Use of a real-time PCR to explore the intensity of *Plasmodium* spp. infections in native, endemic and introduced New Zealand birds

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SUMMARY

Avian malaria, caused by *Plasmodium* spp., is an emerging disease in New Zealand (NZ). To detect *Plasmodium* spp. infection and quantify parasite load in NZ birds, a real-time polymerase chain reaction (PCR) (qPCR) protocol was used and compared with a nested PCR (nPCR) assay. A total of 202 blood samples from 14 bird species with known nPCR results were tested. The qPCR prevalences for introduced, native and endemic species groups were 70, 11 and 21%, respectively, with a sensitivity and specificity of 96·7 and 98%, respectively, for the qPCR, while a sensitivity and specificity of 80·9 and 85·4% were determined for the nPCR. The qPCR appeared to be more sensitive in detecting lower levels of parasitaemia. The mean parasite load was significantly higher in introduced bird species (2245 parasites per 10 000 erythrocytes) compared with endemic species (31·5 parasites per 10 000 erythrocytes). In NZ robins (*Petroica longipes*), a significantly lower packed cell volume was found in birds that were positive for *Plasmodium* spp. compared with birds that were negative. Our data suggest that introduced bird species, such as blackbirds (*Turdus merula*), have a higher tolerance for circulating parasite stages of *Plasmodium* spp., indicating that introduced species are an important reservoir of avian malaria due to a high infection prevalence and parasite load.

Key words: avian malaria, New Zealand, *Plasmodium*, PCR, real-time, robin, Waimarino.

INTRODUCTION

Avian malaria, caused by *Plasmodium* spp., is an emerging disease in New Zealand (NZ). To date, 17 distinct *Plasmodium* lineages have been described in the country and infections have resulted in morbidity and mortality in several of NZ's bird species (Schoener *et al*. 2014). Various studies have explored the prevalence of infection in NZ bird populations, the *Plasmodium* lineages involved, and the geographical spread of the disease (Baillie and Brunton, 2011; Castro *et al*. 2011; Marzal *et al*. 2011; Ewen *et al*. 2012*a*; Howe *et al*. 2012). However, no quantitative molecular analysis of parasite load in individual NZ birds has been performed to study the parasite load for *Plasmodium* spp. infections.

When birds become infected with *Plasmodium* spp., an acute phase of infection with high levels of parasitaemia is followed by a chronic phase where parasites are either absent or present in low numbers in the peripheral circulation (Atkinson *et al*. 2000; Valkiunas, 2005). The parasite load during the acute as well as chronic phase of infection tends to vary between avian host species and between different *Plasmodium* lineages, consequently leading to a varying severity of pathological signs (Atkinson *et al*. 2000; Valkiunas, 2005). High acute phase infection intensities have been associated with high mortality (Zehtindjiev *et al*. 2008; Asghar *et al*. 2011), while low levels of parasitaemia during the chronic phase of infection have been shown to have more subtle effects on individual host fitness, such as influencing reproductive success or life span (Asghar *et al*. 2011, 2015). Parasite dynamics and disease expression are influenced by environmental factors, parasite virulence and host factors such as a bird's susceptibility, and its levels of resistance or tolerance towards infections with *Plasmodium* spp. (Palinauskas *et al*. 2008; Atkinson *et al*. 2013; Atkinson *et al*. 2014; Dimitrov *et al*. 2015).

The addition of parasite load data to infection prevalence studies will lead to a deeper understanding of the pathogen dynamics of various *Plasmodium* lineages in different avian species. This is of special interest in NZ, where introduced passerines and endemic bird species inhabit the same ecological systems. Introduced European species like blackbirds (*Turdus merula*) and song thrushes (*Turdus philomelos*) have, over a long period of time, coevolved in their native range with *Plasmodium* lineages and are now frequently found in NZ (Ewen *et al*. 2012*a*). Higher survival rates and

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higher parasite loads in surviving birds have been found in bird species that have co-evolved with *Plasmodium* species resulting in a higher parasite tolerance (Atkinson *et al*. 2013). In contrast, it is likely that NZ's endemic bird species were not exposed to these parasites until the late 19th century, when European passerines were introduced to NZ (Laird, 1950; Duncan, 1997). Therefore, NZ's endemic bird species are thought to have only recently been exposed to introduced *Plasmodium* species (Ewen *et al*. 2012*a*) potentially leading to a lower tolerance and survival following infection (Atkinson *et al*. 2013). Chronic infection among healthy-looking introduced blackbirds, song thrushes and starlings (*Sturnus vulgaris*) is common, with prevalence ranging from 50% to over 90% described in birds from the North Island (Tompkins and Gleeson, 2006; Ewen *et al*. 2012*a*; Howe *et al*. 2012; Sijbranda *et al*. 2016). In contrast, studies report a low-to-moderate prevalence of avian malaria in many endemic bird species; a few examples include a prevalence of 4% in North Island robins (*Petroica longipes*) (Sijbranda *et al*. 2016), 7% in whiteheads (*Mohoua albicilla*) (Ewen *et al*. 2012*a*), 10% in hihi (*Notiomystis cincta*) (Ewen *et al*. 2012*a*, *b*), 13% in bellbirds (*Anthornis melanura*) (Baillie and Brunton, 2011), but also 17% in South Island saddlebacks (*Philesturnus carunculatus*) (Alley *et al*. 2010), 6–33% in North Island saddlebacks (*Philesturnus rufusater*) (Castro *et al*. 2011; Ewen *et al*. 2012*a*) and a high 41% in red crowned parakeets (*Cyanoramphus novaezelandiae*) (Ortiz-Catedral *et al*. 2011). Sporadic mortality in endemic species has been demonstrated in brown kiwi (*Apteryx mantelli*), great spotted kiwi (*Apteryx haastii*), hihi (*N. cincta*), yellowheads (*Mohoua ochrocephala*) and saddlebacks (Howe *et al*. 2012), indicating that *Plasmodium* lineages that are assumed to be of relatively low pathogenicity in exotic species, could have serious implications for endemic species.

To date, determining parasite load is largely dependent on screening of blood smears. While the examination of blood smears enables determination of parasite load and morphologic characterization and detects co-infections, the process is time consuming, may miss low-intensity parasitemias and relies on good quality smears and an experienced operator (Valkiūnas *et al*. 2006). Thus, this study describes the validation and use of a real-time polymerase chain reaction (qPCR) protocol targeting the conserved large subunit ribosomal-RNA (LSU-rRNA) gene described by Friedl and Groscurth (2012) to detect and assess infection intensity with *Plasmodium* spp. in NZ birds. The effect of avian host species on the level of parasitaemia was examined, and physiological correlates of infection were evaluated by assessing packed cell volume (PCV) and body condition index (BCI).

MATERIALS AND METHODS

Samples

The samples included in this study were collected from 14 bird species during a previous avian malaria research project in and around the Waimarino Forest of NZ (39°26′17·66″S, 175°8′24·76″E) as described by Sijbranda *et al*. (2016). Briefly, body mass, tarsometatarsal length and wing chord were measured and a physical health check was performed. Blood was collected in heparinized tubes and used to determine PCV on the day of collection with the remainder stored for later DNA processing. DNA was extracted from 201 blood samples from NZ robins ($n = 100$), blue ducks ($n = 14$) and 87 birds belonging to other passerine species. These samples were analysed by a nested PCR (nPCR) protocol using the primer sets NF1/NR3 and F/R2 (Table 1) as described by Sijbranda *et al*. (2016).

Real-time PCR

To calculate the quantity of *Plasmodium* parasites in blood samples from the qPCR assay, standard curves were generated, using samples with known quantities of a fragment of the plastid-like LSU-rRNA gene. A 595 bp fragment of the LSU-rRNA gene from a *Plasmodium* (Huffia) *elongatum*-positive blood sample was amplified using the forward and reverse primers L1 and L2 (Table 1), as described by Tan *et al*. (1997). These fragments were ligated into vectors using the pGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions and *Escherichia coli* JM109 high-efficiency competent cells (Promega, Madison, Wisconsin, USA) were transformed. Transformed colonies were selected using blue/white selection and grown overnight at 37 °C in 1 mL Luria-Bertani broth containing 100 μg mL^{-1} Ampicillin sodium salt (Gibco®, Life technologies, New York, New York, USA). DNA was extracted using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen, Life technologies, Carlsbad, California, USA) according to the manufacturer's instructions. Resulting plasmid DNA was quantified using a Nanodrop 2000 (ThermoFisher, Wilmington, Delaware, USA). To prepare standard samples with known quantities of vector DNA copies, purified vector DNA was diluted in Tris-EDTA (TE) buffer to a concentration of 1 ng μL^{-1}. Of this 1 ng μL^{-1} solution, seven 10-fold standard dilutions in TE buffer were made ranging from 1 ng μL^{-1} ($2{\cdot}61 \times 10^{8}$ copies of the target sequence per μL) to 1×10^{-7} ng μL^{-1} (26 copies of the target sequence per μL). Prepared dilution standards were stored at 4 °C and consequently used within a day of preparation.

To detect *Plasmodium* sp. DNA in 201 DNA samples and assay standards, an 85 bp product of the

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Table 1. Primers used for *Plasmodium* spp. screening of 202 blood samples from birds of the Waimarino Forest

Primers	Sequence (5′–3′)	Target gene	Size (bp)	Reference
Related to nested polymerase chain reaction (PCR) testing				
HaemNF1	5′-CATATATTAAGAGAAITATGGAG-3′	Cytochrome b gene	619	Hellgren *et al.* (2004)
HaemNF3	5′-ATAGAAAGATAAGAAATACCATTC-3′			
HaemF	5′-ATAGAAAGATAAGAAATACCATTC-3′		480	Bensch *et al.* (2000)
HaemR2	5′-GCATTATCTGGATGTGATAATGGT-3′			
Related to real-time PCR testing				
L1	5′-GACCTGCATGAAAGATG-3′	Plastid-like large subunit ribosomal-RNA gene	595	Tan *et al.* (1997)
L2	5′-GTATCGCTTTAATAGGCG-3′			
Plasmo474 for	5′-AGGCTAATCTTTTCCGAGAGTCC-3′		85	Friedl and Groscurth (2012)
Plasmo558rev	5′-ACATACTGCTTTAGGATGCGA-3′			

LSU-rRNA gene, which is known to be conserved across a range of *Plasmodium* and *Haemoproteus* species, was amplified using the primers Plasmo474 for and Plasmo558rev (Table 1) as described by Friedl and Groscurth (2012) with the following modifications. Each 20 μL qPCR reaction comprised 10 μL of 2× Perfecta SYBR Green Fastmix® (Quanta biosciences, Gaithersburg, Maryland, USA), 0·25 μM of the primers Plasmo474for and Plasmo558rev and 100 ng of sample DNA or 1 μL of a standard DNA template. The qPCR protocol, performed on the Eco™ Real-Time PCR system (Eco™ Software V4.0.07; Illumina Inc., San Diego, California, USA), included initial steps of 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a pre-melt conditioning at 95 °C for 15 s and a 0·3° incremental melt curve over a 55–95 °C temperature range with a 5 s hold. All seven standard dilutions were run with each round of qPCR. The concentration of *Plasmodium* DNA in each test sample was calculated from the standard curves. It was assumed that each *Plasmodium* parasite contains one copy of the LSU-rRNA gene, so that the amount of copies of the target sequence in an unknown sample correlates directly with the amount of *Plasmodium* parasites in that sample (Wilson *et al*. 1996). Additionally, a DNA sample from a yellow-eyed penguin (*Megadyptes antipodes*) known to contain *Leucocytozoon* sp (Hill et al, 2010) and a DNA sample from a bellbird (*A. melanura*) was known to contain *Haemoproteus* spp. by sequencing were used to test primer specificity.

Parasite load was defined as the number of *Plasmodium* parasites per 10 000 avian blood cells. For the purpose of calculation, it was assumed that the average genome size of passerines is 2·8 pg (Tiersch and Wachtel, 1991), so that each 1 ng of total DNA in unknown samples reflects 357 cells. Thus, the amount of avian blood cells in each reaction was calculated by multiplying the total amount of DNA in ng by 357.

Statistics

The apparent *Plasmodium* prevalence and 95% confidence interval for various groups and species of birds was determined using the Epitools software following the Wilson binomial approximation (Brown *et al*. 2001). The χ^2 tests (Preacher, 2001) were used to analyse differences in *Plasmodium* prevalence between various groups and species of birds. In analyses where frequencies were below 5, Fisher's exact tests were used (Preacher and Briggs, 2001).

To generate a quantitative BCI, body weight in grams was divided by tarsus length in mm. The BCI was assessed for blackbirds, silvereyes and NZ robins. Because data for BCI and PCV in the various bird species were not normally distributed, a non-parametric analysis (Mann–Whitney U tests,

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SPSS, version 22, IBM Statistics) was used to determine whether there was a significant difference in these parameters between *Plasmodium*-negative and -positive birds. The correlation between parasite load and the physical parameters PCV and BCI was explored using linear regression (Microsoft Excel 2010).

Analyses of the effect of bird species group (introduced, native or endemic birds) on quantitative parasite load were performed. The parasite load data were not normally distributed, so a log transformation of the data was applied before evaluating differences in parasite load between introduced, native and endemic birds groups using a one-way analysis of variance (one-way ANOVA), followed by *post hoc* Bonferroni comparisons.

Latent class analysis to estimate sensitivity and specificity of qPCR and nPCR

Markov chain Monte Carlo latent class analysis (LCA) was conducted in R software (R Core Team 2013) to generate estimates of the true prevalence and determine the sensitivity and specificity of the nPCR and qPCR to detect *Plasmodium* spp. DNA for the groups of introduced, native and endemic species, assuming the tests were conditionally independent. The LCA methods were based on those created for analysis of imperfect diagnostic tests (Branscum *et al*. 2005). This routine allows posterior testing for differences in prevalence estimations between populations. The apparent prevalence (AP) estimates of *Plasmodium* spp. infection in the three bird groups were used for input into the LCA model. Prior estimates of test sensitivity and specificity and of infection prevalence were derived from studies of *Plasmodium* diagnostics in human medicine (Roth *et al*. 2016) and were specified using β distributions calculated in Betabuster software (http://betabuster.software.informer.com/). Stability of each model was tested using uninformative priors [β(1,1)], and posterior distributions were compared with models containing informative priors. Pairwise tests for differences in prevalence of *Plasmodium* spp. infection among the three bird groups were performed using a Bayesian statistical probability for estimating differences in prevalence between populations, where values close to 0 and 1 indicate potential significant differences. Plots of true and AP estimates were created in the ggplot2 package (Wickham, 2009).

RESULTS

Detection sensitivity of the LSU-rRNA gene qPCR assay

The LSU-rRNA qPCR detected the target DNA sequence of *Plasmodium* in all standard samples containing amounts of copies of the target DNA ranging from $2{\cdot}61 \times 10^8$ to 261 copies, with Cq-values ranging from 7·4 to 35·0 thermo-cycles, while using a normalized fluorescence threshold of 0·020. All standard curves were highly reproducible and had R^2-values of >99% (Fig. 1). Melt curve analysis showed a consistent dissociation temperature of 78·4 °C for the cloned standard controls, which originated from a *P*. (Huffia) *elongatum* clinical sample. DNA samples extracted from the blood of a yellow-eyed penguin, known to contain *Leucocytozoon* spp., and a bellbird, known to contain *Haemoproteus* spp. were also amplified using these primers; however, melt curves for both amplicons showed higher dissociation temperatures of 84·2 and 82·8 °C, respectively, confirming the ability of these primers to provide genera discrimination.

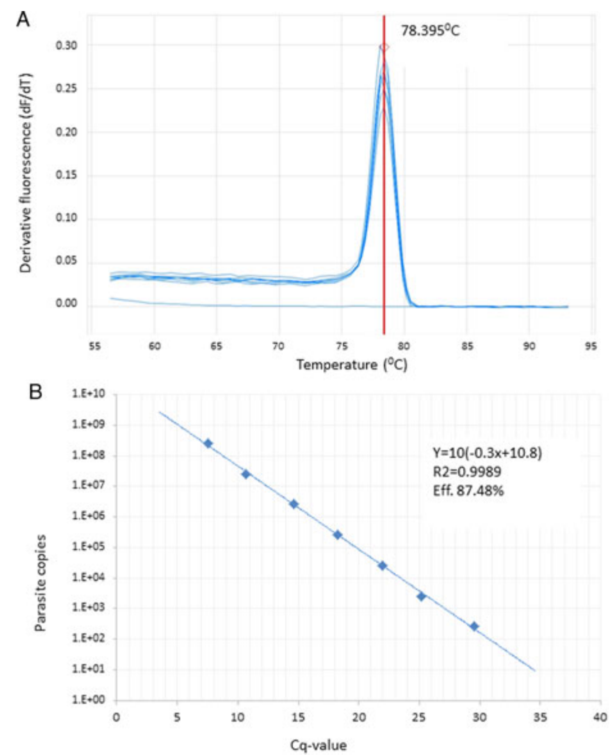

Fig. 1. Real-time polymerase chain reaction (qPCR) derivative melt curves (A) and standard curve (B) of seven standard samples with a dilution factor of 10 and a starting quantity of $2{\cdot}61 \times 10^8$ copies of the qPCR target DNA sequence (Friedl and Groscurth, 2012). Nuclease-free H_2O was used as a negative control.

Infection prevalence based on qPCR

Blood samples of 14 bird species from two orders, including five introduced, two native and seven endemic species, were tested by qPCR during this study (Table 2). The previous nPCR data showed an overall prevalence of *Plasmodium* spp. for all tested birds of 20·3% (Sijbranda *et al*. 2016) (Table 2). qPCR samples were considered positive for the presence of *Plasmodium*-specific DNA when

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Table 2. The estimated true prevalence of *Plasmodium* spp. infection based on nested polymerase chain reaction (nPCR) and real-time PCR (qPCR) results including 95% confidence intervals (CI)

Species	New Zealand (NZ) status[a]	n^b	Prevalence nPCR	95% CI	Prevalence qPCR	95% CI
Blackbird *Turdus merula*	I	34	0·82	0·67–0·92	0·77	0·60–0·88
Song thrush *Turdus philomelos*	I	3	1·00	0·51–1·00	0·67	0·21–0·04
Chaffinch *Fringilla coeleps*	I	1	0·00	0·00–0·79	0·00	0·00–0·79
Dunnock *Prunella modularis*	I	1	1·00	0·21–1·00	0·00	0·00–0·79
Goldfinch *Carduelis carduelis*	I	1	0·00	0·00–0·79	0·00	0·00–0·79
Total for all introduced species	**I**	**40**	**0·81**	**0·66–0·90**	**0·70**	**0·55–0·82**
Silvereye *Zosterops lateralis*	N	33	0·21	0·11–0·38	0·12	0·05–0·27
Fantail *Rhipidura fuliginosa*	N	4	0·00	0·00–0·49	0·00	0·00–0·49
Total for all native species	**N**	**37**	**0·19**	**0·10–0·08**	**0·11**	**0·04–0·25**
NZ robin *Petroica longipes*	E	100	0·04	0·02–0·10	0·22	0·15–0·31
Tomtit *Petroica macrocephalatoitoi*	E	3	0·00	0·00–0·56	0·67	0·21–0·04
Whitehead *Mohoua albicilla*	E	3	0·00	0·00–0·56	0·00	0·00–0·56
Grey warbler *Gerygone igata*	E	2	0·00	0·00–0·66	0·00	0·00–0·66
Bellbird *Anthornis melanura*	E	1	0·00	0·00–0·79	0·00	0·00–0·79
Tui *Prosthemadera novaeseelandiae*	E	1	0·00	0·00–0·79	0·00	0·00–0·79
Blue duck *Hymenolaimus malacorhynchos*	E	14	0·00	0·00–22	0·14	0·04–0·40
Total for all endemic species	**E**	**124**	**0·03**	**0·01–0·08**	**0·21**	**0·15–0·29**
Total for all sampled birds (*I*+*N* + *E*)	**I + N + E**	**201**	**0·22**	**0·17–0·28**	**0·29**	**0·23–0·36**

Bold items are summative group results.
[a] NZ status for each bird species is indicated as introduced (*I*), native (*N*), or endemic (*E*) to NZ.
[b] *n* is the total number of bird captured of a particular species.

results revealed dissociation temperatures within the range of 78·4 ± 1 °C (to account for *Plasmodium* spp. variation). The qPCR showed an overall prevalence of *Plasmodium* infection of 29% (58/201); the prevalence for introduced, native and endemic species groups was 70% (0·55–0·82), 11% (0·04–0·25) and 21% (0·11–0·29), respectively (Table 2). Of note, 20/58 samples were positive by the qPCR, but negative using nPCR. Conversely, nine of the qPCR negative samples were positive by nPCR, one of which had been confirmed to be *P. elongatum* by sequencing. The difference in prevalence between native and endemic species was not significant, while introduced species showed a significantly higher prevalence compared with native ($\chi^2 = 27{\cdot}7$, D.F. = 1, $P < 0{\cdot}001$) or endemic species ($\chi^2 = 33{\cdot}7$, D.F. = 1, $P < 0{\cdot}001$).

Latent class analysis

Discrepancies in infection prevalence were found between LSU-rRNA qPCR and the previously performed cytochrome *b* gene nPCR. For example, in NZ robins, the results of the two assays differed significantly, with the qPCR showing a higher prevalence of infection when compared with the nPCR ($\chi^2 = 14{\cdot}28$, D.F. = 1, $P < 0{\cdot}001$). To estimate the difference in sensitivity and/or specificity of these two tests, a LCA was performed. The resulting overall estimated true prevalence for nPCR was 22·3% (95% CI 17·1–28·5%) and for qPCR 28·7% (95% CI 22·9–35·3%). A higher sensitivity and specificity were found for the qPCR (96·7 and 98·0%) than for the nPCR (80·9 and 85·4%). Pairwise comparison found significant differences in prevalence of *Plasmodium* spp. infections between introduced, native and endemic species. The infection prevalence in introduced species was significantly higher than in native ($P < 0{\cdot}001$) or endemic species ($P < 0{\cdot}001$); the difference between native and endemic species was also significant ($P = 0{\cdot}005$).

Infection intensity

The infection intensity (parasite load) in LSU-rRNA qPCR-positive samples ranged from 0·08 to $1{\cdot}61 \times 10^4$ *Plasmodium* DNA copies per 10 000 avian blood cells. For those samples that were also positive on cytochrome *b* gene nPCR, parasite loads ranged from 0·3 to $1{\cdot}61 \times 10^4$ *Plasmodium* DNA copies per 10 000 avian blood cells. Therefore, the lower detection limit for the qPCR was close to one *Plasmodium* DNA copy per 100 000 cells, compared with three DNA copies per 1000 avian blood cells for the nPCR. This shows that the LSU-rRNA qPCR is better at detecting low levels of parasitaemia than the nPCR, which explains the higher sensitivity of qPCR compared with the nPCR, as was indicated by LCA. Consequently the qPCR detected low parasitaemias in 22 samples of endemic bird species with negative nPCR results.

The mean infection intensity was determined for the groups of introduced, native and endemic bird species (Table 3). A significant difference in parasite load was present between these groups (D.F. = 2,

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Table 3. Estimated true prevalence based on real-time polymerase chain reaction results with 95% confidence interval (95% CI), mean parasite load, parasite load range (PL-range) and standard error of mean (S.E.M.) for *Plasmodium* spp (infections in introduced, native and endemic species, defined as number of *Plasmodium* DNA copies per 10, 000 avian cells)

Bird group	Prevalence	95% CI	Mean parasite load	PL-range	S.E.M.
Introduced	0·70	0·55–0·82	2245	1·23–16 148·31	700
Native	0·11	0·04–0·25	2·8	0·27–8·47	1·7
Endemic	0·21	0·15–0·29	31·5	0·08–455·29	18·4
All birds	0·29	0·23–0·36	1156	0·08–16 148·31	385

Table 4. Mean packed cell volume (PCV) and body condition index (BCI) ± standard errors of means (S.E.M.) for *Plasmodium* spp (positive and negative birds based on qPCR results)

	PCV		BCI	
Species	Positive birds	Negative birds	Positive birds	Negative birds
Blackbird	43·65 ± 0·89	44·88 ± 1·70	2·57 ± 0·11	2·70 ± 0·05
Silvereye	51·00 ± 3·18	47·07 ± 1·48	0·75 ± 0·09	0·63 ± 0·01
Robin	43·79 ± 0·89	46·74 ± 0·58	0·85 ± 0·01	0·83 ± 0·01

$F = 33{\cdot}7$, $P < 0{\cdot}001$). The mean parasite load was significantly higher in introduced bird species compared with endemic species ($P < 0{\cdot}001$) and native species ($P < 0{\cdot}001$). There was no significant difference in infection intensity between native and endemic species ($P = 1{\cdot}0$).

Effect of infection status and parasite load on BCI and PCV

Mean PCV and BCI values were determined for introduced, native and endemic birds (Table 4). Based on qPCR results, robins infected with *Plasmodium* spp. showed a significantly lower PCV than robins that were uninfected ($n = 98$, $U = 534$, $P = 0{\cdot}029$). In addition, no significant relationship was found between total parasite load and BCI or PCV in blackbirds, silvereyes or NZ robins using linear regression.

DISCUSSION

Our study determined levels of parasitaemia in qPCR-positive blood samples between 0·08 and $1{\cdot}61 \times 10^4$ *Plasmodium* DNA copies per 10 000 avian blood cells, which means that the lowest infection intensity that tested positive with LSU-rRNA qPCR was close to one DNA copy per 100 000 cells. The limit of detection of this assay is consistent with what was reported by Friedl and Groscurth (2012) and also with the results from the cytochrome *b* gene nPCR as reported by Hellgren *et al*. (2004), where positive amplifications were seen in blood samples diluted to one parasite per 100 000 host cells. However, in the study by Hellgren *et al*. (2004), the initial infection intensity was determined by microscopic observation of blood smears, the accuracy of which could influence the accuracy of his findings. Our study showed a higher sensitivity of the LSU-rRNA qPCR to detect low-level parasitaemias compared with the cytochrome *b* gene nPCR, with which a lowest infection intensity of three DNA copies per 1000 avian blood cells (based on corresponding qPCR data) was identified. Therefore, compared with the LSU-rRNA qPCR, the nPCR appeared to result in more false-negative results for samples with a low parasitaemia, which also aligned with previous findings by Fallon *et al*. (2003) when the same gene targets were examined. On the other hand, nine samples that were positive following the cytochrome *b* gene nPCR were negative in the LSU-rRNA qPCR; however, due to poor amplification of the nPCR target sequence, eight of these samples were not positively confirmed as *Plasmodium* spp. by DNA sequencing, meaning that the nPCR results for these eight samples were potentially false positives. False-positive nPCR results would contribute to a lower specificity of the nPCR. However, it should be noted that Fallon *et al*. (2003) reported that a PCR using the same LSU-rRNA gene target as our study failed to detect visually positive samples. Regardless, LCA confirmed a higher sensitivity and specificity of the LSU-rRNA qPCR compared with the cytochrome *b* gene nPCR. To increase the reliability of these results, duplicate samples should be tested; due to money and time restraints, this was not done in this initial trial. In addition, the use of an internal extraction control, such as an avian housekeeping gene, should be used in future

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experiments to confirm DNA quality and whether the DNA can be successfully amplified using qPCR techniques (Nolan *et al*. 2015).

Interestingly, the prevalence as well as the parasite load of *Plasmodium* spp. was significantly higher in introduced passerines than in native and endemic species. This finding further supports previous studies that have suggested that introduced species like blackbirds and song thrushes function as an important reservoir of infection and are able to sustain *Plasmodium* lineages within an ecological unit or to introduce them to other sites (Tompkins and Gleeson, 2006; Ewen *et al*. 2012*a*, *b*; Schoener *et al*. 2014).

To gauge the potential impact of avian malaria on the fitness of different bird species, PCV and BCI were compared between *Plasmodium* infected and uninfected blackbirds, silvereyes and NZ robins. As the evaluation of BCI within bird groups containing more than one species is not appropriate (Labocha and Hayes, 2012), evaluation of these parameters within the overall groups of introduced native and endemic bird species was avoided. Based on qPCR results, *Plasmodium*-positive NZ robins had a significantly lower PCV than *Plasmodium*-negative ones (Table 4). This implies that in NZ robins, chronic avian malaria may have a negative effect on the concentration of red blood cells. Although anaemia and impairment of BCI are common during the acute phase of infection (Atkinson *et al*. 2000; Valkiunas, 2005), birds in this phase tend to be less active and either succumb to predation or simply do not get caught in nets (Yorinks and Atkinson, 2000; Moller and Nielsen, 2007). Therefore, it is possible that birds caught in nets were most likely in the chronic, low parasitaemic phase of infection. Previous studies have reported recovery to the original PCV and BCI during this phase (Paulman and McAllister, 2005; Motta *et al*. 2013). Repeat studies would be needed to confirm the finding of reduced PCV values in *Plasmodium*-positive NZ robins, especially since no significant relationship was found between total parasite load and PCV using linear regression. The reduction in red blood cells did not appear to have a clinical impact on the overall fitness of NZ robins, as no decrease in BCI was found in any of the tested bird species.

The chronic effects of *Plasmodium* infections on reproduction, or on physical parameters during times of high energy demand, reduced food intake or co-infection with multiple *Plasmodium* spp., have not been evaluated in wild NZ bird species. Multiple examples from abroad illustrate the importance of including these factors in future NZ studies. For example, a negative correlation between parasitaemia and BCI was previously demonstrated in incubating female Kestrels (Dawson and Bortolotti, 2000). In addition, Marzal *et al*. (2008) found that chronic infection with a single *Plasmodium* lineage did not have a significant impact on body mass or PCV, while infection with multiple strains did. A negative correlation between the level of parasitaemia and the number of fledged offspring has also been demonstrated (Marzal *et al*. 2005; Asghar *et al*. 2011). Furthermore, a reduced lifespan combined with a reduced number and quality of offspring was reported in chronically infected great reed warblers (Asghar *et al*. 2015). A potential explanation for the observation, that in most of the bird groups, there was no significant impact of *Plasmodium* spp. infection on PCV or BCI may be due to the tested birds being in a chronic stage of infection and breeding females were excluded. Alternatively, the birds in our study had reached a balance between tolerance and immune response towards the parasite that allowed normal physiological function.

Based on the findings, speculations regarding disease dynamics of avian malaria in various NZ bird species and groups can be made. While *Plasmodium* prevalence can be widely influenced by environmental factors, such as temporal and spatial distribution of the parasite and host, infection intensity is largely influenced by the genetic make-up of host and parasite (Westerdahl, 2012). Immuno-alleles in the genetic make-up of birds can either lead to qualitative or quantitative resistance against *Plasmodium*, or result in a higher susceptibility to infection (Westerdahl, 2012). Additionally, over time host–parasite relationships can co-evolve towards a higher tolerance and moderated immune response of the host against the micro-organism, avoiding damaging inappropriate inflammatory reactions and allowing hosts to carry parasites without severe pathological signs (Rook, 2009; Atkinson *et al*. 2013). These two hypotheses are likely to explain some of the differences that were found between bird species or groups during this study. Differences in resistance, immunity and tolerance to *Plasmodium* lineages between various bird species are easier to demonstrate when both parasite load and prevalence of infection are evaluated in these species. Introduced species are expected to show higher rates resistance, tolerance and immunity as they have had a greater evolutionary time difference to genetically adapt to the parasites (Ewen *et al*. 2012*a*). The combined high prevalence and high parasitaemia seen in introduced birds with a healthy appearance supports the hypothesis that these birds have co-evolved with their respective introduced *Plasmodium* species resulting in increased parasite tolerance (Atkinson *et al*. 2013). To survive infection, susceptibility and tolerance must go hand in hand with a quantitative resistance at a level sufficient to maintain a healthy condition in most of these birds. The fact that high parasitaemia values were not found in endemic or native species could mean that these species have a lower load

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tolerance towards *Plasmodium* spp., meaning that in case of high parasitaemia, a cascade of inflammatory and pathological reactions to fight the infection causes too much damage to the birds to survive (Atkinson *et al*. 2013). Another explanation could be that endemic and native birds are less susceptible to infection or are able to lower their parasite numbers more than introduced passerines through more efficient immunological responses (higher quantitative resistance) (Westerdahl, 2012). The fact that the prevalence of infection in endemic species is often significantly lower than in introduced species supports the first explanation over the second. If endemic species were well equipped with an immune response to lower parasite numbers, many birds would survive the acute phase of infection and higher prevalence of chronically infected birds with low parasitaemia would be expected in these species (Westerdahl, 2012). Based on this hypothesis, *Plasmodium* infections are potentially more pathogenic in endemic bird species; however, the presence of a low number of chronically infected individuals with a low parasitaemia also shows that a certain level of quantitative resistance is present in endemic birds. Higher sample sizes and long-term studies are needed to further clarify the differences in prevalence and parasite load between various bird species and the effects of these findings on the health and reproduction of these species.

There is also limited knowledge regarding immuno-alleles in NZ bird species, or how they associate with our findings. To prove Westerdahl's (2012) hypothesis regarding quantitative resistance due to immuno-alleles, these genetic features have to be studied. For a complete understanding of disease dynamics, serological data demonstrating which birds were infected in the past and have recovered from infection, as well as controlled ongoing studies into parasite loads in birds of various ages, sexes and in various seasons would be needed. Due to a lack of variability in the qPCR target region of the used assay, the resulting qPCR-positive samples that were negative in the nPCR were not able to be used to identify the amplified *Plasmodium* species or lineages by either DNA sequencing or by dissociation temperature. Extrapolation of previously acquired DNA sequencing results for nPCR products (Sijbranda *et al*. 2016), which reflect only one *Plasmodium* lineage in case of co-infection with multiple lineages in a single host, to qPCR results, which reflect the total parasite load in a single host, is not appropriate. Therefore, exploration of the use of lineage-specific qPCR primers for *Plasmodium* sp. will be useful for ongoing studies (Perandin *et al*. 2004; Zehtindjiev *et al*. 2008; Asghar *et al*. 2011).

Over the last 15 years, there has been a rapid increase in the prevalence of avian malaria in NZ and shifts in the geographical spread of vectors for avian malaria; thus, studying disease dynamics in our NZ birds is of utmost importance to plan efficient future wildlife management strategies for our endemic bird species.

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