is applied in the screening of BFDV to determine if viral inclusion bodies are present. Techniques include light and electron microscopy.	28	Kiatipattanasakul-Banlunara et al., 2002; Shearer et al., 2008.
Immunohistochemical tests	Immunohistochemistry (IHC) is a technique used to observe the physical characteristics of antibodies, their concentration and distribution within host tissue. In screening for BFDV specimens are stained using avidin-biotin complex (ABC) immunoperoxidase technique and then exposed to a primary antibody.	5	Ramis et al. 1994; Robino et al. 2014
Quantitative (Real Time) PCR	Quantitative (or real-time) polymerase chain reaction (qPCR) is a technique used to both amplify and quantify target DNA through the use of either: non-specific fluorescent dyes that intercalate with double-stranded DNA, or with a sequence-specific fluorescent probe that hybridizes with the target.	6	Eastwood et al. 2014; Regnard et al. 2015b
Standard PCR	Polymerase chain reaction (PCR) is a technology used to amplify a piece of DNA across several orders of magnitude through a process of thermal cycling in combination with oligonucleotide probes synthesised to bind to the target region and a DNA polymerase enzyme.	41	Ypelaar et al. 1999; Kondiah, Albertyn & Bragg 2005
Viral Purification	Viral purification allows for the careful study of viral synthesis within cells by combining ultracentrifugation, adsorption chromatography, electrophoresis, and partition in liquid phases to separate virions from incomplete protein fragments and cell debris.	3	Ritchie 1990; Raidal & Cross 1994
Whole genome sequencing	Whole genome sequencing is a laboratory process that determines the complete DNA sequence of an organism's genome at a single time and can be used for multiple tissue types and when only very small quantities of a full DNA copy are present.	22	Ortiz-Catedral et al. 2010; Sarker et al. 2013a

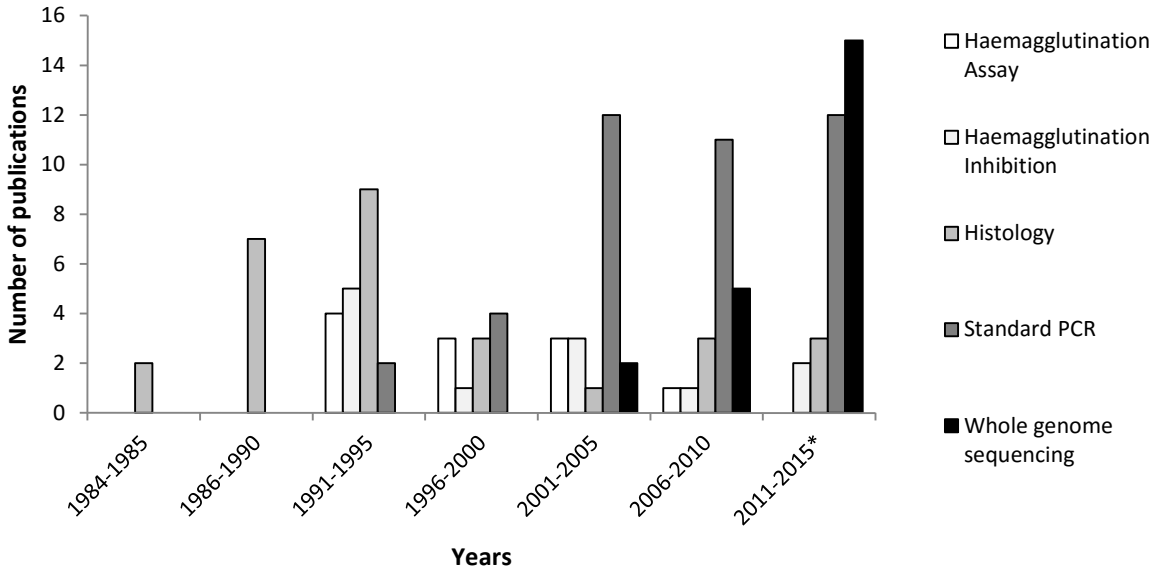

Figure 1.4 The five most common screening and diagnostic methods used from 1984 to July 2015 in BFDV and PBFD based screening publications.

CHAPTER 2: Optimisation of the standard PCR diagnostic test for the Beak and Feather Disease Virus.

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ABSTRACT

Accurate screening when assessing the prevalence and impacts of infectious disease in wildlife populations is crucial for the construction of management guidelines. However, the data collected and used in these circumstances often vary in assessment method with imperfect diagnostic tests providing an incomplete or biased insight into infection incidence within a population. One such pathogen of concern to conservationists is the Beak and Feather Disease Virus (BFDV); responsible for Psittacine Beak and Feather Disease (PBFD). This study aims to reduce uncertainty by quantifying the role that DNA concentration has in detection accuracy when screening for BFDV using the standard PCR assay. No significant differences were present in BFDV detection between highly variable undiluted blood extractions and those screened at standardised concentrations of approximately 50 ng/µl by NanoDrop, or approximately 25 ng/µl by Qubit. At lower concentrations the margin of error surrounding detection is too large to provide an accurate assay of infection at the individual level or within the affected study population. If DNA is quantified prior to screening more reliable results can be used for modelling overall prevalence. This is important when assessing reintroduction and recovery programmes of threatened wild-populations, the management of captive flocks for conservation and the trade in companion birds.

KEY WORDS BFDV, test prevalence, uncertainty, quantification, diagnostic odds ratio

2.1 INTRODUCTION

Population biologists recognize infectious disease as an integral and constant mechanism within natural populations, alongside processes such as predation and competition (Lyles & Dobson 1993). Pathogens act as powerful agents of natural selection and ecosystem regulation, altering whole species compositions and host population genetic diversity (Altizer, Harvell & Friedle 2003). However, emerging infectious diseases (EIDs), defined as those caused by newly discovered pathogens or with increased incidence or range expansion (Daszak, Cunningham & Hyatt 2001; Dobson & Foufopoulos 2001; Morens, Folkers & Fauci 2004) may increase the risk of extinction for vulnerable species and populations, thereby reducing global biodiversity (Lips et al. 2006). Pathogens generally have short generation times which allow them to adapt rapidly to novel hosts (Altizer, Harvell & Friedle 2003), and amongst these, single-stranded DNA viruses have some of the highest rates of mutation (Duffy, Shackleton & Holmes 2008). Consequently many EIDs are caused by viruses which are infectious across a wide host range (Altizer, Harvell & Friedle 2003).

Assessing the prevalence and impacts of infectious disease in free-living wildlife populations can be challenging, particularly during the outbreak of a novel pathogen (Artois et al. 2001). Reliable and accurate screening of individual infection status is crucial in the construction of management guidelines. However, the data collected and used in these circumstances often vary in sampling or assessment method, frequently with imperfect diagnostic tests, therefore providing an incomplete or biased insight into infection incidence within a population (Williams & Moffitt 2010; Lachish et al. 2012). For example, some pathogens, such as the bacterium responsible for bovine tuberculosis (bTB), have limitations to detection in vivo at the individual level (Schiller et al. 2010). This is problematic when trying to manage affected endangered wildlife populations like the Iberian lynx, *Lynx pardinus*. Remaining populations of this species are small, divided, in decline and only remain in isolated areas of Spain and Portugal which makes them difficult to monitor (Briones et al. 2000). Others, such as amphibian chytridiomycosis, are comparatively easy to detect in an infected individual through a variety of screening techniques (Kriger, Hero & Ashton 2006) but many of these are time consuming, relatively expensive or impractical when working with small or endangered populations (Knapp & Morgan 2006). Additionally

conservationists need to correctly interpret the value of pathogen prevalence results and apply them to a managed system, but this is not straightforward as infection incidence and the presentation of clinical disease are fundamentally different (McCallum & Dobson 2002). An individual can be infected with a pathogen without being adversely affected by it (Cunningham 1996), which may make prevalence a poor indicator of impact (Tollington et al. 2015). Modelling is often used to estimate the overall prevalence (proportion of individuals in a population that are infected), but assumes that data derived from screened individuals consists of results assigned to a category of pathogen-positive or -negative without misclassification error (Conn & Cooch 2009). Conservation managers need to both acknowledge and incorporate these uncertainties into their planning processes and management approach instead of simply assigning a classification status to an individual.

One such pathogen of concern to conservationists is the Beak and Feather Disease Virus (BFDV); responsible for Psittacine Beak and Feather Disease (PBFD) which is the most common viral disease in wild psittaciformes (Khalesi et al. 2005). The disease has been implicated in the decline of free-living parrot populations in Australia (Peters et al. 2014) and Mauritius (Kundu et al. 2012) and has now been listed by the Australian government as a "Key Threatening Process" to biodiversity (Eastwood et al. 2015). PBFD, first described in the 1970s, originated in the South Pacific (Ritchie et al. 1989a; Latimer et al. 1991; Heath et al. 2004) and subsequently spread rapidly across the world. Globally, parrots are one of the most vulnerable avian taxa with over a quarter of all extant species classified within threatened categories by the International Union for Conservation of Nature; 75% of which are in population decline (IUCN 2015). BFDV has been reported in a total of 88 species, both Old and New World (Chapter 1), including many Endangered or Critically Endangered species such as Cape parrots (*Poicephalus robustus*) (Regnard et al. 2015), swift parrots (*Lathamus discolor*) (Sarker et al. 2013), orange-bellied parrots (*Neophema chrysogaster*) (Peters et al. 2014), and Mauritius parakeets (*Psittacula eques*) (Kundu et al. 2012).

BFDV belongs to the family Circoviridae, comprising a circular, single-stranded, approximately 2000 nucleotide long DNA genome and lacking a non-coding region (Ritchie et al. 1989b). It contains a highly conserved replication associated protein (*replicase* gene) (Kondiah, Albertyn & Bragg 2006; Kundu et al. 2012; Peters et al. 2014) and a *capsid* protein responsible for viral encapsidation and host cell penetration (Heath et al. 2004; Kundu et al.

2012). PBFD is typically characterized by chronic symmetrical feather abnormalities and dystrophy but can also induce severe claw and beak deformities (Latimer et al. 1991; Bassami et al. 1998; Heath et al. 2004; Kondiah, Albertyn & Bragg 2006) and immunosuppression (Ritchie et al. 1989a; Ritchie, Anderson & Lambert 2003; Kondiah, Albertyn & Bragg 2006; Peters et al. 2014). BFDV has demonstrated high environmental persistence owing to its ability to shift between closely related host species (Peters et al. 2014) and is transmissible both horizontally; through contact with contaminated feather dust, surfaces or objects (Ritchie, Anderson & Lambert 2003), and vertically; from a female to her offspring (Ritchie et al. 1989a; Kundu et al. 2012). Whilst PBFD can be fatal and most frequently affects birds up to three years of age (Ritchie et al. 1989a), infected individuals frequently recover from acute presentation of the disease; which usually only lasts for a number of months (Todd 2000).

Screening for BFDV across global psittacine species has been conducted using a variety of tissue types; including blood, skin, faeces and feathers, as well as various methods such as histology (Shearer et al. 2008), haemagglutination (HA) (Raidal & Cross 1994) and haemagglutination inhibition assays (HI) (Khalesi et al. 2005), standard and quantitative real-time polymerase chain reaction (PCR and qPCR respectively) (Ypelaar et al. 1999; Shearer et al. 2009). A combination of both tissues and methods are commonly used for BFDV detection (for examples see Raue et al. 2004; Khalesi et al. 2005; Robino et al. 2014) but in wild populations this may be problematic as this often relies on presentation of the disease through feather lesions or dystrophy, shedding of infected feather dust or diarrhoea. Whilst samples from wild populations may be easier and require less veterinary expertise to obtain through non-invasive techniques, such as the collection of feathers, there is a higher risk of cross contamination between samples (Taberlet, Waits & Luikart 1999) and thus may increase the proportion of false-positives when screening. Also, as a primary symptom of PBFD is feather loss the collection of dropped feathers (for example from a roost site) may further bias the estimated proportion of infected individuals. Conversely, non-invasive sampling may also provide a far lower estimate of prevalence as some individuals remain asymptomatic despite carrying the virus (Ritchie et al. 1989a). This makes results obtained in screening for BFDV difficult to interpret and often a combined methodological approach is used to try to provide greater detection accuracy.

The standard PCR assay is the most frequently used single method for the detection of BFDV and is also one of the most commonly used tools in mixed methods studies (Chapter 1). Usually, for a standard PCR assay a master mix is made up consisting of target primers, $MgCl_2$, NH_4 buffer, dNTP and a polymerase catalyst which is then added to template DNA prior to amplification (Nichols, Bruford & Groombridge 2001; Raue et al. 2004; Kundu et al. 2012). However, new and improved pre-made master mixes that lack only the target primers are now readily available on the market; claiming to produce higher yields of amplified target genes for better results (for example MyTaq™ HS Red Mix from Bioline and AmpliTaq Gold® from Applied Biosystems®). The difference in detection accuracy between these two methods when screening for BFDV has not yet been compared. Whilst steps have recently been taken to improve the standard PCR protocol by quantifying DNA extraction concentrations prior to screening (Eastwood et al. 2015), also lacking in the literature is an assessment of detection accuracy at variable DNA concentrations and how this impacts the repeatability of a result. This would ensure greater confidence in results, with the ability to estimate the probability of obtaining a false-negative test result for extrapolation purposes. To date only three publications have reported confidence intervals surrounding a viral prevalence in wild populations (Ha et al. 2007; Massaro et al. 2012; Jackson et al. 2015), two of which were based on an assumed estimate of test specificity and none were provided on the basis of repeated screenings to determine test variation.

Accurate screening methods and confidence in a negative result are important when trying to understand the impacts and management of disease in a wide variety of contexts, managers of captive breeding facilities used in the pet-trade may want to increase the confidence in diagnostic tests to prevent the introduction of infected individuals into healthy collections. Similarly conservation managers may require better detection accuracy in order to increase the probability of success in establishing a disease-free base population when undertaking translocations in species recovery and reintroduction programmes. Consequently this study aims to reduce uncertainty by: testing the variation in protocols currently used in the screening for BFDV using the standard PCR assay and quantifying the role that DNA concentration has in detection accuracy. Ultimately this will provide a more reliable and repeatable diagnostic test for use across psittacine populations.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection and DNA extraction

Opportunistic blood samples were drawn from fledged Mauritius parakeets from 1993 to 2004 and then from all offspring produced annually since 2005 as part of ongoing species monitoring and management. This has provided a unique opportunity to analyse BFDV prevalence and evolution prior to, during and after an outbreak event in the 2004/05 breeding season (Kundu et al. 2012; Raisin et al. 2012; Tollington et al. 2015). These samples are collected by the by the Mauritian Wildlife Foundation's parakeet field team from 45 day old Mauritius parakeet nestlings, assigned a studbook ID and systematically recorded with standard data regarding nest site, parents and number of offspring.

Prior to screening for BFDV, an ammonium acetate DNA extraction method was used to extract both bird and BFDV DNA (Bruford et al. 1998). In brief, approximately 50 to 100 µl of whole blood was used from each sample and digested in 250 µl of DIGSOL lysis buffer with 10 µl of 10 mg/mL proteinase K. Virus specific primers were then used to determine presence of viral DNA within that of the host.

2.2.2 Determining the best master mix

The first measure undertaken to standardise and improve upon prior protocols was conducted on individuals from the 2009/10 breeding season of known BFDV infection status (S. Kundu, unpublished data). A simple comparison was made between results obtained from using a PCR master-mix consisting of a standard combination of $MgCl_2$, NH_4 buffer, dNTP and Taq polymerase, as set out by Kundu et al. (2012), and using MyTaq^{TM} HS Red Mix (Bioline; henceforth Red Mix) as a substitute. Eight individuals were tested once each using both solutions through PCR assay targeting a 717-bp region of the *replicase* gene (Ypelaar et al. 1999) comprising 1 µl of extracted DNA template, 5 µl master mix, 0.2 µl each of the forward and reverse primers at 10 pmol/µl and made up to 10 µl with double-distilled water. PCR conditions replicated those of Kundu et al. (2012) with annealing temperature set to 57^{o}C for 30 cycles and products were visualized on a 1.5% agarose gel. A negative control was included in each PCR batch to ensure no contamination was present. To ensure that the bands visualized on a gel represented a BFDV-positive result, a subset of PCR

products were sent to Macrogen Europe (Amsterdam) to obtain the sequence of the amplified *replicase* gene fragment.

2.2.3 DNA concentration standardisation

A study of repeatability was conducted on a matrix of 15 individuals of known BFDV infection status from the 2009/10 breeding season (S. Kundu, unpublished data) in which: each blood sample was extracted for DNA in four separate batches, with six PCR screenings run on each extraction, at three different concentrations: undiluted and unstandardised DNA template and two lower, standardised concentrations. Extraction concentrations were quantified using both a NanoDrop and a Qubit Fluorometer (Qubit dsDNA Assay Kit). The Qubit system is considered to be far more accurate than the simple UV spectrophotometric NanoDrop as it is able to distinguish between DNA, RNA and other contaminants that may present in the extraction through the use of photosensitive dyes (O'Neill et al. 2011). However, the NanoDrop technology attempts to account for this issue through the 260/280 and 260/230 absorbance ratios which are an indication of the ratio of proteins or peptide bonds to DNA present in solution. To determine the presence of any significant variation in the values obtained via each quantification tool, a paired t-test was performed in R3.2.1. (R Core Team 2015)

2.2.4 Tests of repeatability

The repeated sample matrix thus consisted of 24 repeat BFDV screenings performed per individual at each of the three concentrations (Undiluted, Dilution H and Dilution L; Table 2.1), totalling 1080 data points (Figure 2.1). The PCR products were run out on a 1.5% agarose gel and analysed through visual inspection and the presence/absence results recorded. During visual interpretation of the gels it became apparent that there was notable variation in band intensity and based on this results were classified into: "dark" positives which were invariably present at every concentration, "light" positives which were clear but much fainter bands of variable intensity and "absent" (Figure 2.2). To ensure that the light, variable bands represented a BFDV-positive result, a subset of PCR products were sent to Macrogen Europe (Amsterdam) to obtain the sequence of the amplified *replicase* gene fragment.

Mark-Recapture models have been used in other viral repeatability studies as a method of determining the probability of recording a false-negative (for example see

Lachish et al. 2012). However, this approach was not a suitable means of analysis for the dataset because of low encounter rates (high number of zero values) recorded at lower DNA concentrations. It was common to observe no "capture" events across all 24 repeats for an individual; valuable data that would have been discounted to make the model possible and consequently reducing the false-negative estimate. Instead, results were analysed in R3.2.1. (R Core Team 2015) using a logistic regression (1 = Positive, 0 = Negative) to determine whether viral detection at each of the two standardised concentrations differed significantly from a baseline of the higher, but variable concentration undiluted DNA template. The probability of obtaining a negative PCR result was calculated for each of the standardised concentrations as follows where: P is the probability of obtaining a negative result at a given concentration, α is the *Y* intercept and β is the regression coefficient at each concentration.

$$\mathrm{P}(\mathit{Negative}|\mathit{Concentration}) = \frac{e^{\alpha + \beta_1 \dot{X}_1 + \beta_2 \dot{X}_2}}{1 + e^{\alpha + \beta_1 \dot{X}_1 + \beta_2 \dot{X}_2}}$$

The results were then converted into a diagnostic odds ratio (Deeks 2001) of increased likelihood of recording a negative BFDV result at each standardised concentration where: Act. Prop. Neg. is the cumulative proportion of negatives present when the undiluted DNA template was screened.

$$\mathrm{OR}_{\mathrm{Conc.1}} = \frac{\mathrm{P(Negative|Conc.1)} \,/\, 1 - \mathrm{P(Negative|Conc.1)}}{\mathrm{Act.\ Prop.\ Neg.} \,/\, 1 - \mathrm{Act.\ Prop.\ Neg.}}$$

2.3 RESULTS

Red Mix was found to produce consistently clearer BFDV-positive bands than a manually produced master mix when visualized on an agarose gel post-amplification. This made the assignment of infection status less ambiguous and consequently all further screenings were conducted using Red Mix.

When assessing the differences between quantification tools it was found that the extraction concentrations provided by the NanoDrop were consistently significantly lower than those produced by the Qubit Fluorometer across samples (Table 2.1).

The logistic regression determined that the number of BFDV-positive results recorded at standardised concentration Dilution L were significantly lower than in variable

undiluted DNA template, with the odds of recording a false-negative calculated to be 1.66 times higher. Whilst the number of negatives recorded at standardised concentration Dilution H was also lower, this difference was not significant as the odds of recording a false-negative at these standardised concentrations was only 1.04 times higher than when undiluted (Table 2.2, 2.3). For example: in a sample of 100 individuals where the actual number of positives is approximately 93 individuals, when DNA concentrations are standardised to Dilution L you would expect to obtain approximately 31 positives (95% CI: 22 – 40) as opposed to 48 (95% CI: 39 – 58) in variable undiluted DNA template. Where DNA concentrations are standardised to Dilution H you would expect to obtain approximately 47 positives (95% CI 37 – 56).

2.4 DISCUSSION

Conservationists and managers of wildlife populations use BFDV screening results to improve and inform processes and protocols. For example, the Mauritian Wildlife Foundation has recently translocated the first cohort of a new sub-population of Mauritius parakeets to the east of the island. However this was undertaken only after carefully monitoring fledglings for clinical signs of PBFD and screening individuals selected for translocation before being moved and four months after (D. F. personal observation). Careful selection and screening protocols are required as PBFD is suspected to have been responsible for the failure of a reintroduction attempt in 2004/2005 when 32 out of 36 released individuals disappeared after presenting with clinical signs (Tollington et al. 2013; 2015). Similarly, in New Zealand, the management and translocation of red-crowned parakeets (*Cyanoramphus novaezelandiae*) is also heavily dictated by the understanding of BFDV prevalence and impacts on the island, as well as the potential reservoir species in which it occurs (Jackson et al. 2015). In situations such as these, screening results are a major component upon which decisions are based – a system that could hinder overarching recovery objectives if uncertainty was so high that results were uninformative.

2.4.1 Use of a pre-made master mix

The use of a pre-made master mix specifically designed for higher specificity and better amplification does appear to produce consistently clearer results. Not only would using a pre-made master mix reduce the ambiguity surrounding a lighter band on a gel, but also the time taken to set up the reaction in the laboratory and the risk of contamination.

Added benefits of only using a single solution of reagents that has already been designed with specificity in mind is that further optimization of NH_4 and $MgCl_2$ concentrations are not required, thus also reducing the risk of human error each time a PCR is run.

2.4.2 DNA yields and concentration standardisation

The significant differences in BFDV detection between DNA concentrations highlight an area of concern with PCR screening protocols currently used by researchers and practitioners working within BFDV affected systems. Extracted DNA samples can vary greatly in yield depending on the type and amount of tissue used. Feathers typically produce very low genomic DNA yields, particularly when extracted from those that are cut off from the blood supply once fully grown (De Volo 2008), only representing viral incidence during the initial growth phase. Whole (avian) blood samples, however, can produce good quality, high concentration extraction products: often in excess of 200 ng/µl, depending on the initial sample volume (D. F. personal observation). Concentrations can considerably affect the sensitivity of PCR assay (Khalesi et al. 2005) as the amount of viral DNA obtained from any sample will be dependent on the infection level within the host at the time of sampling (Kriger, Hero & Ashton 2006; Knowles et al. 2011; Lachish et al. 2012), making higher DNA yields preferable to increase the probability of detection. False-negatives from PCR assays can also occur if the quality of the extraction is low and other biological materials interfere with amplification (Khalesi et al. 2005). Whilst feathers from birds shedding contaminated feather dust can be used to accurately screen for BFDV due to high viral load (Hess, Scope & Heincz 2004), this may not always be suitable, particularly in the case of nestlings or asymptomatic individuals.

As there was no significant difference in BFDV detection between highly variable undiluted blood extractions and those screened at standardised concentrations of approximately 50 ng/µl by NanoDrop, or approximately 25 ng/µl by Qubit, we recommend that all standard PCR assays should be conducted on quantified, standardised DNA template at this concentration. At lower concentrations the margin of error surrounding detection is too large to provide an accurate assay of infection at the individual level or within the affected study population. If DNA extractions are quantified prior to BFDV screening and the quality or concentration is low, it provides the researcher with a solid basis on which to either discount that individual from population estimates, re-extract if sample volume

allows, or repeat screen the individual to gain confidence in any result obtained. However, as the Mauritius parakeet population represents a unique system from which it is currently relatively easy to obtain blood samples from all annually produced offspring, we acknowledge that this protocol may not be readily applicable to other wild study populations. A similar repeatability study should thus be conducted in future on quantified DNA extractions from feather samples of both symptomatic and asymptomatic individuals and paired with blood screening results to provide estimates in detection error when only this tissue type is available for analysis.

To remove some of the uncertainty surrounding standard PCR based assays quantitative (real-time) PCR techniques are now being more regularly applied to determine individual viral load (Shearer et al. 2009, Eastwood et al. 2015, Regnard et al. 2015). Probe-based assays are able to detect viral DNA at much lower concentrations than recorded by the naked eye when visualizing a gel. However, the reagents and equipment required for screening through standard PCR are currently substantially cheaper than those used for probe-based assays and are thus likely to have continued wide-spread use for the purpose of general BFDV screening.

2.4.3 Conclusion

There are still elements of the standard PCR screening and analysis process that require refinement, particularly regarding repeatability of results when screening tissue types that produce lower DNA extraction yields than blood. Despite the generally high DNA yield from blood samples, the detection of BFDV in 100 samples at the variable undiluted concentrations used for this study is still approximately 50% lower than the actual prevalence. Consequently, if standardised protocols surrounding DNA quantification prior to screening are applied within the laboratory more reliable results can be used for modelling overall prevalence within a host population and provide greater confidence in the margin of error when assigning infection status to an individual. This information is particularly important when assessing reintroduction and recovery programmes of threatened wild-populations but is also valuable when considering the management of captive flocks for conservation, as well as the breeding of and trade in companion birds.

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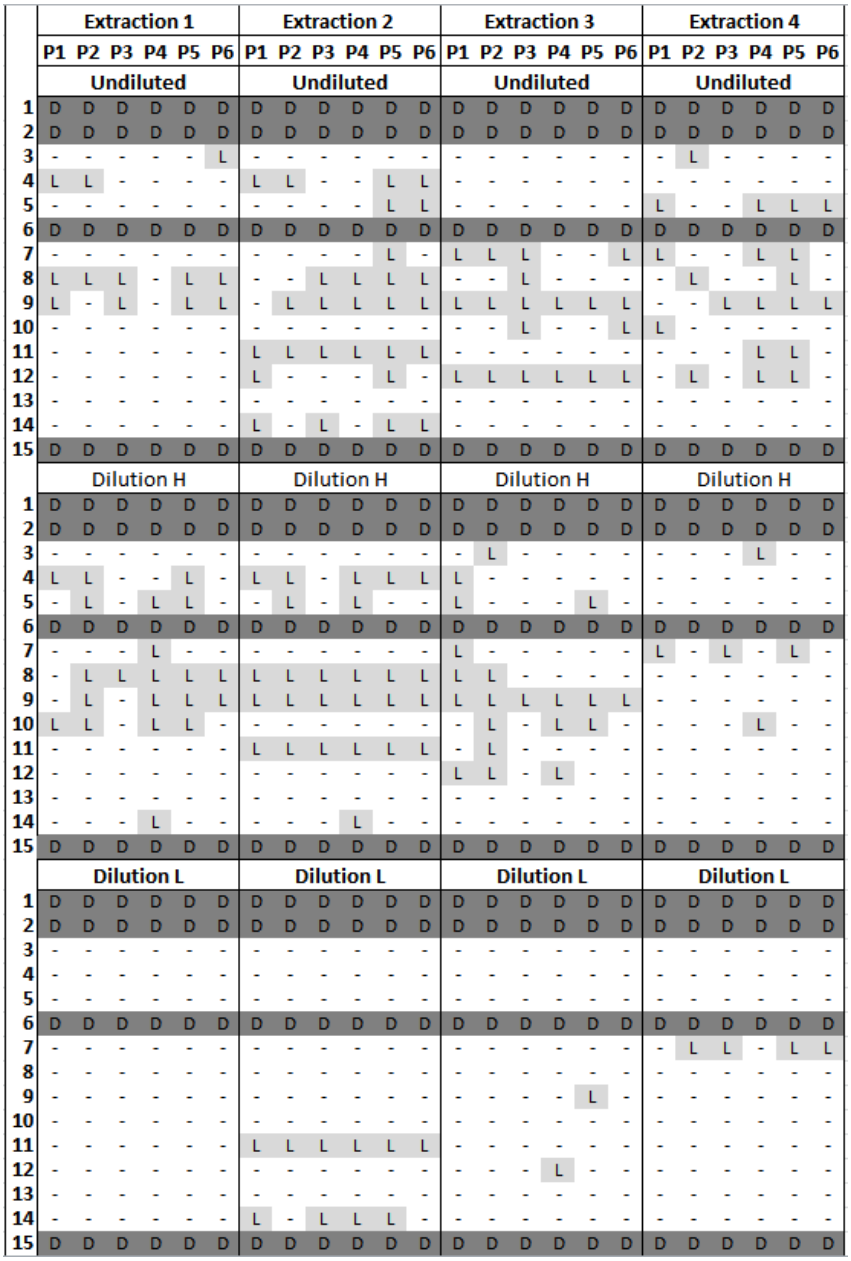
2.6 FIGURES AND TABLES

Table 2.1 Mean DNA extraction concentrations from blood according to the NanoDrop and Qubit Flourometer when undiluted and at two standardised diluted concentrations where Dilution H represents the higher standardised concentration and Dilution L represents the lower standardised concentration.

Concentration	Ẋ NanoDrop	Ẋ Qubit	δ NanoDrop	δ Qubit	df	p
Undiluted	169.70	61.37	94.45	37.20	58	<0.001
Dilution H	53.77	24.23	2.66	18.97	58	<0.001
Dilution L	15.56	7.448	2.79	6.02	58	<0.001



	Extraction 1						Extraction 2						Extraction 3						Extraction 4					
	P1	P2	P3	P4	P5	P6	P1	P2	P3	P4	P5	P6	P1	P2	P3	P4	P5	P6	P1	P2	P3	P4	P5	P6
	Undiluted						Undiluted						Undiluted						Undiluted					
1	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
2	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
3	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-
4	L	L	-	-	-	-	L	L	-	-	L	L	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	L	L	-	-	-	-	-	-	L	-	-	L	L	L
6	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
7	-	-	-	-	-	-	-	-	-	-	L	-	L	L	L	-	-	L	L	-	-	L	L	-
8	L	L	L	-	L	L	-	-	L	L	L	L	-	-	L	-	-	-	-	L	-	-	L	-
9	L	-	L	-	L	L	-	L	L	L	L	L	L	L	L	L	L	L	-	-	L	L	L	L
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	L	L	-	-	-	-	-
11	-	-	-	-	-	-	L	L	L	L	L	L	-	-	-	-	-	-	-	-	-	L	L	-
12	-	-	-	-	-	-	L	-	-	-	L	-	L	L	L	L	L	L	-	L	-	L	L	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	L	-	L	-	L	L	-	-	-	-	-	-	-	-	-	-	-	-
15	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
	Dilution H						Dilution H						Dilution H						Dilution H					
1	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
2	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
3	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	L	-	-
4	L	L	-	-	L	-	L	L	-	L	L	L	L	-	-	-	-	-	-	-	-	-	-	-
5	-	L	-	L	L	-	-	L	-	L	-	-	L	-	-	-	L	-	-	-	-	-	-	-
6	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
7	-	-	-	L	-	-	-	-	-	-	-	-	L	-	-	-	-	-	L	-	L	-	L	-
8	-	L	L	L	L	L	L	L	L	L	L	L	L	L	-	-	-	-	-	-	-	-	-	-
9	-	L	-	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	-	-	-	-	-	-
10	L	L	-	L	L	-	-	-	-	-	-	-	-	L	-	L	L	-	-	-	-	L	-	-
11	-	-	-	-	-	-	L	L	L	L	L	L	-	L	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	L	L	-	L	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	L	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
	Dilution L						Dilution L						Dilution L						Dilution L					
1	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
2	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	L	-	L	L
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	L	L	L	L	L	L	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	L	-	L	L	L	-	-	-	-	-	-	-	-	-	-	-	-	-
15	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D

Figure 2.1 Screening results matrix of 15 individuals of known BFDV infection status using Undiluted, Dilution H and Dilution L DNA extraction concentrations, where each individual was screened six times over each of four repeat DNA extractions from the same blood sample. “D” – Dark Positive, “L” – Light Positive, “-“ – Negative.

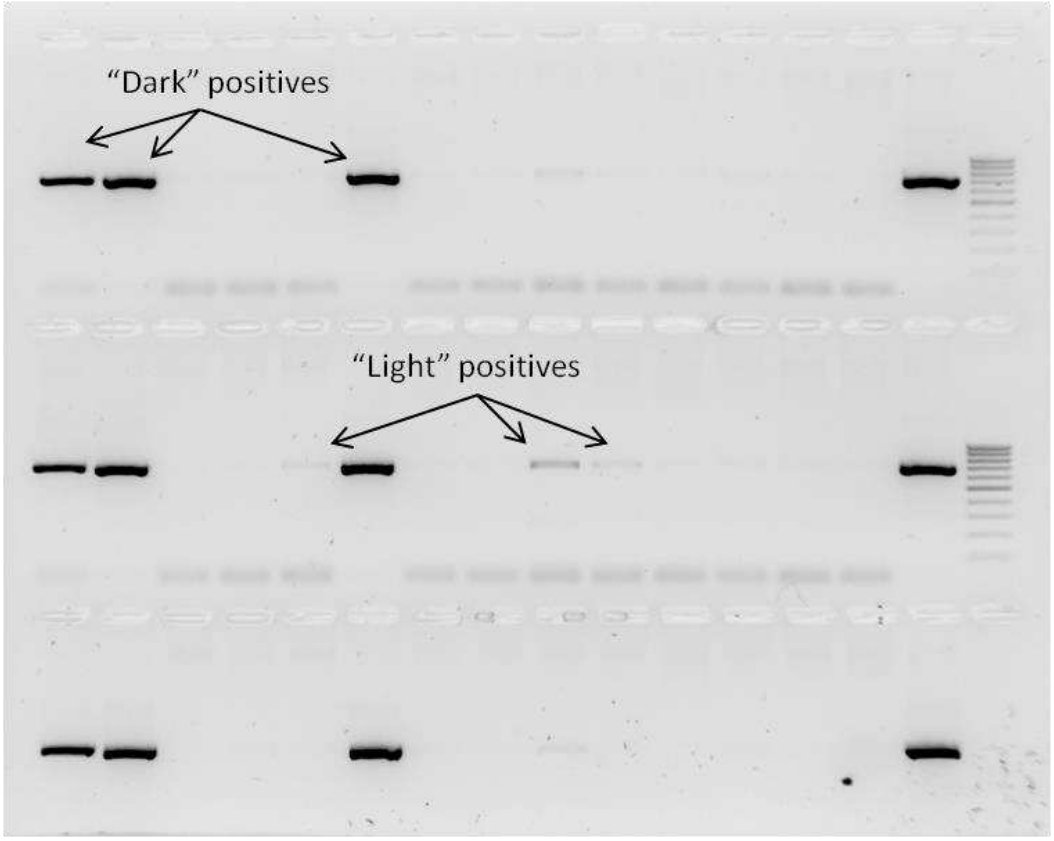


Figure 2.2 Visualisation of PCR products on an agarose gel depicting the classification of BFDV screening results into "dark" positives which were invariably present at every concentration and "light" positives which were clear but much fainter bands of variable intensity.

Table 2.2 Cumulative BFDV screening results of 24 repeat PCRs for each of 15 individuals at three DNA concentrations where: Dilution H is approximately 50 ng/µl (NanoDrop) or 25ng/µl (Qubit) and Dilution L is approximately 16 ng/µl (NanoDrop) or 7ng/µl (Qubit).

Concentration	No. Positives	No. Negatives	Proportion Positives	Proportion Negatives
Undiluted	175	185	0.49	0.51
Dilution H	168	192	0.47	0.53
Dilution L	112	248	0.31	0.69

Table 2.3 Logistic regression analysis of the deviation in BFDV detection from the Constant (undiluted extracted DNA) when DNA template is diluted to approximately 50 ng/µl (Dilution H - NanoDrop) or 25ng/µl (Dilution H - Qubit) and approximately 16 ng/µl (Dilution L - NanoDrop) or 7ng/µl (Dilution L - Qubit).

Predictor	**β**	**SE β**	**Z**	**p**
Constant (Undiluted)	0.056	0.105	-0.527	0.598
Dilution H	0.078	0.149	-0.522	0.601
Dilution L	0.739	0.155	-4.765	<0.001

CHAPTER 3: To clean or not to clean? Experimental evaluation of transmission prevention protocols for a highly infectious viral pathogen in an endangered species

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ABSTRACT

Psittacine Beak and Feather Disease (PBFD), the most common viral disease in wild parrots (Psittaciformes), currently affects numerous endangered island endemics including the Mauritius parakeet (*Psittacula eques*). An outbreak of PBFD in Mauritius interrupted active management for the recovery of the parakeet population and a set of standard biosecurity protocols was implemented in an attempt to reduce human-facilitated viral transmission. This study is the first to assess the efficacy of Beak and Feather Disease Virus (BFDV) biosecurity protocols in a parrot population. We investigated relationships between population management tools, cleaning protocols and viral prevalence in annually produced nestlings; through both a reciprocal design experiment and in the context of a 10-year temporal dataset on viral prevalence. Proximity of nests to a supplemental feeding hopper, alongside differences between subpopulations, explained annual viral prevalence. Reciprocal field experiments revealed that cleaning protocols successfully reduced viral prevalence in nestlings, and particularly in nests further away from feeding hoppers. Our findings indicate that the disinfection of nest sites between breeding seasons and wearing disposable medical barrier when accessing nest sites may reduce viral prevalence in the Mauritius parakeet population. Similar adaptive management solutions may be necessary for the recovery of other endangered species affected by infectious disease.

KEY WORDS Biosecurity, PBFD, population management, parrot, hygiene protocols, experiment

3.1 INTRODUCTION

Psittaciformes (parrots) are one of the most vulnerable avian taxa with over a quarter of all extant species Red-listed as in need of conservation efforts by the International Union for Conservation of Nature, 75% of which are in population decline (IUCN 2015). One major threat to parrots has been the emergence and global spread of Psittacine Beak and Feather Disease (PBFD), the most common viral disease in wild parrots. Caused by the Beak and Feather Disease Virus (BFDV), the disease has been implicated in the decline of wild parrot populations and has now been listed by the Australian government as a "Key Threatening Process" to biodiversity (Eastwood et al. 2015). PBFD was first described in the mid-1970s, originating in the South Pacific (Ritchie et al. 1989a; Latimer et al. 1991; Heath et al. 2004) and spreading rapidly across the world. Affected parrot species include many endangered island endemics such as the kakapo (*Strigops habroptilus*) and kaka (*Nestor meridionalis*) of New Zealand (Massaro et al. 2012), the Australian orange-bellied parrot (*Neophema chrysogaster*) (Peters et al. 2014) and the Mauritius (or "echo") parakeet (*Psittacula eques*) (Kundu et al. 2012). Small, isolated populations such as those of island endemics are considered to be particularly vulnerable to emerging infectious diseases as their populations frequently have low genetic diversity (Wikelski et al. 2004; Carrete et al. 2009; Trinkel et al. 2011) and have usually evolved in the presence of an impoverished pathogen community (Wikelski et al. 2004; Carrete et al. 2009; Spurgin et al. 2012).

PBFD is typically characterized by chronic symmetrical feather abnormalities and dystrophy but can also induce severe claw and beak deformities (Latimer et al. 1991; Bassami et al. 1998; Heath et al. 2004; Kondiah, Albertyn & Bragg 2006) and its immunosuppressant nature increases host susceptibility to secondary infection (Ritchie et al. 1989a; Ritchie, Anderson & Lambert 2003; Kondiah, Albertyn & Bragg 2006; Peters et al. 2014). BFDV belongs to the family Circoviridae and it comprises a circular, single-stranded DNA genome, which is approximately 2000 nucleotides long and lacks a non-coding region (Ritchie et al. 1989b). It contains a highly conserved replication associated protein (*replicase* gene) (Kondiah, Albertyn & Bragg 2006, Kundu et al. 2012; Peters et al. 2014) and a *capsid* protein responsible for viral encapsidation and host cell penetration (Heath et al. 2004; Kundu et al. 2012). BFDV is considered to demonstrate high environmental persistence owing to its ability to infect a broad range of closely related host species (Peters et al. 2014) and is transmissible both horizontally (through contact with contaminated feather dust,

surfaces or objects; Ritchie, Anderson & Lambert 2003), and vertically (from a female to her offspring; Ritchie et al. 1989a; Kundu et al. 2012). Whilst PBFD can be fatal and most commonly affects birds up to three years of age (Ritchie 1989a), infected individuals frequently recover from acute presentation of the disease, which usually lasts only for a number of months (Todd 2000). Other individuals may not display any clinical signs of infection despite carrying the virus (Ritchie 1989a).

The Mauritius parakeet was once the world's rarest parrot (Jones 1987; Kundu et al. 2012). The species experienced a severe population bottleneck after declining to fewer than 20 individuals in the early 1980s (Duffy 1993; Lovegrove, Nieuwland & Green 1995) due to introduced predators and habitat loss (Tatayah et al. 2007). Intensive conservation management has been conducted on the population since the mid-1970s in an attempt to rapidly increase population numbers, intensifying in 1987 through the collaborative efforts of the Mauritian Wildlife Foundation (MWF), the National Parks and Conservation Service (NPCS), Durrell (Jersey, UK) and a number of other external organisations (Tatayah et al. 2007; Raisin et al. 2012). Intensive management included brood manipulation, supplemental feeding, provision of artificial nest sites, captive-breeding, reintroduction, and control of invasive alien predators (Tatayah et al. 2007; Taylor & Parkin 2010). This management successfully resulted in a steady population increase to approximately 600 individuals and 102 known breeding pairs by the 2013/14 breeding season (Henshaw et al. 2014). However, these efforts were interrupted by a severe outbreak of PBFD in 2005. Subsequent screening of historical samples using molecular probes for BFDV confirmed its presence in the population prior to this outbreak (Kundu et al. 2012). This study also detected a selective mutation in the *replicase* gene of the virus which may have enhanced its virulence, with its capacity to spread facilitated by recovery and management efforts at the time (Kundu et al. 2012). .

Following this outbreak, PBFD was considered a severe threat to the parakeet's recovery, prompting the immediate cessation of some elements of the recovery such as the transfer of individuals and eggs between nest sites, whilst the provision of artificial nest boxes, control of alien predators, the use of supplemental feeding hoppers and a minimal regime of visits to nest sites for monitoring purposes remained in place (Tollington et al. 2013). However, managers are concerned that these ongoing field activities are promoting

horizontal transmission of the virus. Two management activities are considered high risk: the provision of supplemental food in fixed feeding hoppers and visits by field staff to access nesting sites for banding and nest box maintenance. Therefore, since 2005, the field team has attempted to reduce or eliminate any potential human-mediated transmission of BFDV by restricting activities to weekly disinfection of feeding hoppers with Virex (Kilco) and a rigorous biosecurity and hygiene protocol at nest sites. This nest site protocol comprises two elements: (i) wearing medical barrier suits whilst accessing nests (disinfected with Virex between nest visits and reused about 3 times each prior to disposal), and (ii) disinfecting all nest boxes with Virex at the end of each season. However, these mitigation strategies are difficult, increasingly time consuming (with the annual addition of new nest sites to be monitored as the population grows) and expensive to implement, and it is currently not known whether they reduce overall prevalence of the virus in the population.

Here, we test whether these mitigation strategies reduce transmission of BFDV in the Mauritius parakeet population. To our knowledge this is the first study of any parrot population (both wild and captive) to assess the efficacy of BFDV transmission mitigation protocols. In this study we focus on nest site management but we account for the influence of feeding hoppers on the results. We achieve this in two steps. First we use long-term systematic sampling of the population across ten years to quantify annual fluctuations in BFDV. We predict that despite mitigation protocols there will be some variation in annual BFDV prevalence associated with climatic or environmental parameters and perhaps further viral mutation. Second, we determine experimentally whether prevention protocols are effective by using a reciprocal design experiment implemented by MWF conducted during the two most recent consecutive breeding seasons, 2013/14 and 2014/15. We predict that mitigation protocols will reduce prevalence of BFDV in nestlings from nests where current management is in place and, conversely there will be higher BFDV prevalence in nestlings from nests where no where no protocol was enforced.

3.2 MATERIALS AND METHODS

3.2.1 Longitudinal field sampling and experimental design

Blood samples have been drawn opportunistically from post-fledged Mauritius parakeets since 1993 and routinely from all 45 day old nestlings produced annually since 2005 as part of ongoing species management. Each nestling is assigned a Studbook ID which is recorded by the Mauritius parakeet field team alongside standard data regarding nest site, parents and number of offspring per nest during each breeding season (September to May). This sample regime provides a unique opportunity to screen the offspring from each breeding season for BFDV and to examine temporal trends in the host-pathogen dynamic prior to, during and after the outbreak (Kundu et al. 2012; Tollington et al. 2015) and in doing so provides important context for the field experiment.

Experimental groups were allocated based on geographic separation. Prior to the effects of intensive population management, the Mauritius parakeet individuals surviving in the Bel Ombre region (Figure 3.1, group i) represented a genetically distinct group due to their relative geographic isolation from the rest of the population (Figure 3.1, group ii) (Raisin et al. 2012). With little migration observed between these two subpopulations this distribution provided a natural division to use for the experiment. For the 2013/14 breeding season the field team wore medical barrier suits whilst accessing the nests of group ii and disinfected these nest boxes with Virex at the end of the breeding season (henceforth “treatment”), with group i used as a control where no measures were taken to reduce BFDV spread. All other processes remained as normal including blood sampling and data collection. Over the 2014/15 breeding season these groups were reversed to complete the reciprocal design of the experiment.

3.2.2 Laboratory analysis

Where present, viral DNA was extracted from 50 to 100 µl of host whole blood using a combination of DIGSOL extraction buffer and 10 mg/mL proteinase K. Extractions were quantified using a Qubit dsDNA Assay Kit and standardised to approximately 25 ng/µl prior to screening for BFDV (Chapter 2). Infection of an individual with BFDV was assessed using PCR following the protocols detailed in (Kundu et al. 2012). In brief, the PCR assay targeted a 717-bp region of the r*eplicase* gene (Ypelaar et al. 1999) and comprised 1 µl of extracted

host DNA template, 5 µl MyTaq^{TM} HS Red Mix (Bioline), 0.2 µl each of the forward and reverse primers at 10 pmol/µl and was made up to 10 µl with double-distilled water. PCR annealing temperature was set to 57^{o}C for 30 cycles and products were visualized on a 1.5% agarose gel. A negative control was included in each PCR batch to ensure no contamination was present.

3.2.3 Data analysis

3.2.3.1 Analysis of breeding season data

A total of 887 samples were screened for BFDV across all breeding seasons from 2009/10 to 2014/15 and added to an existing viral prevalence dataset initiated by Kundu et al. 2012. Combined, these data resulted in a cumulative 10-year viral prevalence dataset for all annually screened nestlings together with their corresponding life-history data. To eliminate any potential inflation in viral prevalence caused by the experiment, only infection data from the subpopulation with continued treatment were included for the 2013/14 and 2014/15 breeding seasons.

We investigated the relationships between viral prevalence in nestlings and four fixed factors in R 3.1.2 (R Core Team 2015) using the *AICcmodavg* package (Mazerolle 2012). General linear mixed models (GLMMs) were run using a binomial response variable accounting for the proportion of infected nestlings per nest site (number of positives, number negatives) and setting a binomial error distribution and a logit link function. We evaluated a set of candidate models investigating the effects of combinations of year, nest type (cavity or artificial box), distance to the nearest feeding hopper and subpopulation (Bel Ombre or Camp, henceforth BO or CA) on BFDV prevalence. Whilst supplemental feeding status of the parents of each brood is also annually recorded only one hopper is watched daily, thereby making this variable imperfectly observed. An independent-samples t-test found a significant difference in the mean distance to feeding hopper between subpopulations where adults nesting at CA (M = 0.8 km, SD = 0.86) had on average shorter distance to travel from nest-site to feeding hopper than those nesting at BO (M = 2.4 km, SD = 0.95); $t(240) = 18.06$, $p < 0.001$. Therefore the interaction between subpopulation and distance to nearest feeding hopper was also included in the candidate model set to account for this difference. Female parent was used as a random effect to account for both the vertical and horizontal viral transmission pathways, as females generally nest at the same

site year on year. In a second set of GLMMs we also considered year as a second random effect to control for the substantial annual variation in BFDV prevalence. This parameter was a means to explore the effects of the remaining management-related fixed factors as this variation is likely a result of unmeasured environmental or climatic factors. In both model sets we compared the most parsimonious models based on the AIC_c; models within 2 ΔAIC_c's were considered equally plausible.

3.2.3.2 Analysis of transmission prevention protocol experiment

In the analysis of the protocol experiment all 2013/14 and 2014/15 breeding season data were isolated from the long-term dataset. We used GLMMs with binomial errors and a logit link function to explore the effect of four factors on the proportion of BFDV infected nestlings per brood, with female included as a random effect. The experiment was designed to explore the effectiveness of treatment but our longitudinal analysis highlighted three additional factors that may directly influence or interact with its effectiveness. The candidate GLMMs therefore evaluated the relationship between BFDV prevalence and subpopulation, distance to the nearest feeding hopper, year and treatment (binary). The interactions between subpopulation and distance to nearest feeding hopper as well as treatment and distance to nearest feeding hopper were also included in the candidate model set. Year, subpopulation and treatment are all inherently linked due to the experimental design and therefore the interaction between these three factors could not be included. We selected the most parsimonious models based on the lowest AIC_c and determined AIC_c weights (*AICcmodavg* package; Mazerolle 2012); models within 2 ΔAIC_c's were considered equally plausible.

3.3 RESULTS

3.3.1 Annual fluctuation in viral prevalence

Figure 3.2a shows annual fluctuation in BFDV prevalence. The proportion of infected nestlings in the 2010/11 (38.7%) and 2013/14 (39.5%) breeding seasons was comparable to the 2005/06 (39.4%) breeding season, the first annual systematic sampling period. The model evaluating the effect of year on the proportion of infected nestlings, using the initial "outbreak" 2005/06 breeding season as a baseline, indicated significantly lower incidence in five subsequent breeding seasons: 2006/07, 2008/09, 2011/12, 2012/13 and 2014/15 (Table 3.1).

Year was the only significant predictor of viral prevalence when included as an explanatory variable (AIC_c weight = 1). However when this was evaluated as a random variable instead, BFDV prevalence was most influenced by distance to the nearest feeding hopper, subpopulation and the interaction between these two factors (Table 3.2). Distance to the nearest feeding hopper was the primary predictor of viral prevalence (Table 3.3) as females breeding closer to supplemental feeding hoppers had a significantly higher proportion of nestlings infected with BFDV. Subpopulation was significant as nestlings at BO have experienced a consistently lower viral prevalence than those at CA over the last decade (Figure 3.2 b and c). The significance of the interaction between distance and subpopulation is explained by the increased proximity of nest sites to feeding hoppers in BO. The differences in nest-type (artificial box or natural cavity) were not found to be related to viral prevalence.

3.3.2 Transmission prevention protocol experiment

The models including all factors of treatment, distance to nearest hopper, subpopulation and year had the lowest AIC_c values. The top 9 models all included the factor of year which was highly correlated with viral prevalence (Table 3.4; 3.5) as there was a drop in average proportion of infected nestlings from 42.2% in 2013/14 to 15.5% in the 2014/15 breeding season. The disease prevention protocol was the next most important predictor of BFDV prevalence (Table 3.5) where treated sites had significantly lower proportions of infected nestlings than those in the control population ($\beta = -1.189$, SE = 0.417, $\chi 2 = -2.853$, $p < 0.01$).

When the interaction between treatment and distance to nearest feeding hopper was evaluated independently, there was a highly significant negative effect of treatment on viral prevalence with increasing distance between nest site and feeding hopper ($\beta = -0.763$, SE = 0.260, $\chi 2 = -2.934$, $p < 0.01$). Within each subpopulation, whilst both showed a significant effect of treatment on reduction in viral prevalence, the effect was greater at BO ($\beta = -3.108$, SE = 0.790, $\chi 2 = -3.934$, $p < 0.001$) than at CA ($\beta = -0.723$, SE = 0.296, $\chi 2 = -2.443$, $p < 0.05$).

3.4 DISCUSSION

3.4.1 Annual fluctuation in viral prevalence

The prevalence of BFDV on Mauritius has fluctuated cyclically between periods of high and low viral prevalence since the first widespread outbreak in 2005. Cyclical seasonal or annual fluctuations in prevalence of infectious diseases in wildlife have been attributed to climatic and environmental factors or changes in behaviour of an infected host. For example, the prevalence and transmission of West Nile virus is dependent on the suitability of climatic factors for the persistence of mosquito vectors and parasitic replication (Campbell et al. 2002). For other pathogens, such as Mycoplasmal conjunctivitis, the social nature of house finches and their propensity to aggregate at domestic bird feeders for extended periods in the winter months has been linked to increased transmission (Hotchkiss et al. 2005). BFDV is thought to be highly environmentally stable (Ritchie 1995; Todd 2000; Jackson 2014) and cleaning recommendations to reduce infectivity include disinfectants such as glutaraldehyde (Raidal 2012) but it is currently unknown whether, for example, a high rainfall year would in itself reduce environmental build-up on feeding hoppers and on perches within field aviaries. The effect of reduced movement to and from feeding hoppers in wetter years should also be considered as the plumage of most birds loses the ability to shed water over a period of prolonged exposure, reducing body temperature and easy flight (Kennedy 1970). This may decrease the number of individuals using supplemental feeding hoppers if it requires flying a long distance, consequently lessening the opportunity for infection to spread. The future incorporation of climatic variables into a GLMM in combination with management related factors would allow for a better assessment of whether annual BFDV prevalence is affected by seasonal changes or the severity of rainfall events. However, this is dependent on the availability of these data from weather stations in close enough proximity to each subpopulation for reliable analysis.

The annual variation in BFDV prevalence within the Mauritius parakeet population may plausibly be attributed to a “boom-and-bust” epidemic pattern similar to other infectious diseases such as Avian influenza (Breban et al. 2009), with host-pathogen genotypic combinations playing a key role in prevalence and symptomatic presentation of disease (Thrall, Godfree & Burdon 2003). The selective sweep of a unique viral mutation in the *replicase* gene within the Mauritius parakeet population during the 2005 PBFD outbreak

may be indicative of this dynamic (Kundu et al. 2012). Further analysis of how the virus evolves on Mauritius could provide insight as to whether the peaks in viral prevalence post-2005 are associated with the accumulation and persistence of specific mutations within the genome.

BFDV can be shed through feather dust and faecal matter, making the accumulation of both in nest sites and around feeding hoppers a likely source of constant re-infection of individuals when there is an inadequate and irregular cleaning regime in place. Duck populations in North America have experienced recurrent outbreaks of Avian influenza in 2-4 year intervals due to both direct contact between individuals and virion build up in the environment through faecal matter (Breban et al. 2009). Additionally, house finches infected with Mycoplasmal conjunctivitis have been observed to remain at feeding hoppers for longer durations due to reduced feeding efficiency, allowing for a longer contact period with the surrounding environment and thereby increasing the risk of transmission to other susceptible individuals (Hotchkiss et al. 2005). The higher prevalence of BFDV in nestlings produced in nests closer to feeding hoppers is therefore unsurprising. Those individuals breeding closer to supplemental feeding hoppers are likely to feed more regularly, thus repeatedly encountering accumulated virus present in the environment directly surrounding the stations and experiencing regular contact with high viral load individuals. The shorter distances between nest sites and hoppers dispersed throughout the forest habitat at CA allows more individuals to exploit supplemental food sources on a regular basis, accounting for the higher viral prevalence year-on-year within this subpopulation.

3.4.2 Efficacy of transmission prevention protocols

The repeated measures experiment conducted from 2013-2015 confirmed that disinfection reduces BFDV prevalence, implying that cleaning of nest sites should continue after the completion of each breeding season. The treatment appears to be particularly important for those nest sites situated further away from feeding hoppers, the interpretation being that parents feeding nestlings at these nests are less likely to be frequent users of supplemental food sources. Consequently this result has important implications for the management of the BO subpopulation as they have a larger proportion of nest sites situated far away from the single feeding hopper near the field station. To continue to maintain consistently lower BFDV prevalence within this subpopulation it will be

necessary to maintain a rigorous cleaning regime and continue to wear protective suits when visiting nest sites during the breeding season. Whilst the treatment appears to be having a lesser effect within the CA subpopulation, the reduction in prevalence is still significant but there are perhaps further implications with regards to the positioning of feeding hoppers within this subpopulation. Future research focus should be placed on quantifying food taken, both in and out of breeding season, with an assessment made as to whether any of the hoppers dispersed throughout the forest inhabited by the CA subpopulation could be removed without too detrimental an impact on survival or recruitment. This could potentially aid a reduction in viral prevalence at CA as the average distance to nearest feeding hopper would be increased, making cleaning protocols more effective for a larger number of nest sites.

3.4.3 Implications for conservation

We have demonstrated that, against a 10-year backdrop of viral prevalence in an endangered bird population infected with a BFDV, cleaning protocols significantly reduce viral prevalence depending on proximity to feeding hoppers. Crucially this finding is contrary to assumptions made to date that BFDV, like chicken anaemia virus, is resistant to disinfection (Ritchie 1995; Jackson et al. 2014). These findings are not only valuable for the future management of the Mauritius parakeet population but also for the recovery of other endangered or vulnerable psittacine species. For example, the recent outbreak of a novel virulent BFDV genotype in the last remaining Australian orange-bellied parrot population has been a cause for major concern to conservationists (Peters et al. 2014). More broadly, our study illustrates how endangered species' recovery programmes can provide experimental test-beds for answering key management questions. Faced with outbreaks of infectious disease in wild populations, wildlife managers should not disregard the effectiveness of disease prevention protocols for mitigating the spread of infection.

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3.6 FIGURES AND TABLES

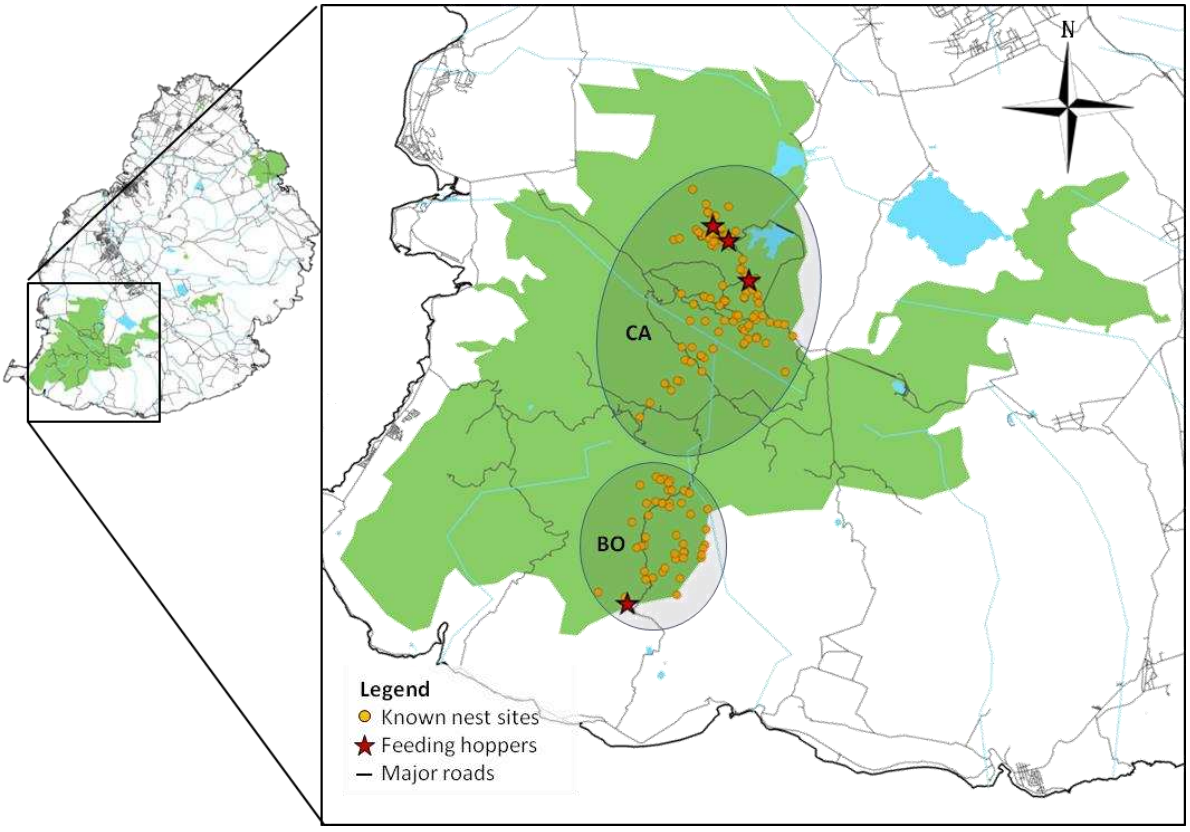

Figure 3.1 The Black River Gorges National Park with the two Mauritius parakeet subpopulations. BO = Bel Ombre (Experimental group i) and CA = Camp (Experimental group ii). Inset: location of the remaining Mauritius parakeet breeding populations in the south-west of Mauritius.

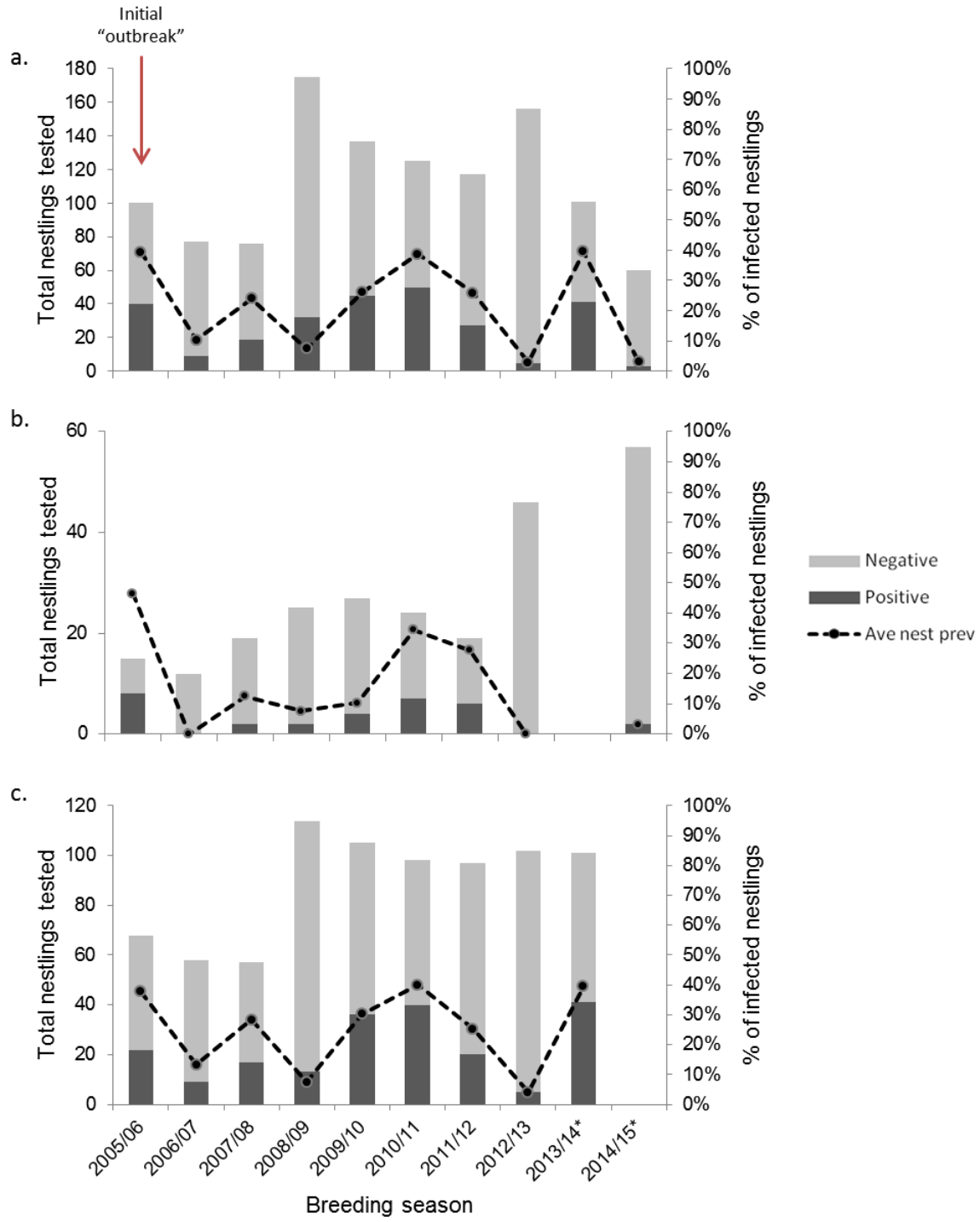

Figure 3.2 (a) Total number of 45 day old nestlings screened for BFDV from 2005 to 2015, (b) total number of 45 day old nestlings within the Bel Ombre subpopulation screened for BFDV from 2005 to 2015 (excluding the 2013/14 experimental season) and (c) total number of 45 day old nestlings within the Camp subpopulation screened for BFDV from 2005 to 2015 (excluding the 2014/15 experimental season). All categorized by positive or negative status (stacked columns), as well as the average proportion of infected nestlings per nest in each breeding season (dashed line). The initial "outbreak" breeding season has been indicated with a red arrow. *Nest site prevalence reported for 2013/14 and 2014/15 reflects only nestlings screened from sites with the cleaning protocol in place.

Table 3.1 The effect of year on the proportion of infected nestlings where the "outbreak" 2005/06 breeding season used as the baseline prevalence.

Breeding season	**β**	**SE β**	**Z**	**p**
Constant (2005/06)	-0.628	0.249	-2.521	< 0.05
2006/07	-1.377	0.438	-3.142	< 0.01
2007/08	-0.508	0.367	-1.387	0.17
2008/09	-1.592	0.368	-4.320	< 0.001
2009/10	-0.321	0.315	-1.022	0.31
2010/11	0.128	0.312	0.410	0.68
2011/12	-0.693	0.336	-2.060	< 0.05
2012/13	-2.649	0.486	-5.455	< 0.001
2013/14	0.205	0.326	0.628	0.53
2014/15	-2.767	0.767	-3.606	< 0.001

Table 3.2 A comparison of the 14 general linear mixed effect candidate models analysing the prevalence of BFDV in 45 day old Mauritius parakeet nestlings across the viral prevalence dataset extending from 2005/06 to 2014/15 (only cleaned nest sites for 2013/14 and 2014/15). Factors related to viral prevalence include: distance to the nearest feeding hopper (NH), subpopulation (SP) and nest type (NT) based on Akaike's information criterion corrected for finite sample size (AIC_c) and weights (AIC_c weights). All models were run with nesting female and year as random factors. K denotes the number of parameters in each model and models are ranked according to their ΔAIC_c.

Rank	Model	K	AIC_c	ΔAIC_c	AIC_c weights
1	NH	4	773.02	0	0.36
2	NH + SP + NH*SP	6	774.48	1.46	0.17
3	SP*NH	6	774.48	1.46	0.17
4	NH + SP	5	774.73	1.71	0.15
5	SP	4	776.53	3.51	0.06
6	NH + SP + NT + NH*SP	8	777.54	4.52	0.04
7	NH + SP + NT	7	777.8	4.77	0.03
8	NT	5	781.98	8.96	0

Table 3.3 Model average estimates for the effects of subpopulation (SP) and distance to the nearest feeding hopper (NH) on the longitudinal variation in the proportion of annually produced Mauritius parakeet nestlings infected with BFDV.

Factor	Model average estimate	SE	95% CI
SP	-0.29	0.71	-1.68 – 1.09
NH	-0.39	0.24	-0.86 – 0.09

Table 3.4 A comparison of the 23 general linear mixed effect candidate models analysing the prevalence of BFDV in 45 day old nestlings over the 2013/14 and 2014/15 Mauritius parakeet breeding seasons. Factors related to viral prevalence include: treatment (T), distance to the nearest feeding hopper (NH), subpopulation (SP) and year (Y) based on Akaike's information criterion corrected for finite sample size (AIC_c) and weights (AIC_c weights). All models were run with the nesting female as a random factor. K denotes the number of parameters in each model and models are ranked according to their ΔAIC_c.

Rank	Model	K	AIC_c	ΔAIC_c	AIC_c weights
1	T + Y + SP	5	300.69	0	0.49
2	T + NH + Y + SP	6	302.57	1.88	0.19
3	T + NH + Y + SP + T*NH	7	303.3	2.61	0.13
4	T + NH + Y	5	303.85	3.16	0.1
5	T + NH + Y + T*NH	6	305.71	5.02	0.04
6	T + Y	4	306.18	5.49	0.03
7	Y + SP	4	309.74	9.05	0.01
8	Y	3	311.28	10.59	0
9	NH + Y + SP	5	311.77	11.08	0
10	T*NH	5	322.88	22.19	0
11	T + NH + SP + T*NH	6	325	24.31	0
12	NH	3	329.52	28.83	0
13	SP	3	329.64	28.95	0
14	NH + SP	4	331.19	30.5	0
15	T + NH	4	331.24	30.55	0
16	T	3	331.64	30.95	0
17	T + NH + SP	5	333.01	32.32	0

Table 3.5 Model average estimates for the effects of treatment (T), distance to the nearest feeding hopper (NH), subpopulation (SP) and year (Y) on the experimental 2013/14 and 2014/15 Mauritius parakeet breeding seasons.

Factor	Model average estimate	SE	95% CI
Y	-1.91	0.48	-2.85 – -0.97
T	-1.26	0.55	-2.34 – -0.17
SP	1.04	0.49	0.09 – 1.99
NH	-0.20	0.21	-0.60 – 0.21

CHAPTER 4: Molecular phylogeographic analysis of the ancestral origins of Beak and Feather Disease Virus in parakeets on Mauritius implicates the global pet trade

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ABSTRACT

Psittacine Beak and Feather Disease (PBFD), caused by the Beak and Feather Disease Virus (BFDV), originated in the South Pacific in the 1970s before spreading rapidly across the world due to the pet trade in companion birds. An outbreak of PBFD in Mauritius in 2005 interrupted active management for the recovery of the endangered Mauritius parakeet. Time-calibrated phylogenies suggest the BFDV viral strain present in both the endemic Mauritius parakeet and invasive rose-ringed parakeet populations on the island was introduced in the late 1980s. It is therefore unlikely that the feral Asian rose-ringed parakeets that were introduced in the 1880s were the source of infection on the island. Mauritian viral isolates are instead most closely related to those originating from wild Senegalese rose-ringed parakeets, which may be evidence of the impact of the global pet bird trade. Nucleotide networks indicate that *replicase* isolates on Mauritius have diverged into 67 known haplotypes since 1993, seven of which have occurred over multiple breeding seasons in more than five Mauritius parakeet hosts and only one of which is shared by both Mauritius parakeet and rose-ringed parakeet hosts. BFDV haplotypes found within Mauritius parakeet hosts are shared across the one captive and three wild subpopulations indicating a level of human-facilitated transmission despite strict hygiene protocols. This study also presents the first published report of BFDV in wild rose-ringed parakeets within their African and Asian native ranges, providing an important backdrop for our finding of a relatively recent introduction of BFDV to Mauritius. Ongoing transport of infectious disease around the world due to global pet trade risks introducing novel BFDV isolates into wild populations of vulnerable species.

KEY WORDS PBFD, BFDV, infectious disease, pet trade, demographic expansion, viral recombination

4.1 INTRODUCTION

Emerging infectious diseases (EIDs), defined as those caused by newly discovered pathogens or with increased incidence or range expansion (Daszak, Cunningham & Hyatt 2001; Dobson & Foufopoulos 2001; Morens, Folkers & Fauci 2004) may increase the risk of extinction for vulnerable species and populations, thereby reducing global biodiversity (Lips et al. 2006). Pathogens generally have short generation times which allow them to adapt rapidly to novel hosts (Altizer, Harvell & Friedle 2003), and amongst these, single-stranded DNA viruses have some of the highest rates of mutation (Duffy, Shackleton & Holmes 2008). Consequently many EIDs are caused by viruses which are infectious across a wide host range (Altizer, Harvell & Friedle 2003).

Psittacine Beak and Feather Disease (PBFD), the most common viral disease in wild psittaciformes (Khalesi et al. 2005), is caused by the Beak and Feather Disease Virus (BFDV). BFDV belongs to the Circoviridae family and comprises a circular, single-stranded, approximately 2000 nucleotide long DNA genome which lacks a non-coding region (Ritchie et al. 1989a). Both its size and structure make BFDV a relatively simple pathogen for studying molecular variation in the context of disease ecology and drivers of spread (Sarker et al. 2014a). The genome consists of a highly conserved replication associated protein (*replicase* gene) (Kondiah, Albertyn & Bragg 2006; Kundu et al. 2012; Peters et al. 2014) and a *capsid* gene responsible for viral encapsidation and host cell penetration (Heath et al. 2004; Kundu et al. 2012). BFDV is transmissible horizontally, through contact with contaminated feather dust, surfaces or objects (Ritchie, Anderson & Lambert 2003), and vertically, from a female to her offspring (Ritchie et al. 1989b; Kundu et al. 2012).

PBFD was first described in the 1970s (Pass & Perry 1984), originating in the South Pacific (Ritchie et al. 1989b; Latimer et al. 1991; Heath et al. 2004) and is considered to have spread rapidly across the world owing to its high environmental persistence and ability to shift between closely related host species (Peters et al. 2014; Sarker et al. 2014b). All *Psittaciformes* are considered to be susceptible to infection (Sarker et al. 2014a) and to date BFDV or PBFD has been recorded in a total of 88 species (26 New World and 62 Old World) and five subspecies globally (Chapter 1). Small, isolated host populations such as those of island endemics are considered to be particularly vulnerable to emerging infectious diseases as their populations frequently have low genetic diversity (Wikelski et al. 2004; Carrete et al. 2009; Trinkel et al. 2011) and have usually evolved in the presence of an impoverished pathogen community (Wikelski et al. 2004; Carrete et al. 2009; Spurgin et al. 2012). Island species are also increasingly at risk due to human-facilitated biological invasions

and the alteration of an often already limited habitat (Vitousek 1998), with the number of bird species introduced to oceanic islands being roughly equal to the number of species extirpated from them (Sax, Gaines & Brown 2002).

The endangered Mauritius parakeet (*Psittacula eques*), confined to the Black River Gorges National Park in the south west of the island, was once the world's rarest parrot (Jones 1987; Kundu et al. 2012) but by 2014 had been recovered to approximately 600 individuals and 102 known breeding pairs (Henshaw et al. 2014) through intensive captive breeding and reintroduction (Tatayah et al. 2007; Raisin et al. 2012). BFDV was first recorded in the Mascarene Islands in the early 1990s (Pers. Comm. R. Bristol) and low viral prevalence was detected in blood samples taken on an *ad hoc* basis from 1993 to 2004. However in the 2005/06 breeding season an outbreak of PBFD swept through the population, coinciding with a viral mutation in the *replicase* gene (Kundu et al. 2012). Since the outbreak, blood samples have been taken from all annually produced offspring, providing a unique opportunity to analyse the temporal evolution of BFDV on Mauritius over the last two decades and before, during and after the outbreak (Kundu et al. 2012; Tollington et al. 2015).

Alongside interest in how the virus has evolved on Mauritius, it has also become crucial in the management of this EID to understand when, and from where the virus first reached the island. Historically there have been 56 exotic bird introductions to Mauritius including rose-ringed parakeets (*Psittacula krameri*) and Madagascar lovebirds (*Agapornis cana*). The Madagascar lovebirds were introduced in 1739 but were extirpated circa 1950 (Jones 1980, 1996). Asian rose-ringed parakeets were introduced over a century later in the 1880s (Jones 1996), primarily due to the influx of Indian migrant labourers between 1834 and 1910 (Christopher 1992) and their descendants still persist on the island today (Jackson et al. 2015a). The rose-ringed parakeet is the world's most widely introduced parrot with breeding populations in approximately 35 countries across five continents (Tayleur 2010). To date no BFDV or PBFD screening has been conducted on any free-living populations across its extensive native range (Chapter 1). However, populations within both its invasive range and captive individuals have tested positive for BFDV (Kundu et al. 2012; Julian et al. 2013; Sa et al. 2014), implying that the virus is also likely to be present in wild flocks.

It has been suggested that the introduction of BFDV to Mauritius occurred in 1959 (Kundu et al. 2012, 95% HPD 1920 and 1988), considerably later than the establishment of either the rose-ringed parakeets or Madagascar lovebirds on the island. Whilst the most likely source of BFDV

introduction on Mauritius is assumed to be the Indian subcontinent its true origin remains unknown. By applying phylogenetic analysis to viral haplotypes present in both the endemic Mauritius parakeet and invasive rose-ringed parakeet populations on the island, and alongside other globally affected psittacine species, we aim to infer the origin of the BFDV viral genotyopes present on Mauritius. We examine patterns of viral diversification that have occurred in isolation on Mauritius since 1993, look for evidence of viral recombination and compare the rate of mutation of the *replicase* gene on Mauritius to that found in other global regions. Our findings regarding the origin of BFDV on Mauritius have important implications for current trade and import of companion birds to other regions of high psittacine biodiversity. Importations may pose a threat to the ongoing conservation of the Mauritius parakeet, the last remaining endemic parrot of the Mascarene islands (Hume 2007).

4.2 MATERIALS AND METHODS

4.2.1 Mauritius parakeet sampling and DNA extraction

Blood samples were drawn by the Mauritius parakeet field team from all 45 day old nestlings produced each breeding season (September to May) since 2005 and opportunistically from post-fledged birds since 1993 as part of ongoing management of this endangered species. Each fledgling was assigned a Studbook ID which was recorded alongside standard data regarding nest site, parents and number of offspring in the nest. For this study a total of 887 samples were screened for BFDV across all breeding seasons from 2009/10 to 2014/15 and added to the standing Mauritius parakeet viral prevalence dataset (Kundu et al. 2012), now spanning over two decades. Prior to screening for BFDV, an ammonium acetate DNA extraction method was used to extract both bird and BFDV DNA (Bruford et al. 1998). In brief, approximately 50 to 100 µl of whole blood was used from each sample and digested in 250 µl of DIGSOL lysis buffer with 10 µl of 10 mg/mL proteinase K. Extractions were quantified using a Qubit dsDNA Assay Kit and standardized to approximately 25ng/µl prior to screening for BFDV (Chapter 2).

Virus specific primers were then used to determine presence of viral DNA within that of the host. Screening was carried out through PCR assay targeting a 717-bp region of the *replicase* gene (Ypelaar et al. 1999). Reactions comprised 1 µl of extracted DNA template, 5 µl MyTaq^{TM} HS Red Mix (Bioline), 0.2 µl each of the forward and reverse primers at 10 pmol/µl and made up to 10 µl with double-distilled water. PCR annealing temperature was set to 57^{o}C for 30 cycles and products were

visualized on a 1.5% agarose gel. A negative control was included in each PCR batch to ensure no contamination was present. All positive PCR products were sent to Macrogen Europe (Amsterdam) for sequencing.

4.2.2 Rose-ringed parakeet sampling and DNA extraction

Wild rose-ringed parakeet blood samples from both the African and Asian native ranges sent to DICE as part of a separate whole-genome sequencing study were obtained from Pakistan, Bangladesh, Senegal and the Gambia. Additionally, ad hoc blood samples were taken from invasive wild individuals from Mauritius between 2009 and 2011 and a tissue extraction was provided from an individual from the introduced United Kingdom (UK) population by the Institute of Zoology, Zoological Society of London. All but the UK sample were extracted, quantified, screened and sequenced as described for the Mauritius parakeet samples.

4.2.3 Sequence analysis

Geneious 8.1.7 (Kearse et al. 2012) DNA editing software was used to align and edit all DNA sequences. Whole or partial BFDV genome sequences from both wild and captive strains sequenced globally were obtained and imported into Geneious from GenBank for phylogenetic comparison and analysis (Table 4.1). *Replicase* was selected for analysis as a selective mutation in this gene was identified to be the most likely cause for the "outbreak" of PBFD on Mauritius in 2005 (Kundu et al. 2012). Additionally, a positive result was far more frequently obtained from *replicase* PCR assay products than when conducting an assay using primers for *capsid*. The dataset was therefore larger enabling a finer-scaled analysis.

No recombination events were present among the Mauritian BFDV isolates when the dataset was analysed using RDP4 (Martin et al. 2010) with default settings and the RDP (Martin & Rybicki 2000), GENECONV (Padidam et al. 1999), BootScan (Martin et al. 2005), MaxChi (Smith 1992), Chimaera (Posada & Crandall 2001) and SiScan (Gibbs, Armstrong & Gibbs 2000) methods. Due to the lack of recombination, all Mauritian data could be used for molecular network and Bayesian analyses.

4.2.3.1 Global phylogeny

The GenBank accession numbers for all global BFDV sequences used in these analyses are listed in Table 4.1. The programme jModelTest 2.1.7 (Posada 2008) was used to infer the best fit

nucleotide substitution model. A transition model with gamma distributed rate variation and a proportion of invariable sites (GTR+I+G) was favoured. Evolutionary rates were then determined using the programme Beast v1.8.2 (Drummond et al. 2012). The constant population size coalescent demographic model was fitted to the data as the choice of coalescent prior has been shown to have little effect on estimates of viral movement dynamics (Lemey et al. 2009). Like Harkins et al. (2012) and Sarker et al. (2014a, b) we chose to employ the uncorrelated lognormal relaxed-clock model (Drummond & Suchard 2010). Tracer v1.6 was used to ensure thorough model mixing and that a reasonable effective sample size (ESS>200) had been reached for all parameters. Ten independent Monte Carlo-Markov chains (MCMC) were implemented for 100 million generations each, with trees sampled every 10 000 generations. TreeAnnotator v1.8.2 was used to obtain the tree with the highest clade credibility (Drummond et al. 2012) and FigTree v1.4.2 was then used to generate the consensus tree (Andrew 2009).

4.2.3.2 Mauritius parakeet BFDV haplotypes

Network 4.6.1.3 (Fluxus Technology Ltd. 2014) was used to construct a Median-Joining nucleotide haplotype network for the *replicase* gene sequences obtained from the Mauritius parakeet and rose-ringed parakeet populations, and the output was colour coded according to year of sampling. A second Median-Joining model was constructed from only the Mauritius parakeet BFDV dataset and colour coded according to subpopulation.

We used Arlequin 3.5.2.2 (Excoffier & Lischer 2010) to examine whether the Mauritius BFDV population had experienced historical demographic changes. Departures from mutation–drift equilibrium were tested using Tajima's D (Tajima 1989) and Fu's F_S (Fu & Li 1993). These tests were applied to the haplotypes present across the entire *replicase* dataset used in this study and the smaller Mauritius dataset analysed by Kundu et al. (2012), to determine whether a viral diversification event was detectable since the proposed selective sweep that was thought to have occurred in 2005.

4.3 RESULTS

4.3.1 Dating the introduction of BFDV to Mauritius

The Bayesian analysis of *replicase* sequences isolated from both Mauritius parakeet and feral rose-ringed parakeet hosts on Mauritius indicates that this lineage is monophyletic (Figure 4.1). The

Mauritius clade was determined to be mostly closely related to the first BFDV sequences isolated from wild rose-ringed parakeets in Senegal, both of which clustered with an isolate obtained from a captive Zambian Black cheeked lovebird. The BFDV strain present on Mauritius was determined to have diverged from the Senegalese clade in 1986 or 1987, approximately 28 years before the 2014/15 breeding season. The divergence between the Mauritius/Senegal clade and the Zambian isolate occurred approximately 3.5 years prior to that event, in 1983 or 1984. When all Mauritius *replicase* data were phylogenetically analysed alongside an outgroup BFDV sequence from Senegal, Zambia and South Africa, the time since divergence was in agreement with the global phylogeny (Figure 4.2); determined to be approximately 34 years prior to the 2014/15 breeding season. The estimated introduction date of BFDV to Mauritius was 27 years prior to the 2014/15 breeding season, in 1987 or 1988. All *replicase* sequences obtained from Mauritian hosts between 2009 and 2015 have been deposited in GenBank (KT753406 – KT753526).

Following its introduction to Mauritius, the BFDV isolates appeared to have diverged into four main clades (see Figure 4.2; shaded in grey, purple and blue and unshaded), only one of which currently persists on the island (Figure 4.2; grey shading). This clade included only one isolate originating from a rose-ringed parakeet, in the 2006/07 breeding season, whilst all other rose-ringed parakeet isolates were determined to fall within terminal lineages. The second clade (Figure 4.2; purple shading) predominantly consisted of the isolate determined to be the outbreak haplotype from the 2005/06 breeding season (Kundu et al. 2012). The third clade (Figure 4.2; blue shading) consisted predominantly of isolates derived from rose-ringed parakeets, with Mauritius parakeet isolates within this group occurring most recently in the 2013/14 breeding season. This observed pattern is indicative of recent viral transmission between these two host species, with the most recent divergence between them having occurred in approximately 2008 or 2009. The unshaded clade consisted entirely of isolates originating from feral rose-ringed parakeets. Mean nucleotide substitution rates for the *replicase* gene for the BFDV strain present on Mauritius were estimated to be 1.92×10^{-3} per site per year (Table 4.2).

4.3.2. Evolution of BFDV on Mauritius

Since the first detection of BFDV in Mauritius parakeets in the 1993/94 breeding season the *replicase* isolates have diverged into 67 observed haplotypes (Figure 4.3). Of these, 55 haplotypes occurred in just single host individuals whilst seven occurred on more than five occasions and persisted over multiple breeding seasons. The distribution of these more persistent haplotypes

formed two evolutionary clusters, with the most abundant of these haplotypes (A in Figure 4.3) isolated from 56 Mauritius parakeet individuals and last detected in 2010/11. Subsequently, two predominant haplotype clusters emerged (B and C in Figure 4.3), isolated from 27 and 14 Mauritius parakeet individuals respectively. Both of these haplotypes occurred since the 2011/12 breeding season, with only a single nucleotide variation between haplotypes A and B, haplotypes B and C and two base pair changes between haplotypes A and C. Haplotype E consisted of 21 individuals and was the only viral isolate shared by Mauritius and rose-ringed parakeets. Haplotype G was the only haplotype to consist solely of isolates from rose-ringed parakeet hosts, first appearing in 1995 and occurring in 16 individuals in the 2006/07 breeding season.

The results of both the Tajima's D and Fu's F tests suggested that viral population present on Mauritius between 1993/94 and 2014/15 analysed in this study underwent a recent selective sweep or genetic bottleneck (Simonsen, Churchill & Aquadro 1995) before expanding (Tajima's D = -1.81, $p < 0.01$, Fu's $F_S = -24.59$, $p < 0.001$). Whilst the significant decrease in viral diversity indicative of directional selection was identified by Kundu et al. (2012), the subsequent diversification in haplotypes that we report here was not detectable within the less extensive sample-set from 1993/94 to 2008/09 (Table 4.3).

4.3.3. Haplotype distribution between Mauritius parakeet subpopulations

The distribution of haplotypes within only the Mauritius parakeet subpopulations is presented in Figure 4.4. Haplotypes A, E and F all comprised *replicase* sequences isolated from fledglings produced in both wild subpopulations, "Bel Ombre" and "Camp", as well as the Gerald Durrell Endemic Wildlife Sanctuary (GDEWS), which was used as a captive breeding facility during intensive management. Haplotypes B and C both comprised isolates from the "Camp" and "Bel Ombre" subpopulations, as well as from those fledglings translocated to Le Vallée de Ferney after the culmination of the 2014/15 breeding season.

4.3.4 Multiple variants of BFDV from single hosts

Sporadic re-sequencing was conducted on Mauritius parakeet BFDV positive PCR products (n=10) across breeding seasons to ensure repeatability. During this process, two different *replicase* haplotypes were detected within single samples drawn from two individuals from the 2010/11 breeding season. No other instances of multiple infections were recorded.

4.3.5 BFDV in other native and introduced populations of rose-ringed parakeets

This study presents the first published report of BFDV in wild rose-ringed parakeets within their African and Asian native ranges. The isolates obtained from wild Bangladeshi rose-ringed parakeets within their Asian native range were found to be most closely related to one another and also to a single isolate originating from a wild Gambian individual from within the African native range (Figure 4.1). The isolates from Pakistan grouped with one another and formed a sister clade to the Bangladesh-Gambia clade. As a group, these isolates diverged from a clade consisting of a viral strain found to occur predominantly in captive hosts sampled in Poland in approximately 1996. This Polish clade also included the isolate obtained from the single feral UK rose-ringed parakeet sample included in this study. All *replicase* sequences obtained from rose-ringed parakeet hosts analysed in this study have been deposited in GenBank (KT725790 – KT725803).

4.3.5. Wider global phylogenetic patterns

The global tree consisted of a high proportion of clades containing sequences that were monophyletic by location including groups of isolates obtained from captive flocks in Thailand, another from captive flocks in South Africa, and a number of clades from Australasia and Oceania (Figure 4.1). Unlike captive hosts, those clades consisting of isolates from wild birds were predominantly monophyletic and did not display high divergence. The most basal global clade consisted of a monophyletic cluster of isolates originating from wild New Caledonian Rainbow lorikeets and their native subspecies, which were sister to captive Australian Rainbow lorikeets and a wild Moluccan red lory from Indonesia. Our study is the first to include an isolate originating from a wild Indonesian BFDV host. This basal group was determined to have diverged from the more recently evolved BFDV lineages in approximately 1951. Sequences from captive hosts of Polish, South African and Australian origin were widely dispersed throughout the phylogenetic tree. Mean nucleotide substitution rates for the *replicase* gene across all BFDV strains were estimated to be 1.70 x 10^{-3} per site per year (Table 4.2).

4.4 DISCUSSION

4.4.1 Dating the introduction of BFDV to Mauritius

Unlike the patterns of divergence on other islands such as New Zealand (Jackson et al. 2015b) the monophyletic nature of the BFDV strain present within both rose-ringed and Mauritius

parakeet hosts on Mauritius is indicative of a single introduction to the island. When Mauritius BFDV isolates were analysed alongside the global dataset, the date of introduction to the island was estimated to have occurred approximately 38 years later than when these data were analysed previously by Kundu et al. (2012). Our TMRCA estimate of 27.9 years before the 2014/15 breeding season (between 1987 and 1988) falls within the upper bounds of their prior estimate of between 1920 and 1988 (Kundu et al. 2012) and has a much narrower confidence interval. Due to both the relatively recent global establishment of BFDV and the close phylogenetic relationship between isolates from Senegal, Zambia and those on Mauritius, it is likely that the feral Asian rose-ringed parakeets that were introduced in the 1880s (Jones 1996) were not the source of infection on the island. As a more likely scenario, there have been 354 recorded instances of live *Psittaciforme* imports on to Mauritius from 13 different countries since 1975 (CITES 2015), any of which may have acted as the source as they fall within the timeframe suggested by our time-calibrated phylogenetic analysis of the viral sequences. Therefore, as has been documented for wild Cape parrots in South Africa infected with BFDV (Regnard et al. 2015), it would seem most plausible that the strain of BFDV present on Mauritius was accidentally introduced as a product of the more recent trade in companion birds.

4.4.2 Evolution of BFDV on Mauritius following introduction

The mutation rate calculated for the viral *replicase* gene on Mauritius of 1.92 x 10^{-3} substitutions per site per year is higher than but comparable to the rates inferred for all global strains when using a lognormal relaxed evolutionary clock. However, this estimate is considerably higher than those produced by both the lognormal relaxed and strict clock estimates for *replicase* nucleotide substitution in Australian cockatoos (Sarker et al. 2014a), and in previous strict clock estimates for Mauritius parakeet hosts (Kundu et al. 2012). This inconsistency is likely due to the much larger dataset analysed in this study, which was inclusive of a period of diversification in BFDV haplotypes across both of the endemic and introduced parakeet host populations on Mauritius since the proposed selective sweep that is thought to have occurred in 2005. The size of a host population has a profound effect on the ability of a pathogen to persist, with a minimum density required for establishment (Lyles & Dobson 1993). As the Mauritius parakeet population has increased in population size through intensive management over the past 28 years (Tatayah et al. 2007; Raisin et al. 2012), so too has the number of hosts available for infection by the virus. Additionally, the ability of a pathogen to establish in a host population has a direct relationship with its virulence and an

inverse relationship with its transmission efficiency (Lyles and Dobson 1993). Whilst BFDV has been found to be widely infectious (Chapter 1) and PBFD is frequently fatal in immature birds up to three years of age (Ritchie et al. 1989b), infected individuals commonly recover from severe clinical presentation of the disease, which usually lasts only for a number of months (Todd 2000). These attributes of BFDV would appear to have allowed it to become highly prevalent in the Mauritius parakeet population (with up to 40% prevalence in annually recruited nestlings; Chapter 3) whilst host numbers have continued to increase (Tollington et al. 2015).

The rose-ringed parakeet population on Mauritius has been infrequently sampled, with no genetic data on viral isolates collected since 2011. The nominal number of shared BFDV haplotypes between the endemic and introduced parakeet species could therefore be indicative of either a relative lack of contact between the two species due to allopatry, or a sampling regime that is unable to detect fine-scale levels of viral transmission between host species on the island.

4.4.3 The occurrence of multiple haplotypes within a single host

Our detection of multiple BFDV viral haplotypes in an infected host individual has also been described in cockatoos (Sarker et al. 2014b). Therefore, whilst our haplotype network generated for the strain of BFDV present on Mauritius displays the viral population dynamics and diversification, it is unlikely to be exhaustive of all variants present on the island. The ability for multiple BFDV infections to persist within a single host, along with its high rate of mutation, allows for the rapid evolution of novel BFDV variants through recombination (Julian et al. 2013). Whilst captive-breeding facilities may have had a positive influence on endangered species' conservation indirectly, the mixing of multiple parrot species from different geographic areas in a captive environment creates an ideal mechanism for viral transmission (Julian et al. 2013; Robino et al. 2014). If these novel BFDV variants leak into vulnerable wild host populations such as Mauritius parakeets, such an event may alter the virus' pathogenicity and subsequently increase the threat imposed by infection (Jackson et al. 2015b).

4.4.4 Shared haplotypes between Mauritius parakeet subpopulations

The sharing of haplotypes between subpopulations of Mauritius parakeets is to be expected. During the intensive management and recovery of this host population, interventions such as brood manipulation, captive-breeding and reintroduction were undertaken to help rapidly increase numbers of parakeets (Tatayah et al. 2007; Taylor & Parkin 2010). The Mauritian Wildlife

Foundation's parakeet field team has attempted to reduce or eliminate any potential human-mediated transmission of BFDV with a rigorous biosecurity and hygiene protocol since 2005 (Chapter 3). However despite these protocols, the recent translocation of parakeets to Le Vallée de Ferney on Mauritius to found a new subpopulation also included the transfer of BFDV to the east coast. The unavoidable regular movement of vehicles, equipment and staff between localities for ongoing species management is the most likely source of continued gene flow between viral populations. Whilst efforts to reduce BFDV transmission have been found to be effective (Chapter 3), the complete elimination of viral transfer between sites may not be possible.

4.4.5 BFDV in other native and introduced populations of rose-ringed parakeets

Alongside the introduction of BFDV to Mauritius, trade in companion birds is the most likely source of introduction of BFDV to the African and Asian native ranges of rose-ringed parakeets. This hypothesis is supported not only by the close phylogenetic relationship we have revealed between isolates from wild hosts in Mauritius and Senegal, but also by the unexpected identification of a Gambian isolate that is most closely related to those originating from Southern Asia. Gambia is geographically encompassed by Senegal, with a contiguous population of rose-ringed parakeets between these two countries (IUCN 2015). This finding confirms that multiple global BFDV strains are present within West and Central Africa. Rose-ringed parakeets are abundant in their native range and the confirmed presence of BFDV within these hosts may be indicative of a high risk of spill over into other vulnerable species with sympatric overlap such as Timneh parrots (*Psittacus timneh*) and Grey parrots (*Psittacus erithacus*) (IUCN 2015). Similarly, the presence of BFDV in rose-ringed parakeets from Bangladesh and Pakistan may act as an abundant reservoir host for infecting vulnerable sympatric species such as Red-breasted parakeets (*Psittacula alexandri*) and Blossom headed parakeets (*Psittacula roseata*) (IUCN 2015).

The inclusion of the feral UK rose-ringed parakeet isolate in one of the many Polish clades dispersed throughout the global phylogeny (Figure 4.1) is most likely indicative of trade from captive breeding facilities in Eastern Europe. BDFV is highly prevalent in Polish breeding facilities (Julian et al. 2013), a large source of both companion birds and likely to add to the spread of infection worldwide (Harkins et al. 2014). Rose-ringed parakeets have successfully established feral populations across Europe (IUCN 2015; Jackson et al. 2015a) and given that captive parrots in Germany, Portugal, Spain, Italy and Poland (De Kloet & De Kloet 2004; Raue et al. 2004; Rahaus et al.

2008; Julian et al. 2013) have tested positive for BFDV it is likely that the virus is also present in other wild flocks outside of the UK.

4.4.6 Implications for the conservation of vulnerable parrot species

The Mauritius parakeet is the last remaining of ten parrot species that once occurred in the Mascarene islands (Hume 2007) and has only recently been recovered from fewer than 20 known individuals (Duffy 1993; Lovegrove, Nieuwland & Green 1995). Despite the initial concern that conservation managers had when PBFD was first detected within the Mauritius parakeet population the species has continued to recover in the face of disease. However, BFDV has evolved rapidly over the last decade, with regular fluctuation in viral prevalence on the island (Chapter 3) and two new haplotypes becoming prevalent in recent breeding seasons. The continued spread of infection between subpopulations on Mauritius as well as the connectivity of BFDV strains around the world highlights how easy the unintentional spread of virions can be, even when a strict hygiene protocol is in place. It has been estimated that aviculturists in South Africa lose up to 20% of their flock to the disease annually, with major economic impacts to breeders (Heath et al. 2004). However the benefits of conserving global parrot biodiversity within their native ranges extend far beyond their captive market value. Consequently we recommend that policy relating to the continued import of *Psittaciforme* species into Mauritius and other known psittacine biodiversity hotspots is re-evaluated. The pet trade substantially increases the risk of introducing novel or recombined BFDV isolates that may prove to have higher pathogenicity than the strain currently present on the island.

The first detection of BFDV in a wild parrot species native to both Asia and Africa highlights the need for further research in these global regions and has implications for the conservation of vulnerable sympatric species. Most of the African continent is data deficient on BFDV incidence as no studies have been published on wild populations north of Zambia (Chapter 1). Similarly, little work has been conducted in Asia outside of South-East Asian cockatoo species. These results, in combination with the presence of BFDV in feral rose-ringed parakeet flocks outside of the species' native range, provide further support for recommendations to assess global breeding and trade in parrots (Jackson et al. 2015a).

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4.6 FIGURES AND TABLES

Table 4.1 Details of all global BFDV *replicase* sequences analysed in this study. AU = Australia, BD = Bangladesh, CN = China, GM = Gambia, DE= Germany, ID = Indonesia, IT = Italy, JP = Japan, MU = Mauritius, NC = New Caledonia, NZ = New Zealand, PK = Pakistan, PL= Poland, PT = Portugal, SN = Senegal, ZA = South Africa, TH = Thailand, GB = United Kingdom, US = United States of America, ZM = Zambia.

Accession #	Host	Year	Country	Captive/Wild	Accession #	Host	Year	Country	Captive/Wild
KJ953861	Adelaide rosella	2005	AU	Wild	JF519618	Red fronted parakeet	2010	NZ	Wild
KJ953867	Adelaide rosella	2006	AU	Wild	JQ782201	Yellow crowned parakeet	2011	NZ	Wild
KF688548	Australian ringneck parrot	1996	AU	Wild	JQ782202	Yellow crowned parakeet	2012	NZ	Wild
KF688549	Australian ringneck parrot	1996	AU	Wild	KT725800	Rose-ringed parakeet	2014	PK	Wild
AF311295	Bluebonnet	2000	AU	Captive	KT725801	Rose-ringed parakeet	2014	PK	Wild
EF457974	Cockatiel	2007	AU	Captive	JX221018	African grey parrot	2007	PL	Captive
EF457975	Cockatiel	2007	AU	Captive	JX221020	African grey parrot	2007	PL	Captive
KJ953854	Crimson rosella	2006	AU	Wild	JX221036	Alexandrine parakeet	2008	PL	Captive
KJ953855	Crimson rosella	2006	AU	Wild	JX221042	Alexandrine parakeet	2009	PL	Captive
AF311297	Eastern long billed corella	2000	AU	Captive	JX221029	Australian king parrot	2008	PL	Captive
AF311298	Galah	2000	AU	Captive	JX221033	Australian king parrot	2008	PL	Captive
KF385401	Gang gang cockatoo	1996	AU	Captive	JX221040	Blue fronted amazon	2008	PL	Captive
KF499132	Gang gang cockatoo	2004	AU	Captive	JX221005	Budgerigar	2010	PL	Captive
KF385402	Gang gang cockatoo	2004	AU	Captive	JX221009	Budgerigar	2011	PL	Captive
KF385408	Glossy black cockatoo	2006	AU	Captive	JX221013	Cape parrot	2003	PL	Captive
AF311300	Major Mitchells cockatoo	2000	AU	Captive	JX221043	Crimson rosella	2009	PL	Captive
KJ866054	Malee ringneck parrot	2014	AU	Wild	JX221006	Crimson rosella	2010	PL	Captive
KC693652	Orange bellied parrot	2011	AU	Wild	JX221035	Eastern rosella	2008	PL	Captive
KF188695	Orange bellied parrot	2013	AU	Captive	JX221021	Orange winged amazon	2007	PL	Captive
KF188681	Orange bellied parrot	2013	AU	Captive	JX221024	Pacific parrotlet	2007	PL	Captive
AF311299	Rainbow lorikeet	2000	AU	Captive	JX221015	Red winged parrot	2006	PL	Captive
JX049195	Rainbow lorikeet	2009	AU	Captive	JX221016	Red winged parrot	2006	PL	Captive
KF385399	Red tailed black cockatoo	2013	AU	Wild	JX221002	Rose-ringed parakeet	2010	PL	Captive

Accession #	Host	Year	Country	Captive/Wild	Accession #	Host	Year	Country	Captive/Wild
KF385400	Red tailed black cockatoo	2013	AU	Wild	JX221007	Rose-ringed parakeet	2011	PL	Captive
KF850537	Regent parrot	2013	AU	Wild	JX221030	Senegal parrot	2008	PL	Captive
AF311296	Rosy faced lovebird	2000	AU	Captive	JX221031	Senegal parrot	2008	PL	Captive
AF311301	Sulphur crested cockatoo	2000	AU	Captive	JX221025	White cockatoo	2007	PL	Captive
AF311302	Sulphur crested cockatoo	2000	AU	Captive	AY521236	African grey parrot	2004	PT	Captive
KF385419	Sulphur crested cockatoo	2013	AU	Unknown	EU810207	African grey parrot	2008	PT	Captive
KF673335	Swift parrot	2004	AU	Wild	KT725797	Rose-ringed parakeet	2015	SN	Wild
KF673336	Swift parrot	2004	AU	Wild	KT725799	Rose-ringed parakeet	2015	SN	Wild
KJ953863	Yellow rosella	2005	AU	Wild	AY450435	African grey parrot	2003	ZA	Captive
KJ953873	Yellow rosella	2005	AU	Wild	HM748920	African grey parrot	2008	ZA	Captive
KT725792	Rose-ringed parakeet	2013	BD	Wild	AY450440	African red bellied parrot	2003	ZA	Captive
KT725793	Rose-ringed parakeet	2013	BD	Wild	HM748924	Amazon	2008	ZA	Captive
GQ386944	Budgerigar	2008	CN	Captive	HM748925	Amazon	2008	ZA	Captive
KT725790	Rose-ringed parakeet	2014	GM	Wild	GQ165756	Budgerigar	2008	ZA	Captive
AY521237	African grey parrot	2004	DE	Captive	KM188453	Cape Parrot	2011	ZA	Wild
KF673337	Moluccan red lory	2005	ID	Wild	KM188454	Cape Parrot	2011	ZA	Captive
KF723384	African grey parrot	2009	IT	Captive	HM748926	Eclectus parrot	2008	ZA	Captive
KF723385	African grey parrot	2009	IT	Captive	AY450441	Jardine	2003	ZA	Captive
AB277746	Budgerigar	2006	JP	Captive	HM748929	Rose-ringed parakeet	2008	ZA	Captive
AB277747	Budgerigar	2006	JP	Captive	AY450439	Ruppells parrot	2003	ZA	Captive
AB514568	Cockatiel	2010	JP	Captive	AY450434	White bellied caique	2003	ZA	Captive
HQ641492	Mauritius parakeet	1993	MU	Wild	AY450436	White cockatoo	2003	ZA	Captive
HQ641491	Mauritius parakeet	1995	MU	Wild	GU015012	African grey parrot	2006	TH	Captive
HQ641493	Mauritius parakeet	1996	MU	Wild	GU015013	African grey parrot	2006	TH	Captive
KT753417	Mauritius parakeet	2009	MU	Wild	GU015014	Alexandrine parrot	2006	TH	Captive
KT753449	Mauritius parakeet	2013	MU	Wild	GU015015	Alexandrine parrot	2006	TH	Captive
HQ641486	Rose-ringed parakeet	2007	MU	Wild	FJ685980	Blue and yellow macaw	2006	TH	Captive
KT753490	Rose-ringed parakeet	2009	MU	Wild	GU015017	Chestnut fronted macaw	2006	TH	Captive

Accession #	Host	Year	Country	Captive/Wild	Accession #	Host	Year	Country	Captive/Wild
JX049213	Eclectus parrot	2011	NC	Captive	GU015019	Eclectus parrot	2006	TH	Captive
JX049214	Eclectus parrot	2011	NC	Captive	GU015020	Eclectus parrot	2006	TH	Captive
JX049220	New Caledonian parakeet	2011	NC	Captive	GU015023	Great green macaw	2006	TH	Captive
KF768545	New Caledonian rainbow lorikeet	2012	NC	Wild	FJ685985	Lovebird	2005	TH	Captive
KF768546	New Caledonian rainbow lorikeet	2012	NC	Wild	GU015022	Palm cockatoo	2006	TH	Captive
JX049196	Rainbow lorikeet	2011	NC	Wild	GU015021	Red and green macaw	2006	TH	Captive
JX049198	Rainbow lorikeet	2011	NC	Wild	GU015018	Red shouldered macaw	2006	TH	Captive
JX049199	Rainbow lorikeet	2011	NC	Wild	FJ685989	Salmon crested cockatoo	2006	TH	Captive
JX049219	Red rumped parrot	2011	NC	Captive	FJ685978	Sulphur crested cockatoo	2005	TH	Captive
JX049221	Rose-ringed parakeet	2011	NC	Captive	FJ685979	Yellow crested cockatoo	2005	TH	Captive
GU936287	Eastern rosella	2008	NZ	Wild	AY521238	African grey parrot	2004	GB	Captive
JQ782196	Eastern rosella	2009	NZ	Wild	KT725791	Rose-ringed parakeet	2012	GB	Wild
JF519619	Eastern rosella	2010	NZ	Wild	AY521235	Rosy faced lovebird	2004	GB	Captive
KF467251	Eastern rosella	2012	NZ	Wild	AY521234	Rose-ringed parakeet	2004	US	Captive
GQ396652	Red crowned parakeet	2008	NZ	Wild	AY450442	Black cheeked lovebird	2003	ZM	Captive
GQ396653	Red crowned parakeet	2008	NZ	Wild					

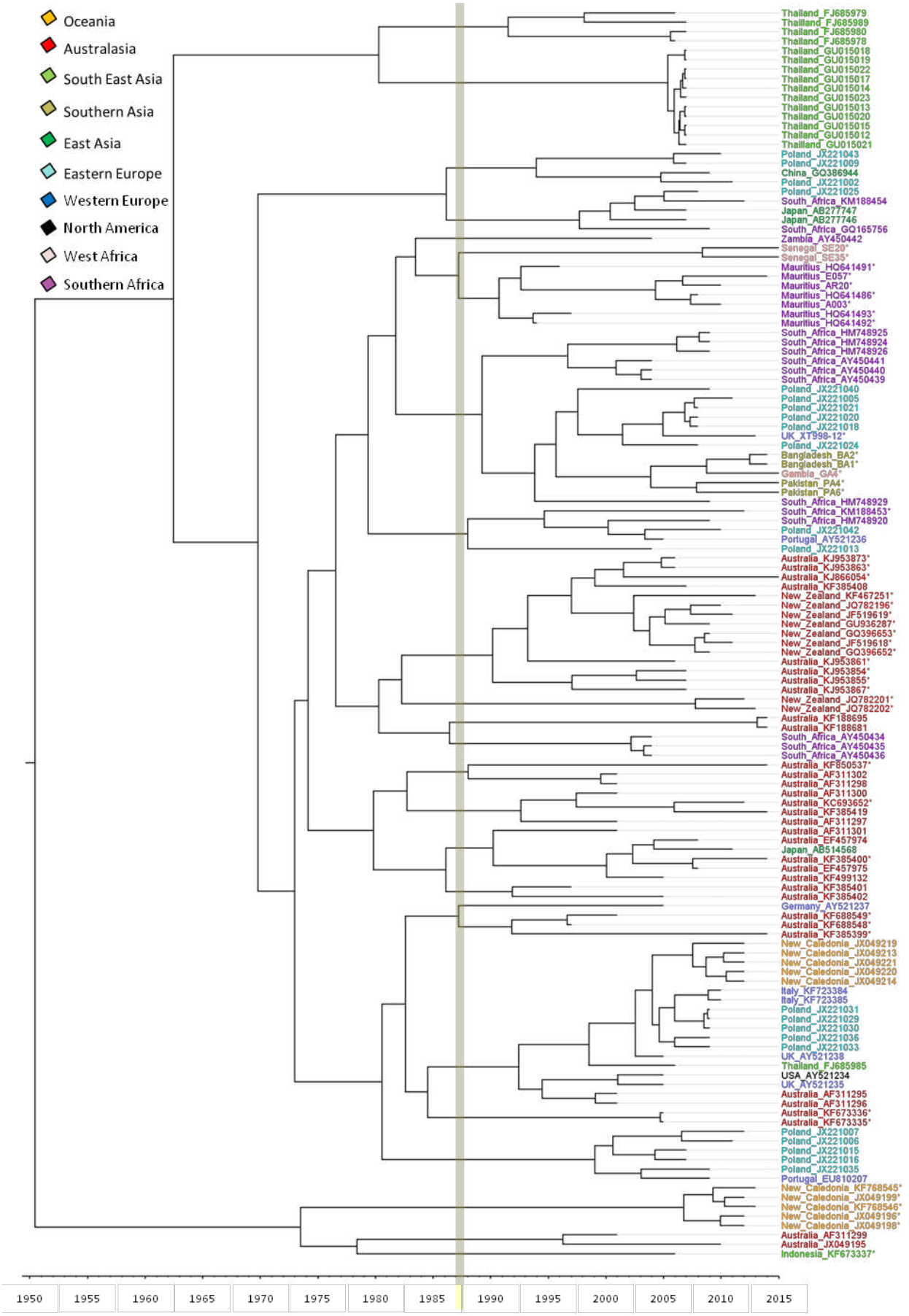

Figure 4.1 Maximum clade credibility tree for the *replicase* dataset under GTR+I+G nucleotide substitution model and a constant population size, relaxed clock evolutionary model. Taxon names are coloured by global region and wild individuals have been marked with a *. The TMRCA for the Mauritius isolates is 1987/88 (indicated by the yellow bar).

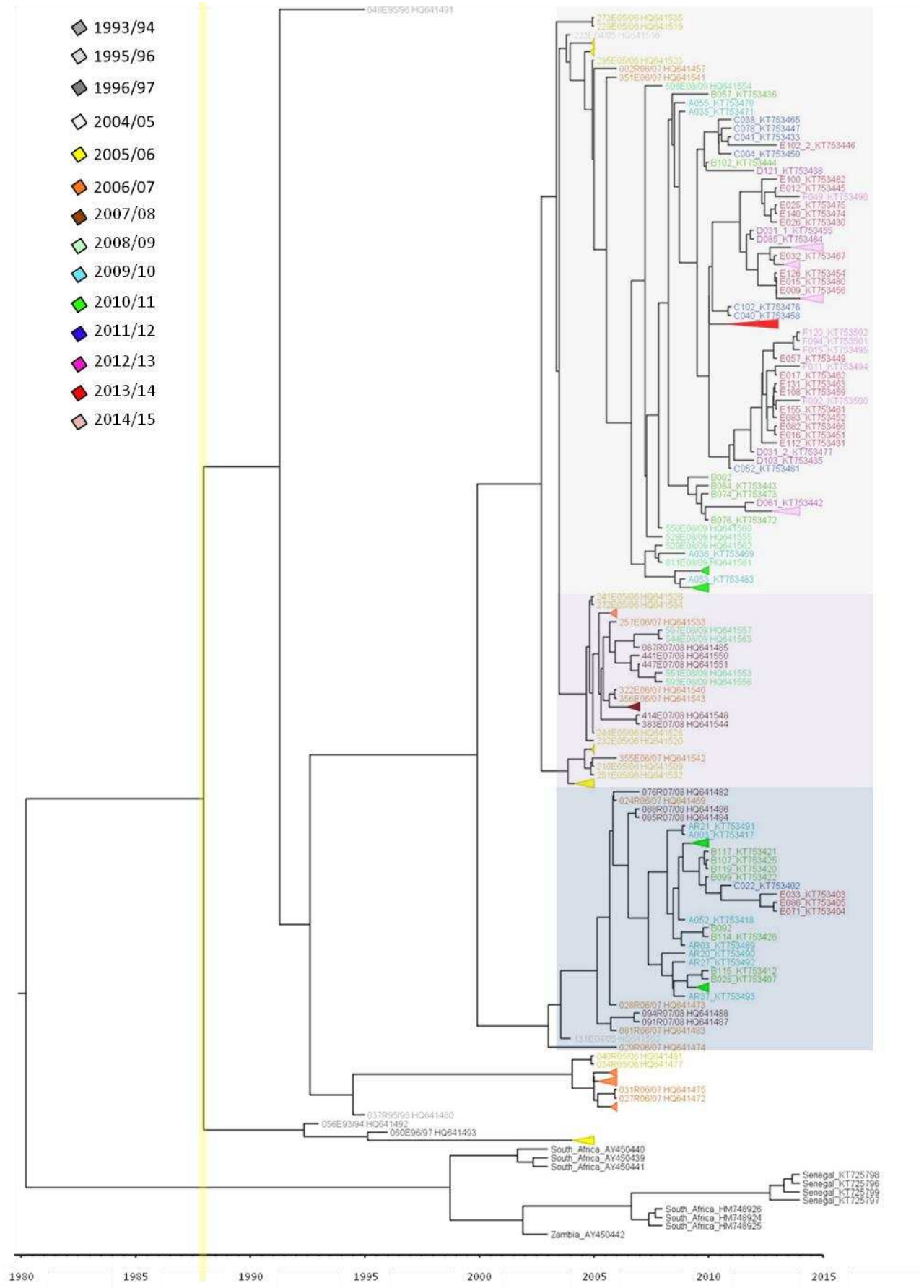

Figure 4.2 Maximum clade credibility tree for the Mauritius *replicase* dataset under GTR+I+G nucleotide substitution model and a constant population size, relaxed clock evolutionary model. Tips are labelled with the Mauritius parakeet breeding season from which the sample was collected. The outgroup comprises Senegal, Zambia and South Africa. The estimated TMRCA for Mauritius isolates is 1987/88 (indicated by the yellow bar, 95% HPD Interval 1980/81 to 1992/93). Distinct clades have been shaded in grey, purple and blue.

Table 4.2 Mean evolutionary rate and the 95% HPD intervals estimated for the *replicase* gene of BFDV isolates from both the global phylogenetic analysis and the combined Mauritius parakeet and feral rose-ringed parakeet data set.

Tree	**Mutation rate**	**95% HPD Interval**
Global	1.70×10^{-3}	1.22×10^{-3}, 2.27×10^{-3}
Mauritius	1.92×10^{-3}	1.26×10^{-3}, 2.64×10^{-3}

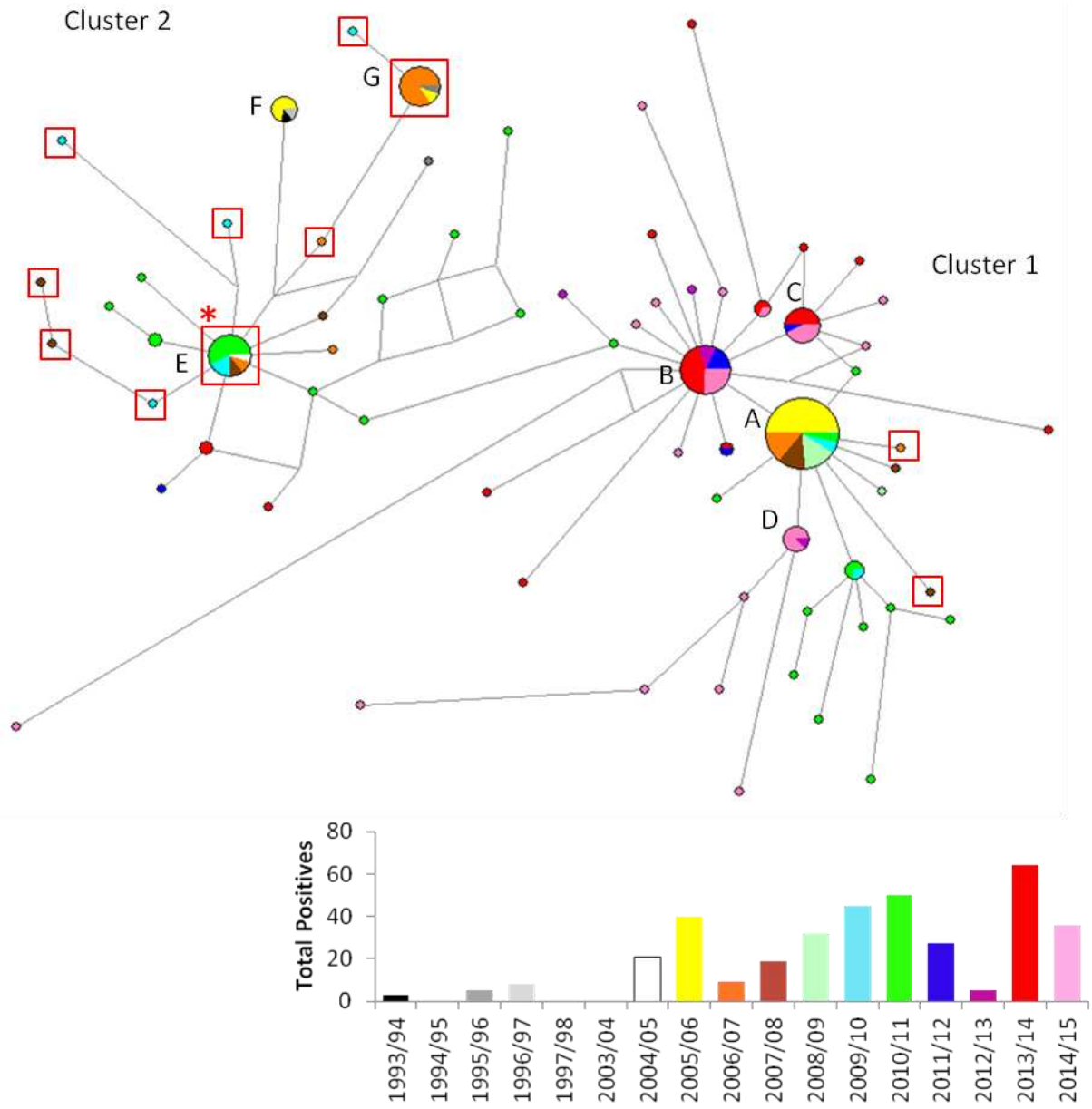

Figure 4.3 Haplotype network displaying the diversity and evolution of BFDV *replicase* haplotypes in both Mauritius parakeet and feral rose-ringed parakeet hosts on Mauritius from 1993 to 2015. The size of each circle is proportional to the number of individuals sharing that viral haplotype and the length of the lines between circles are proportional to the number of base pair changes between each haplotype. Haplotypes containing isolates from rose-ringed parakeets have been demarcated by a red box. The single viral haplotype that is shared by 6 rose-ringed parakeet and 15 Mauritius parakeet hosts has been indicated by a red *.

Table 4.3 The number of haplotypes (H), gene diversity (h) and nucleotide diversity (π) within sites as well as the tests of neutrality to indicate BFDV population stability between the full r*eplicase* dataset analysed in this study and the smaller dataset analysed by Kundu et al. (2012), where $p < 0.05$ is considered significant.

Dataset	**Fu's F (*p*)**	**D (*p*)**	**H**	**h**	**π**
This study	-24.59 (< 0.001)	-1.81 (< 0.01)	67	0.90 (± 0.01)	0.02 (± 0.01)
Kundu et al. 2012	1.99 (0.79)	0.40 (0.69)	15	0.66 (± 0.05)	0.01 (± 0.01)

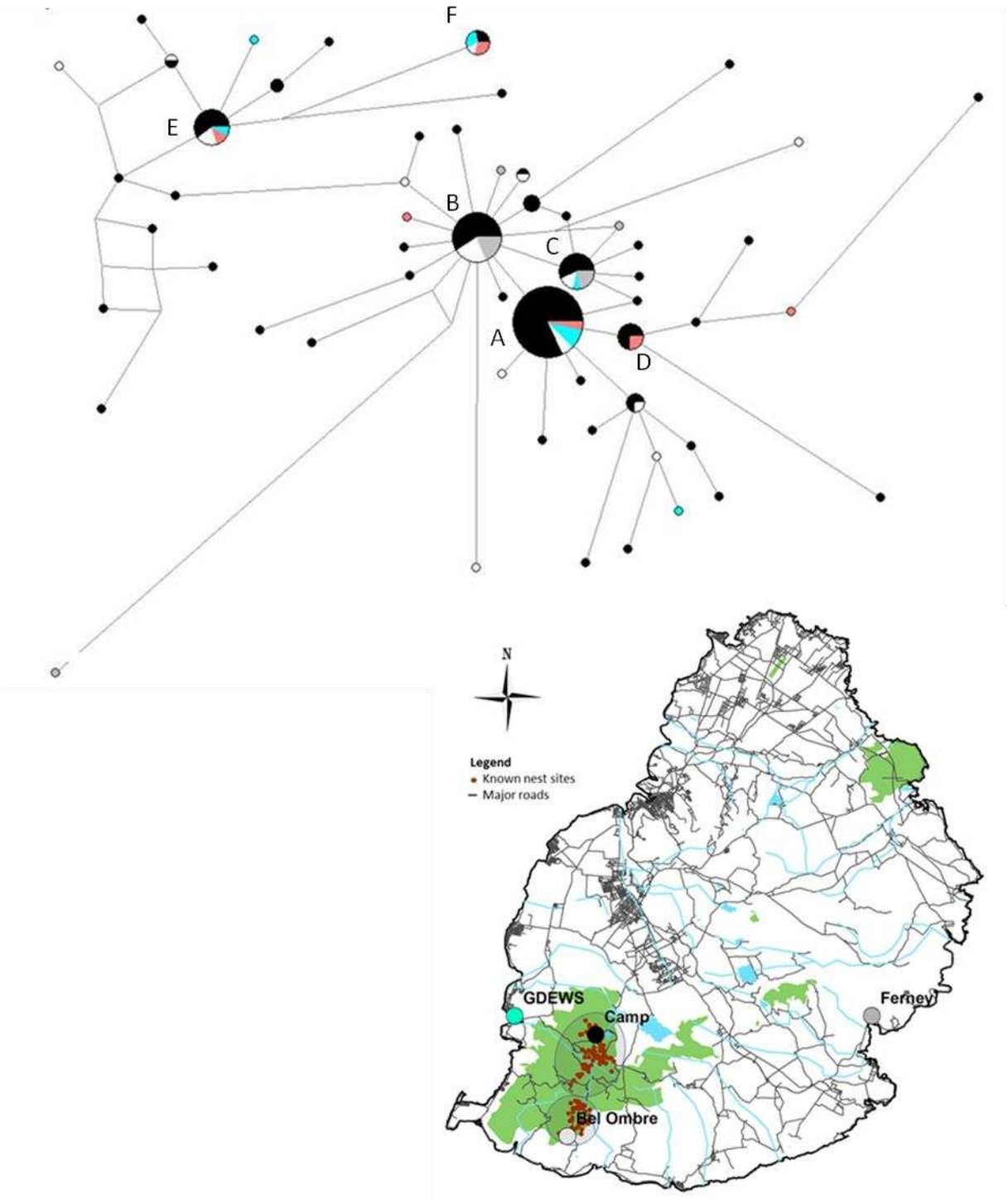

Figure 4.4 *Replicase* nucleotide network displaying the distribution of BFDV haplotypes between Mauritius parakeet subpopulations on Mauritius from 1993 to 2015 where: GDEWS = The Gerald Durrell Endemic Wildlife Sanctuary, Ferney = Le Vallée de Ferney, Camp and Bel Ombre subpopulations reside within the Black River Gorges National Park. Red has been used to indicate infected individuals where their geographic location is unknown.

CHAPTER 5: General discussion and conclusions

Global research on PBFD and BFDV in both wild and captive parrot populations has steadily increased over the last three decades. The focus of this research has encompassed developments into a thorough understanding of the virus, it pathogenicity and its modes of transmission, as well as screening whole populations and using molecular phylogeographic approaches to inform conservation and management strategies. Our research has included many of these elements, with a particular focus on how they affect the continued recovery and management of the Mauritius parakeet population in the face of infectious disease. The review presented in Chapter 1 determined that whilst many advances have been made into the way in which we screen for the presence of BFDV and can interrogate the results, little has been done to standardise these processes across research groups. The variety of optimised diagnostic tests and technologies available for BFDV screening and analysis have improved substantially since its first scientific assessment. However the lack of standardisation complicates the ability to compare prevalence estimates or screening results between different species, captive breeding facilities or global regions. Therefore one of the primary reasons for conducting a series of tests on repeatability in Chapter 2 was to provide the first step to resolving this issue.

Currently 88 species are known to be susceptible to infection with BFDV, many of which are in decline in the wild due to pressures such as habitat loss and introduced species within their native ranges. Standard PCR is one of the most widely used screening techniques and we hope that, with the provision of a known margin of error surrounding a negative result using this method, conservation managers and aviculturists will be better equipped to rear and maintain healthy flocks. A more reliable estimate of individual infection status is of particular value to translocation and reintroduction programmes, highlighted by the previous failure encountered by the Mauritian Wildlife Foundation in 2005 when an entire translocated cohort of Mauritius parakeets were lost to PBFD.

Since the outbreak of PBFD in Mauritius in 2005 a rigorous hygiene protocol was implemented in an attempt to reduce human-facilitated transmission. The continued monitoring and management of this population has provided a unique opportunity to use an endangered species' recovery programme as an experimental platform for answering key

management questions. The results presented in Chapter 3 involving the reciprocal design experiment conducted over two consecutive breeding seasons demonstrated that regular disinfection does reduce the prevalence of BFDV. However we also found that other management related tools such as the provision of feeding hoppers can become a source of reinfection and thus reduce the effectiveness of these disinfection protocols. These findings are widely applicable to the recovery of other endangered or vulnerable psittacine species, such as the Australian orange-bellied parakeet. However many research gaps still exist regarding the true geographic extent of BFDV globally so it is currently unknown how many species would benefit from management strategies inclusive of infectious disease.

The analyses conducted in Chapter 1 highlighted regions of high endemism such as Central and South America, Africa and South East Asia that are currently largely data deficient with regards to the presence of BFDV in both wild and captive parrot populations. The first known BFDV positive results for wild rose-ringed parakeets of both West African and Southern Asian origin provided by our molecular study in Chapter 4 support the need for further investigation into these areas. The widespread abundance of rose-ringed parakeets in both their native and invasive ranges provides an ideal host for viral maintenance and spill over into other sympatric vulnerable species. Surprisingly these two geographic regions were linked by the occurrence of a BFDV strain in a Gambian parakeet host that was most closely related to isolates obtained from the Indian subcontinent. Like other studies into the transmission of BFDV around the world, these results implicate the global trade in companion birds as a vector for disease.

The introduction and persistence of Asian rose-ringed parakeets on Mauritius was thought to be the most likely source of BFDV on the island but our phylogeographic analyses suggest that this is unlikely. Instead we propose that the BFDV strain currently prevalent on the island is the result of a single introduction, between 1987 and 1988, facilitated by the global pet trade due to the isolate's monophyletic nature and genetic similarity to isolates from Senegalese parakeet hosts. Despite the initial concern that conservation managers had when PBFD was first detected within the Mauritius parakeet population the species has continued to recover. However, BFDV has evolved rapidly over the last decade, with a description of the regular fluctuation in viral prevalence on the island demonstrated in Chapter 3. The increased population of Mauritius parakeets due to intensive management

for recovery has also directly increased the ability for BFDV to persist and two new viral haplotypes have become prevalent in recent breeding seasons.

The continued spread of infection between subpopulations on Mauritius as well as the connectivity of BFDV strains around the world highlights how the virus can be unintentionally spread with considerable ease, even when a strict hygiene protocol is in place to prevent such an occurrence. We therefore conclude with the recommendation that policy relating to the continued import of *Psittaciforme* species into Mauritius is re-evaluated, along with broader assessments elsewhere into global breeding and trade in parrots outside of their native ranges. We have demonstrated that with standardised screening, sound hygiene protocols and ongoing research into data deficient global regions, the presence of BFDV and PBFD does not prevent a small or endangered population being successfully recovered from the brink of extinction.