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No T-cell-mediated immune response detected in a red-fronted parakeet (*Cyanoramphus novaezelandiae*) infected with the Beak and Feather Disease Virus (BFDV)

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Abstract Here I report on a small scale study aimed at generating baseline information on the immune response of wild red-fronted parakeets, as assessed by blood cell counts, and subcutaneous challenge with phytohaemagglutinin (PHA), a mitogen that causes swelling at the point of injection. Eleven parakeets captured in mist-nets were injected into the right patagium with 0.5 mg PHA and the resulting swelling measured at 6 hours post-injection. Prior to PHA challenge, feather and blood samples were collected for detection of beak and feather disease virus and *Plasmodium*. Blood smears were also prepared for blood cell counts. Swelling occurred 6 hours post-injection in all but one individual, which tested positive for beak and feather disease virus. In this individual, no measurable swelling was detected. Estimated leucocyte counts, lymphocyte counts and heterophil counts of the same individual were similar to values of beak and feather disease virus negative individuals. *Plasmodium* DNA was detected in 2 individuals and their immune response was similar to that of parakeets testing negative for both beak and feather disease virus and *Plasmodium*. Estimated leucocyte counts, lymphocyte and heterophil counts did not differ between *Plasmodium* infected and non-infected individuals. The fact that the only individual testing positive for beak and feather disease virus showed no immune response to PHA challenge suggests increased susceptibility to other pathogenic infections. Although preliminary, this study highlights the potential damaging consequences of the accidental introduction of beak and feather disease virus in conservation programmes of threatened New Zealand parrots, some of which might already suffer from decreased immunocompetence resulting from reduced genetic diversity.

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Keywords *Cyanoramphus novaezelandiae*; phytohaemagglutinin; Little Barrier Island; leucocyte counts; PHA

INTRODUCTION

The Beak and Feather Disease Virus (BFDV) is the causative agent of Psittacine Beak and Feather Disease (PBFD), an infection of parrots and cockatoos that has been the subject of extensive research since its description (Pass & Perry 1984). The clinical signs of the disease are highly variable among hosts, but include deformed feathers (i.e., clubbed, curled shape), feather lesions (i.e., breaking of emerging shafts, bleeding shafts), abnormal feather loss and feather colouration, and necrosis of the beak (Gerlach 1994a). Viral particles can be confirmed by microscopy of affected tissue, antigen tests, or by PCR detection of viral DNA (Latimer *et al*. 1990; Ramis *et al*. 1994). One advantage of PCR-based diagnosis is the ability to detect viral DNA among individuals not showing symptoms of PBFD, particularly in feather samples (Khalesi *et al.* 2005), which allows for field surveys of the virus in locations of interest (Ortiz-Catedral *et al*. 2009b).

PBFD is considered an immunosuppressive disease due to its damage to the thymus and Bursa of Fabricius and secondary bacterial and fungal infections commonly found in PBFD birds (Gerlach 1994a). For example, long-billed corellas (*Cacatua*

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tenuirostris) experimentally infected with BFDV show reduced estimated leucocyte counts than non-infected individuals (Bonne *et al.* 2009), which indicates a potential decrease in the cell-mediated immune response following viral infection. However, research on immune function and BFDV infection is limited.

Recent studies in New Zealand have highlighted the usefulness of the phytohaemagglutinin (PHA) test to investigate immunocompetence of native species of conservation concern such as Forbes parakeets (*Cyanoramphus forbesi*) (Tompkins *et al*. 2006) and New Zealand robins (*Petroica longipes*) (Hale & Briskie 2007). This study was aimed at producing baseline information about the immunocompetence of a New Zealand endemic: the red-fronted parakeet (*Cyanoramphus novaezelandiae*). Red-fronted parakeets are vulnerable to extinction (www.iucn.org) and currently restricted to islands free of introduced predators and a few mainland sites (Robertson *et al*. 2007).

METHODS

A total of 11 red-fronted parakeets were captured using mist nets on Little Barrier Island (LBI) from 5 to 17 May 2008. All captured parakeets exhibited normal plumage and no external indication of current infection with BFDV. Every captured bird was weighed, measured and given a uniquely numbered metal band following guidelines by the New Zealand Department of Conservation (DOC). Blood was obtained by puncture of the brachial vein. To prepare blood smears, a drop of blood was collected with a non-heparinised capillary tube, transferred onto a microscope slide, and then smeared to a thin layer following the "push-slide" method (Walberg 2001). Slides were air-dried, fixed in 100% methanol (Bennett 1970), and then stained with May Grunwald-Giemsa followed with a phosphate buffer/rinse (Robertson & Maxwell 1990) (Technecult Laboratories Ltd., Napier, New Zealand). White blood cell counts were completed by haematologists at Gribbles Veterinary Pathology (Auckland, New Zealand), and involved counting 10 random fields at 100 x magnification, following the methodology described in Parker *et al*. (2006) to determine estimated leucocyte count, lymphocyte count and heterophil count. Other blood cell types such as monocytes and eosinophils are also found in avian blood, but for this study the focus was on lymphocytes and heterophils due to their role in immune function following Hale & Briskie (2007). In addition to blood, 2 contour feathers from the ventral region were collected with tweezers for molecular determination of sex following the methodology described by Griffiths *et al*. (1998) and for detection of BFDV DNA following the methodology described by Ha *et al.* (2007). Molecular sexing and detection of BFDV DNA was done at the Equine and Parentage and Animal Genetics Centre at Massey University.

Given that immune response can be affected by other pathogens in addition to BFDV, I also tested parakeets for presence of *Plasmodium* DNA in blood samples. *Plasmodium* infection has been confirmed in a number of native and exotic birds species in New Zealand (Sturrock & Tompkins 2008; Tompkins & Gleeson 2006). As some of these bird species occur on LBI (i.e., blackbird *Turdus merula*; song thrush *T. philomelos*), this pathogen was screened also in parakeets. Approximately 70 µl of blood were collected using a heparinized capillary tube. Its ends were sealed with plasticine and kept at 4 °C until PCR analysis 2 days after blood collection. DNA extraction and PCR amplification of *Plasmodium* followed Tompkins & Gleeson (2006) and was completed at Landcare Research, Auckland. The parakeets used in the PHA essay were also tested for *Salmonella*, *Campylobacter* and *Yersinia*, but none of these pathogens was detected (Ortiz-Catedral *et al*. 2009a).

To experimentally test the strength of the immune response, parakeets were injected subcutaneously with 0.5 mg PHA dissolved in 0.1 mL phosphate-buffered saline into the right patagium as described by Tompkins *et al.* (2006). PHA induced swelling was measured using callipers to the nearest 0.05 mm prior to injection, then at 6 hours post-injection (average of 3 measures on the same point). After injection, parakeets were held in card boxes padded with fresh kanuka (*Kunzea ericoides*) branches and provided with food and water *ad libitum*. Food consisted of sliced apple, fresh peas, freshly collected karamu (*Coprosma robusta*) berries, and corn on the cob. Data collection was completed under full approval by DOC (permits AK-22658-FAU, AK-15300-RES, AK-20666-FAU and AK-22857-FAU). Capture, sample collection and handling of parakeets were conducted following approved protocols by the Massey University Animal Ethics Committee (protocols MUAEC 07/138 and 08/24).

Statistical analysis

The amount of swelling after 6 hours post-injection with PHA was compared using the Mann-Whitney U test in StatView version 5.0.1©. Traditionally, PHA assays compare swelling after 24 hours of injection; however, measuring the resulting swelling at 6 hours post-injection has been shown to result in reliable estimate of PHA responsiveness (Smits *et al.* 1999). Further, this methodology has been successfully used in native New Zealand parakeets previously (Tompkins *et al*. 2006). A significance level at $\alpha = 0.05$ was set for comparisons between BFDV negative *vs.* BFDV positive birds. The same statistical approach

was used to compare *Plasmodium* negative *vs.* *Plasmodium* positive individuals. All individuals that tested negative for either *Plasmodium* or BFDV are referred to as the uninfected group.

RESULTS

Of the 11 captured parakeets for this research, only 1 tested positive for BFDV DNA in feather samples. This individual showed no external signs indicative of PBFD; its plumage appeared normal and indistinguishable from the uninfected group (n = 8). In 2 other parakeets, *Plasmodium* DNA was detected. These two parakeets also appeared normal on inspection of plumage. During the PHA test, there was no measurable swelling response 6 hours post-injection in the parakeet where BFDV DNA was confirmed. The swelling in the uninfected group averaged 1.2 ± 0.26 mm. When contrasting the swelling response of the BFDV positive individual and the uninfected group, the difference only approached significance (BFDV positive mean rank: 1.0; uninfected group mean rank: 6.5; Z = -1.5, P = 0.11). Of 11 parakeets sampled, only 9 blood smears were suitable for white blood cell counts. Estimated leucocyte counts did not differ between the BFDV positive individual *vs.* the uninfected group (n = 6) (uninfected group: 7.12 ± 2.13 x $10^{9\text{-}1L}$, BFDV positive individual: 4.6 x $10^{9\text{-}1L}$, Z = - 0.29, *P* = 0.77). Similarly, there was no difference in the lymphocyte count between the uninfected group and the BFDV positive individual (lymphocyte count uninfected group: 75.8 ± 4.15 x$10^{9\text{-}1L}$; BFDV positive individual: 76 x $10^{9\text{-}1L}$; Z = - 0.29, *P* = 0.77). Finally, no difference was detected in heterophil count between the same groups (heterophil count uninfected group: 10.6 ± 3.32 x$10^{9\text{-}1L}$; BFDV positive individual: 4 x$10^{9\text{-}1L}$; Z = -1.46, *P* = 0.14).

The swelling response of parakeets infected with *Plasmodium* averaged 1.82 ± 0.55 mm. Such swelling was not statistically different from the uninfected group (*Plasmodium* positive mean rank: 8.5; uninfected group mean rank: 5.44; *Z* = -1.16, P = 0.24). Estimated leucocyte count did not differ statistically between the *Plasmodium* positive group (n = 2) and the uninfected group (estimated leucocyte count uninfected group: 7.12 ± 2.13 x $10^{9\text{-}1L}$; *Plasmodium* positive group: 4.7 ± 0.5 x$10^{9\text{-}1L}$; Z = -0.39, *P* = 0.69). Likewise, lymphocyte count did not differ significantly between *Plasmodium* infected and uninfected individuals (lymphocyte count uninfected group: 75.8 ± 4.15 x $10^{9\text{-}1L}$; *Plasmodium* positive group: 58.5 ± 6.5 x $10^{9\text{-}1L}$; Z = - 0.77, *P* = 0.44). Lastly, the heterophil count was similar between infected and uninfected parakeets (heterophil count uninfected group: 10.6 ± 3.32 x$10^{9\text{-}1L}$; *Plasmodium* positive group: 12.5 ± 1.5 x$10^{9\text{-}1L}$; Z = 1.16, *P* = 0.24).

DISCUSSION

The recent discovery of PBFD in a natural population of red-fronted parakeets has raised concerns over the potential effects of this viral disease among threatened New Zealand parrots (Ortiz-Catedral *et al*. 2009b). While it is well established that PBFD causes mortality of parrots and allies in aviculture (Heath *et al*. 2004) and in some captive-breeding programmes (Commonwealth of Australia 2005), the effects of the disease in wild populations are not well documented. The results in this study indicate that BFDV occurs in a free-living population of parakeets in New Zealand and that infection may be associated with lack of responsiveness to challenge with PHA. The PHA test has been shown to be a good indicator of acquired T-cell immunocompetence of birds (Tella *et al*. 2008). Thus, BFDV may render the infected individual unable to mount T-cell immunocompetence, even when there are no clinical signs of PBFD, as it is the case for the BFDV positive individual in this study.

The results presented here should be confirmed with a larger group of parakeets, ideally including individuals with sub-clinical as well as clinical BFDV infection to better understand the immunosuppressive effects of the virus in free-living parakeets. It is unclear why infection with either *Plasmodium* or BFDV was not associated with higher heterophil and overall leucocyte counts indicative of current parasitic infection (Campbell 1994), and lower lymphocyte counts, often associated with immunosuppression (Walker *et al.* 1983) and virus infection (Bonne *et al.* 2009). It is likely that the limited sample size used is insufficient to detect such differences. Another possibility is that haematologic changes would be detected as these infections develop. As mentioned previously, the uninfected group and the *Plasmodium* and BFDV infected parakeets were undistinguishable on external inspection. However, since the birds used are free-living nothing is known about infection date and pathogenesis of the infections. One limitation of this study is that only one response of the avian immune system was assessed (T-cell immunocompetence). Another component of the avian immune system is the humoral response (production of immunoglobulins) to antigens (Gerlach, 1994b). Ideally, future studies on the effects of BFDV among New Zealand parrots should include an assessment of humoral immune response.

Although preliminary, the results presented here complement previous studies of BFDV in native and exotic parrots in New Zealand by Ha *et al*. (2007) and Ortiz-Catedral *et al*. (2009b) and strengthen the view of BFDV as a potential further threat for the conservation of New Zealand parrots.

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