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Temporal Characterization of the Viral Load of Psittacine Beak and Feather Disease Virus in Rosy-Faced Lovebirds (*Agapornis roseicollis*)

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Simple Summary: Psittacine beak and feather disease virus (BFDV) poses a significant threat to parrots worldwide. This highly pathogenic virus causes feather and beak abnormalities and can potentially lead to immune system suppression. Unfortunately, there is currently no cure for this disease. Our current knowledge of Psittacine beak and feather disease mainly stems from studying infected individuals exhibiting visible symptoms. However, our understanding of the pathology and the role of asymptomatic individuals in disease transmission is limited. In our study, we aimed to investigate the temporal changes in the viral load in feather and fecal samples from 17 asymptomatic Rosy-faced Lovebirds (*Agapornis roseicollis*) using qPCR. Our findings revealed that most of the studied individuals had very low levels of viral load. Three individuals initially exhibited high viral load, but over the course of the experiment, their viral load showed a decreasing trend in both fecal and feather samples. Surprisingly, we observed that the viral load in one individual dropped from a high level to an undetectable level within three months. This suggests that BFDV infection might not be lethal or highly pathogenic for some individuals. Additionally, our study demonstrated that the viral load in feathers was higher compared to that in feces.

Abstract: Psittacine beak and feather disease virus (BFDV) is a widespread and highly pathogenic virus in parrots. The disease typically presents with feather and beak abnormalities, along with possible immune system suppression. No cure or commercialized vaccine is currently available. Our understanding of the Psittacine beak and feather disease often comes from infected individuals with visible symptoms. Limited knowledge exists regarding the pathology and role of asymptomatic individuals in disease transmission. Asymptomatic individuals could shed the virus in their crop secretion, feces, or feathers. In this study, we investigated the temporal change in the viral load in feather and fecal samples from 17 asymptomatic Rosy-faced Lovebirds (*Agapornis roseicollis*) using qPCR. Our results showed that most of the individuals had very low viral load, while three individuals with high viral load at the beginning of the experiment were observed to exhibit a decreasing trend in viral load in both fecal and feather samples. Surprisingly, the viral load in an individual can drop from a high level to an undetectable level within three months. This suggests that BFDV infection might not be lethal or highly pathogenic for some individuals. We also showed that the viral load in feathers was higher than in feces.

Keywords: avian pathogen; lovebirds; parrots; psittacine beak and feather disease; viral load

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1. Introduction

Psittacine beak and feather disease (PBFD) poses a major threat to both wild and captive parrots worldwide [1–3]. The disease is caused by the highly contagious psittacine beak and feather disease virus (BFDV), a single-stranded DNA circovirus [4,5]. The virus can infect most, if not all, psittacine species, which is particularly concerning due to the endangered status of many parrot species [1,6–9]. The global trade of parrots has been shown

to play a significant role in disseminating the virus across different continents, including Asia, Africa, the Americas, Europe, and Australasia [10–15]. Recent reports also highlighted the increasing BFDV occurrence in non-psittacine species [16]. Although the pathogenicity of BFDV in non-psittacine species remains to be determined, it is also concerning that those infected non-psittacine species could play a role in disease transmission.

The infection outcomes of PBFD in parrots can be severe and lethal. Acute cases were frequently observed in young individuals with a sudden death without visible symptoms [17,18]. Chronic PBFD tends to occur in older individuals with or without manifestation of symptoms. Once infected, the virus will remain in the host for the rest of its life; thus, the infected individual could become a carrier and may shed the virus at any point of its life. Asymptomatic individuals may start developing visible symptoms once their immune system is weakened [5]. Typical symptoms of PBFD include feather dystrophy and abnormal beak and claw growth [19]. Additionally, the virus is immunosuppressing, targeting the immune system, particularly the thymus and bursa of Fabricius, thus leading to the depletion of lymphocytes [17]. Infected individuals often die from secondary infections [17].

To date, despite the discovery of PBFD in Australia in the 1970s, no cure or effective treatment has been discovered for the disease [20]. Vaccines for BFDV have been recently developed but not yet commercialized. In a captive environment, the major measures for controlling BFDV infection are better hygiene and early diagnosis, usually performed using polymerase chain reaction PCR [8,11]. Infected individuals, even asymptomatic, could shed the virus in their crop secretion, feces, or feathers, which contaminate the environment [21]. The transmission mechanisms of the PBFD include direct contact, the fecal–oral route, vertical transmission (i.e., from the parent to the offspring), contaminated water and food, and feather and skin particles [22,23]. Therefore, infected individuals, even asymptomatic, need to be isolated from healthy individuals permanently, which might pose a burden for captive population management, especially for the breeding industry.

Over the three decades, the focus of PBFD research has shifted from disease characterization to BFDV screening, prevalence, evolution, and phylogenetic analysis [1,24–27]. A wealth of data on the BFDV genotypes and prevalence are available [6,14,28–31]. However, our understanding of the infection dynamic, which is critical for designing effective conservation management strategies and clinical treatments, is still limited. For example, numerous studies that use PCR detection have reported a high BFDV prevalence in wild and captive parrot populations, with most individuals being asymptomatic [16,30,32,33]. However, the viral load and shedding patterns in these asymptomatic birds, as well as their relationships with the overall health conditions of the birds, are unclear [16,34]. Such knowledge could help in estimating the transmission rate, identifying high-risk individuals or populations for more targeted actions, and advancing our understanding of the infection dynamic between the virus and the host. Furthermore, our knowledge on BFDV pathology primarily came from acute individuals during autopsy [35–37]. The characteristics of the physiological changes (e.g., anemia, leukopenia, liver necrosis, etc.) in different parrot species have mostly been reported in acute individuals [36]. These pathology studies were limited by a biased sample size, as most of the samples used in previous studies came from clinics where the birds were severely ill. The impacts of the virus on asymptomatic and carrier individuals were completely unknown.

International trading, both legal and illegal, has been identified as the major factor contributing to the fast spread of BFDV [31]. Among all the parrots, lovebirds (*Agapornis*) were the most traded genus according to the record of CITES, with the trade volume of 4,287,540 individuals between 1975 and 2016 [38]. The actual trade volume was even higher, because the Rosy-faced Lovebird (*Agapornis roseicollis*) was removed from the CITES Appendices in 2005. *Agapornis* are small African parrots with nine existing species in the genus [39,40]. Some of them are popular as companion pets, especially *A. roseicollis* [41]. Invasive populations of lovebirds have been reported in the UK, France, Italy, and Spain [41]. The outcomes of BFDV infection in lovebirds ranged from sub-clinical to lethal [42]. Some

veterinarians suggested that most of the infected lovebirds in US are asymptomatic carriers. Given the high international trade volume, the establishment of an invasive population worldwide, and their popularity as a companion pet, lovebirds could serve a major reservoir of the disease [43,44].

To this end, given very limited information on the role of asymptomatic individuals in BFDV transmission, we aimed to investigate the temporal change in the viral load in feather and fecal samples from 17 asymptomatic *A. roseicollis*, as feathers and feces are the primary means of viral shedding. To achieve that, we developed a quantitative PCR (qPCR) assay for BFDV DNA quantification in our infected birds, since the BFDV genome is very variable, with some studies showing host-specific viral sequences [45,46]. We predicted that the viral load in fecal and feather samples would differ, and that the viral load would decrease in asymptomatic individuals during the experiment.

2. Materials and Methods

2.1. Study Species and Sample Collection

Seventeen Rosy-faced Lovebirds (14 males and 3 females) obtained from local breeders were housed in the Centre for Comparative Medicine Research (CCMR) in the University of Hong Kong, and kept in individually ventilated cages (IVC; Tecniplast, West Chester, PA, United States) to avoid cross-infection or contamination. The age at which the birds arrived at the facility varied between 2 and 23 months old. The health of the birds was regularly assessed by monitoring for the occurrence of any disease symptoms. Fresh fecal samples were collected from the bottom of the IVC. Around 7 feathers were plucked from each of following body regions for DNA extraction for three birds (i.e., RAA7-9): back, chest, and down feathers on the rump, using sterilized forceps with clean nitrile gloves. Since the viral loads among feather types were found to be similar after investigating the difference between feather types (see Figure 1), only the feathers from the back were collected and examined for the remaining 14 individuals. In total, 66 fecal samples and 69 feather samples were collected and examined. Samples were collected from each bird once per month on average. All samples were stored in 1.5 mL microcentrifuge tube at −80 °C until DNA extraction. All samples were collected inside a biosafety cabinet.

2.2. DNA Extraction

DNA from feather samples were extracted using DNeasy Blood & Tissue kits (Qiagen, Hilden, Germany) inside a biosafety cabinet. Both enzymatic and mechanistic treatments were included in the extraction processes. The samples were mixed with 360 µL enzymatic buffer (20 mM Tris-HCL, 2 mM sodium EDTA, 1.2% Trition X-100, 20 mg/mL lysozyme, at pH 8.0) and incubated at 37 °C for 1.5 h with 8 rpm rotations. Two-hundred mg glass beads were then added into the samples. Samples were shaken vertically for 5 min at 30 Hz in a TissueLyser (Qiagen), followed by proteinase K treatment (50 µL) at 56 °C for 30 min. Samples were then incubated at 95 °C for 5 min. Next, samples were centrifuged at 9000× *g* for 1 min to remove pellets, and the supernatant was pipetted for DNA purification using a DNeasy Mini spin column according to the manufacturer's protocol. DNA from fecal samples were extracted using a QIAamp Fast DNA Stool Mini Kit (Qiagen) inside a biosafety cabinet, following the manufacturer's protocol. All extracted DNA were stored at −80 °C until further analysis.

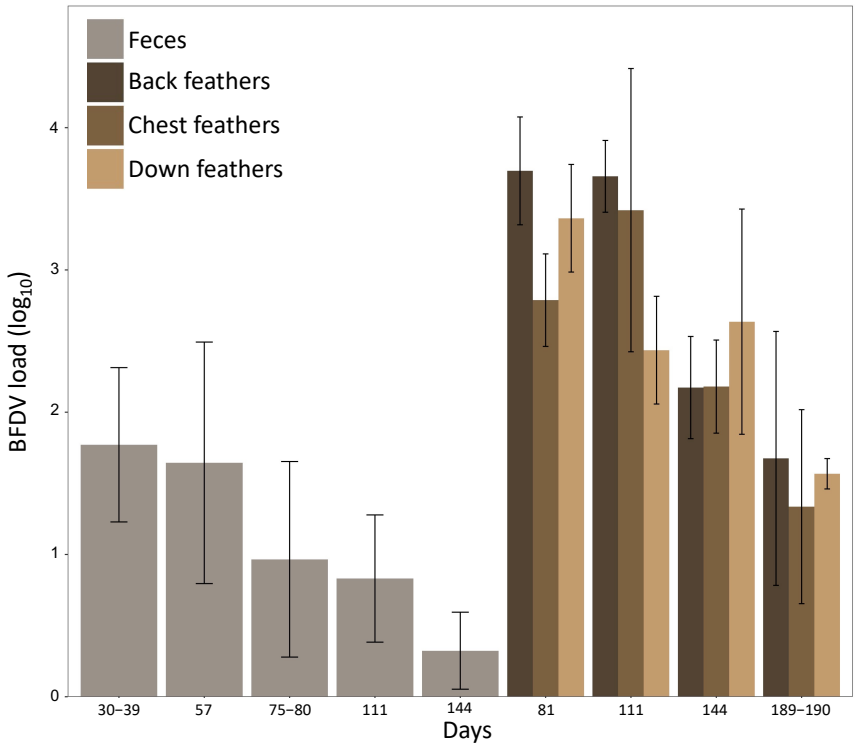

Figure 1. Psittacine beak and feather disease virus (BFDV) viral load of fecal and feather samples in the three *Agapornis roseicollis* individuals that had high viral load at the beginning of the experiment. These three individuals were RAA7, RAA8, and RAA9 (Table 1). The *x*-axis shows the day of sample collection after the birds arrived the facility and were housed in individually ventilated cages. The error bar indicates the standard error. Gray: fecal samples. Dark brown: back feathers. Brown: Chest feathers. Light brown: down feathers.

2.3. Quantitative PCR (qPCR)

Four hundred and twenty BFDV sequences were downloaded from GenBank and aligned using MEGA7, together with another 14 BFDV sequences isolated from our samples. Since the BFDV sequences were highly variable, we focused on the BFDV sequences isolated from our lovebirds (i.e., 20 sequences) for primer design. A pair of primers were designed on the relatively conserved region of the *rep* genes, which is one of the two protein-coding genes in the viral genome [47]. qPCR was performed in duplicate per sample, in a 15 µL reaction mix containing 2X iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.4 µM forward primer (5′-GAGCTGTTGCTGCCGTGAT-3′), 0.4 µM reverse primer (5′-CGCCCATGCCTGACGTAG-3′), and 20 ng gDNA using the CFX96 Torch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The thermal cycle program consisted of 1 cycle of 2 min at 95 °C, and 40 cycles of 5 s at 95 °C and 10 s at 62 °C, ending with a melt curve analysis ramping from 65 °C to 95 °C with 0.5 °C/5 s increment. The melting curve analysis was used to assess the specificity of the amplicons. Only one peak with the same melting temperature (+/−0.5 °C) was observed in all positive samples, which was further confirmed by gel electrophoresis of the qPCR product. The identity of the amplicon was subsequently validated using Sanger sequencing. Next, we used standard curve method to calculate the absolute DNA copy number present in the sample. To construct the standard curve, we first cloned the amplicon into the T-vector pMD19 (TaKaRa Bio, Kusatsu, Shiga, Japan) using a DNA ligation kit (TaKaRa). The resulting plasmids containing the BFDV sequence were linearized using a restriction enzyme ScaI-HF (New England Biolabs, Ipswich, MA, USA) and quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Waltham, MA, USA). Ten-fold dilutions were performed on the linearized plasmids, which were used as the standard curve. Additionally, we also evaluated the qPCR amplification efficiency using BFDV-positive fecal samples with 2-fold serial dilution. The amplification efficiencies of the

designed assay were 88.6% and 98.6% when the linearized plasmids and BFDV-positive fecal samples were used as the template DNA, respectively (Supplementary Figure S1).

2.4. Statistical Analyses

The effect of sex and age on the viral load was analyzed using a generalized linear mixed model (GLMM) with the *R package lme4* [48]. We input sex and age as independent variables and log (DNA copy number + 1) as dependent variable. The bird identity was input as a random factor. To examine the viral load differences between sample types, including feces, back feathers, chest feathers, and down feathers, we conducted an analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) test. *p* values were corrected using the Benjamani–Hochberg procedure. The normality and homogeneity of the variance across groups was tested using the Shapiro–Wilk normality test (W = 0.968, *p*-value = 0.373) and Levene's Test (df = 3, F value = 0.057, $p = 0.981$), respectively. This analysis included samples from three sampling periods (i.e., day 75–81, day 111, and day 144). All statistical analysis were performed in R.

3. Results

3.1. Variation in Viral Loads between Individuals

Among the 17 individuals tested using qPCR, only one individual (i.e., RAA21) was found to be BFDV-negative in both fecal and feather samples (Table 1). Ten individuals were BFDV-positive in both fecal and feather samples. Six individuals were either BFDV-positive in fecal samples (RAA15-16, and 18) or feather samples (RAA22-24).

For the individuals with temporal sampling (12 individuals), three individuals (RAA7-9) were consistently BFDV-positive, with a relatively low Cq values in both fecal and feather samples, indicating a large amount of viral DNA copies (Table 1). These three individuals were considered to contain a high viral load of BFDV.

Other individuals with BFDV-positive samples did not show a consistent result throughout the sampling period and between fecal and feather samples. The positive results for these individuals were occasionally detected with high Cq values (>35 cycles; equivalent to ~1 copy of BFDV DNA), indicating low viral load. The chance of a false positive for these individuals was low because multiple positive results were detected independently. All negative controls (i.e., sampling negative and qPCR negative controls) in the qPCR assays showed no BFDV amplification. These individuals were therefore considered to have a low viral load of BFDV. Yet, despite the differences in the viral load, none of the birds showed any observable PBFD symptoms and they behaved normally throughout the sampling period. In addition, sex and age appeared to have no influence on the viral load (Supplementary Table S1).

Table 1. Summary table of the Psittacine beak and feather disease virus (BFDV) detection results of 17 *Agapornis roseicollis* individuals. A. Fecal samples. **B.** Back feather samples. All samples were tested in duplicate using qPCR. The colored cells indicated positive results, with different colors representing the Cq values of the qPCR result. **C.** Summary of the qPCR results in fecal samples, back feathers, chest feathers, and down feathers. Values inside parentheses indicate the occurrence of the positive results throughout the sampling period.

A

Individual	Week of Arrival	Sampling Period (Week)																				Cq
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
RAA7	−4		+		+			+					+					+			+	<30
RAA8	−4	+	+		+				–				–					+			+	30−35
RAA9	−4	+	+		+				+				+					+			+	>35
RAA11	−4		+		–				+				–					–			–	Negative
RAA12	−4		+		–				–				–					–				
RAA13	−4	+	+		–				–				–					+				
RAA14	−4	+	+		–				+				–					–				
RAA15	−1		+			–				–				–					–			
RAA16	−1		+			+								+					–			
RAA17	−1		–			–				–				+			+	–				

Table 1. *Cont.*

A

Individual	Week of Arrival	Sampling Period (Week)																							Cq
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19					
RAA18	−2		−			−				+				−											
RAA20	9										+				−				−						
RAA21	13														−										
RAA22	16																			−					
RAA23	16																			−					
RAA24	17																			−					
RAA25	17																		+						

B

Individual	Week of arrival	Sampling period (week)																							
		8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29		
RAA7	−4	+				+					+				+										
RAA8	−4	+				+					+				+										
RAA9	−4	+				+					+						+								
RAA11	−4	+									+						+								
RAA12	−4					+					+						+								
RAA13	−4	+				+					+						−								
RAA14	−4	+				+					−				+										
RAA15	−1		−				−					−					−								−
RAA16	−1		−									−													
RAA17	−1		+				−																		
RAA18	−2		−				−																		
RAA20	9											+					+								−
RAA21	13																								−
RAA22	16																					+			
RAA23	16																					+			
RAA24	17																			+					
RAA25	17																			+					

C

Individual	Age (months)	Sex	Feces	Back feather	Chest feather	Down feather
RAA7	16	F	+ (5/5)	+ (4/4)	+ (4/4)	+ (4/4)
RAA8	16	M	+ (4/6)	+ (4/4)	+ (4/4)	+ (4/4)
RAA9	16	M	+ (6/6)	+ (4/4)	+ (4/4)	+ (4/4)
RAA11	23	F	+ (2/5)	+ (3/3)		
RAA12	16	M	+ (1/5)	+ (3/3)		
RAA13	16	M	+ (3/6)	+ (3/4)		
RAA14	16	M	+ (3/6)	+ (3/4)		
RAA15	6	M	+ (2/5)	− (0/5)		
RAA16	10	M	+ (3/4)	− (0/2)		
RAA17	9.5	M	+ (2/6)	+ (1/2)		
RAA18	7.5	M	+ (1/4)	− (0/2)		
RAA20	6	M	+ (1/3)	+ (2/3)		
RAA21	5	M	− (0/1)	− (0/1)		
RAA22	4	M	− (0/1)	+ (1/1)		
RAA23	4.5	F	− (0/1)	+ (1/1)		
RAA24	3	M	− (0/1)	+ (1/1)		
RAA25	3	M	+ (1/1)	+ (1/1)		

3.2. Temporal Viral Load Changes within an Individual

Focusing on the individuals with a high viral load (RAA7-9), we further demonstrated that the viral load in both the fecal and feather samples dropped continuously within 200 days after the birds arrived at the facility (Figures 1 and 2). The trend was similar among all sample types (Figure 1). The average reduction in viral load was similar between fecal and feather samples (−82.32% and −96.58%, respectively). Notably, the fecal viral load in one individual (RAA8) was barely detectable after ~3 months (Figure 2).

3.3. Feathers Had a Higher BFDV Load Than Feces

For the individuals with a high viral load (RAA7-9), there was a significant difference in viral load among body parts (ANOVA; $p < 0.0001$; Supplementary Table S2). The viral loads in all feather samples were significantly higher than those in feces (adjusted $p < 0.001$; Tukey HSD post hoc; Supplementary Table S2). However, there was no significant difference observed among the different feather types.

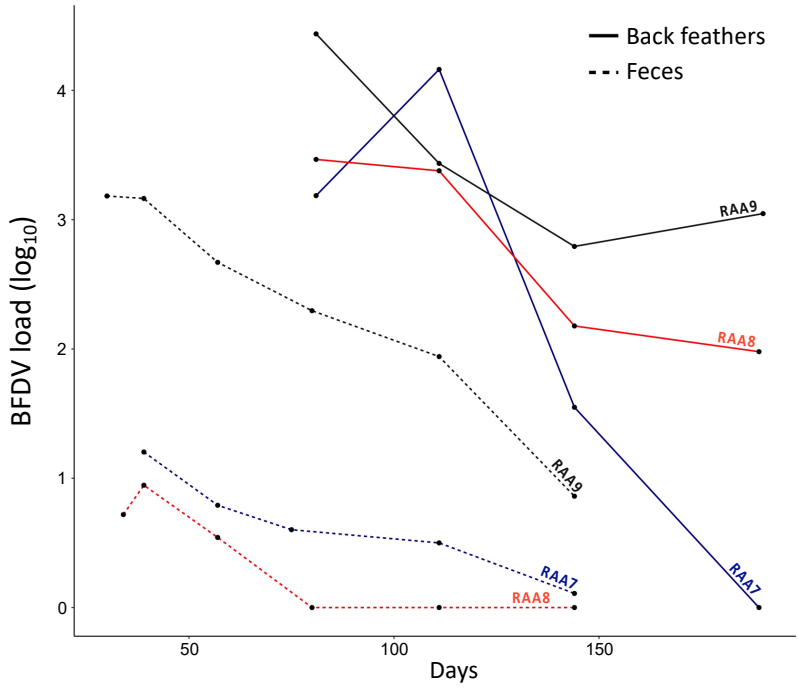

Figure 2. Temporal change in Psittacine beak and feather disease virus (BFDV) viral load in fecal (dashed line) and back feather (solid line) samples in three *Agapornis roseicollis* individuals that had high viral load at the beginning of the experiment. These three individuals were RAA7, RAA8, and RAA9 (Table 1). The *x*-axis shows the day of sample collection after the birds arrived the facility and were housed in individually ventilated cages.

4. Discussion

Most individuals (16 out of 17) in this study were BFDV-positive, but they did not exhibit any observable PBFD symptoms and behaved normally. Notably, despite the high BFDV prevalence, only three individuals had a relatively high viral load, while most individuals had very low levels of viral load. Among the individuals with high viral load, the viral load decreased drastically from a high level to only a few copies detected within 100 days in both the feather and fecal samples. In one individual with high viral load at the beginning of the experiment, the viral loads in its fecal samples became barely detectable after ~3 months.

We observed that feather samples from all three body regions had a higher BFDV load compared to feces. It took longer for the viral load to drop to a relatively low level in feathers than feces. One possible explanation for this finding is that the virus and viral DNA in the feathers represent the historical infection [49]. The virus and viral DNA could persist for a longer period of time in feathers than in the digestive system. Previous studies have shown that viral inclusion bodies in feathers are common during BFDV infection, and feather follicles are one of the major virus replication sites [50]. The virus could accumulate and stay in the feather for a long period, and may only be removed during cleaning (e.g., preening and bathing) or molting. Similar findings were observed in Budgerigars (*Melopsittacus undulatus*) and Crimson Rosella (*Platycercus elegans*) [49,51]. On the other hand, the bursa of Fabricius has been suggested as one of the main sites of BFDV replication [52], and there are sophisticated immunological interactions between the immune cells and pathogens in the gut for the host to clear the invading virus [53]. The viral load in fecal samples, therefore, may better reflect the current BFDV infection status than that in feather samples. As BFDV is known to persist in the environment for a prolonged period, the potential for the virus to persist in feathers for an extended duration can pose a long-lasting infection risk, even if the host has recovered or suppressed the virus. The potential for recovered or tolerant birds, which show no signs of disease, to carry the

virus and shed it into the environment or infect other birds has important implications for conservation and disease management.

Variations in the susceptibility to BFDV infection between different species has been observed [43]. Eclectus Parrots (*Ecletus roratus*) and Gang Gang Cockatoos (*Callocephalon fimbriatum*) suffer severe consequences of BFDV infection, while Cockatiels (*Nymphicus hollandicus*) were said to be less susceptible to the infection [2,46,54]. Some lovebird species are highly susceptible, with some advanced clinical signs [7,43]. A 100% mortality was reported in captive flocks of the Black-cheeked Lovebird (*A. nigrigenis*) and Lilian's Lovebird (*A. lilianae*) [43]. In contrast, we showed that some *A. roseicollis* appear to cope well with BFDV infection, as suggested by the lack of observable signs of disease, low viral loads in most BFDV-positive birds, and fast decrease in viral load in individuals that originally had a high viral load within a short period of time. In a previous study, *A. roseicollis* and *A. fischeri* that were in close contact with diseased birds were observed to show no signs of disease in captivity [43]. Other studies also reported the recovery of lovebirds from BFDV-associated feather abnormality, but it is uncertain whether those individuals developed a chronic form of the disease or became carriers [19,55]. Although no BFDV was found in a wild population of *A. lilianae* in Malawi, South Africa, the virus contact history of the population was unknown [56]. These findings, taken together, suggest that some *Agapornis* species might be more tolerant to BFDV infection than some cockatoos and lorikeets [13].

One limitation of this study is that only three individuals had a relatively high viral load. To comprehensively understand viral persistence across tissue types and how host factors may impact viral load, larger sample sizes are needed. Additionally, we acknowledge that the low Cq value in this study may be due to residual viral DNA copies rather than viable virus. Additional assays, such as a hemagglutination test, are necessary to validate the viral viability. Furthermore, although all birds in this study were infected with BFDV before entering the housing facility, the exact timing of infection was unknown. To investigate the host–virus dynamics, a virus challenge experiment could be conducted in the future.

In conclusion, we showed that the BFDV load can drop from a high level to an undetectable level in the feces of Rosy-faced Lovebirds within a few months, and most of the BFDV-positive birds shed very little virus into the environment. The discovery of higher viral loads in feathers, which can persist for longer periods compared to feces, has provided crucial insights into the potential risk of BFDV infection for conservation and disease management purposes. This knowledge can help inform strategies to mitigate the spread of the virus and protect parrot populations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/birds5030028/s1, Figure S1: Standard curves of the PBFDV qPCR detection assay. Table S1: Generalized linear mixed model testing the effect of age and sex on BFDV viral load. Table S2: BFDV viral load difference among sample types.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials; further inquiries can be directed to the corresponding author.

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