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Linking avian malaria parasitemia estimates from quantitative PCR and microscopy reveals new infection patterns in Hawai'i

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abstract

Plasmodium parasites infect thousands of species and provide an exceptional system for studying host-pathogen dynamics, especially for multi-host pathogens. However, understanding these interactions requires an accurate assay of infection. Assessing Plasmodium infections using microscopy on blood smears often misses infections with low parasitemias (the fractions of cells infected), and biases in malaria prevalence estimates will differ among hosts that differ in mean parasitemias. We examined Plasmodium relictum infection and parasitemia using both microscopy of blood smears and quantitative polymerase chain reaction (qPCR) on 299 samples from multiple bird species in Hawai'i and fit models to predict parasitemias from qPCR cycle threshold (Ct) values. We used these models to quantify the extent to which microscopy underestimated infection prevalence and to more accurately estimate infection patterns for each species for a large historical study done by microscopy. We found that most qPCR-positive wild-caught birds in Hawaii had low parasitemias (Ct scores 35), which were rarely detected by microscopy. The fraction of infections missed by microscopy differed substantially among eight species due to differences in species' parasitemia levels. Infection prevalence was likely 4–5-fold higher than previous microscopy estimates for three introduced species, including Zosterops japonicus, Hawaii's most abundant forest bird, which had low average parasitemias. In contrast, prevalence was likely only 1.5–2.3-fold higher than previous estimates for Himatione sanguinea and Chlorodrepanis virens, two native species with high average parasitemias. Our results indicate that relative patterns of infection among species differ substantially from those observed in previous microscopy studies, and that differences depend on variation in parasitemias among species. Although microscopy of blood smears is useful for estimating the frequency of different Plasmodium stages and host attributes, more sensitive quantitative methods, including qPCR, are needed to accurately estimate and compare infection prevalence among host species.

1. Introduction

Parasites are frequently used to test ecological and evolutionary hypotheses of coevolution, sexual selection, host immunocompetence, and pathogen virulence (Hamilton, 1982; Woolhouse et al., 1997; Otto and Nuismer, 2004; Mayer et al., 2016). Avian haemosporidian blood parasites are a common model system for these studies because they often infect multiple host species (Ricklefs and Fallon, 2002; Beadell et al., 2006; Fecchio et al., 2021), and hosts vary in their responses to infection (Atkinson and van Riper, 1991; Valkiunas, 2005). Some hosts experience high parasite intensities and mortality when parasitized, while others suffer little morbidity and mortality, and many species maintain chronic low intensity infections for a number of years (van Riper et al., 1986; Valkiunas, 2005). Variation in infections and host responses to infections influence transmission, evolution, and disease risk within host communities (Woolhouse et al., 1990, 1997; Regoes et al., 2000). A crucial part of studying host-parasite interactions is accurately measuring a host's infection status and the infection intensity.

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Measuring infection prevalence and intensity is important in determining which host species are more likely to become infected (Beadell et al., 2006; Maria et al., 2009; Svensson-Coelho et al., 2013) and, for vector-borne diseases, estimating host infectiousness for vectors (Komar et al., 2003; Mackinnon and Read, 2004; Kilpatrick et al., 2007; Pigeault et al., 2015). The probability of detecting infection increases when infection intensity is higher, and infection intensity varies both among hosts and over the course of infection (van Riper et al., 1986; Moens et al., 2016). The parasitemia (the fraction of red blood cells infected) for most malaria parasites is highest during the acute phase of infection, which lasts for a few weeks starting approximately 1 week after infection (Atkinson et al., 2000, 2001; Dimitrov et al., 2015), and hosts may suffer morbidity or mortality during this time and thereafter. Subsequently, species and individuals will remain chronically infected, often at much lower infection intensities, for months or years (Jarvi et al., 2002; Valkiunas, 2005). Accurately estimating infection prevalence in a species or population can be challenging because heavily infected hosts can have decreased activity (Yorinks and Atkinson, 2000), which may lead to undersampling, especially in studies that capture flying birds with mist nets (Valkiunas, 2005; Mukhin et al., 2016), and low-level chronic infections can be difficult to document, depending on the detection technique used (Jarvi et al., 2002).

Parasite detection methods vary in their sensitivity (probability of detecting true positives) and in the information they provide. Malaria researchers have employed different detection methods over time, with earlier studies using microscopy of stained blood smears and more recent studies primarily using PCR-based molecular techniques (Garnham, 1966; Fallon et al., 2003; Zehtindjiev et al., 2008). Microscopy provides useful information about the morphology and life stages of haemosporidians (Jarvi et al., 2002; Fallon et al., 2003; Valkiunas et al., 2008), but it generally has lower sensitivity than molecular techniques for detecting infection. This is especially true for low infection intensities, because the number of red blood cells that can be reasonably screened are far less than the numbers in an extracted DNA sample (Jarvi et al., 2002; Fallon et al., 2003; Fallon and Ricklefs, 2008). As a result, many studies now use either PCR (Beadell and Fleischer, 2005) or quantitative PCR (qPCR) to measure infection prevalence and the latter to estimate infection intensity (Neddermeyer et al., 2023; Paxton et al., 2023). However, integrating historical estimates of prevalence and intensity based on microscopy with more recent disease surveys that use qPCR requires a way to accurately integrate the measurements from these two methods.

Here, we develop a statistical relationship between Plasmodium relictum parasitemia quantified by microscopy and by qPCR to integrate past and current studies of avian malaria and examine patterns of infection among species. We analyzed blood samples both from experimentally infected birds and wild-caught birds in Hawai'i (USA), and examined the probability of detecting infection in eight common species found in Hawai'i that differ in parasitemia. We re-examined historical patterns of infection prevalence among these species using a large previous study that assessed infection using microscopy (van Riper et al., 1986). Finally, we combined parasitemia data from experimental infections and wild caught birds to assess whether parasitemias estimated from birds caught with mist nets are biased towards low results because birds with high parasitemias are less likely to be caught and sampled.

2. Materials and methods

2.1. Sample collection

We collected 720 avian blood samples by brachial venipuncture (25–100 lL) from wild birds caught in forested sites between 29 and 2,000 meters above sea level from 15 species on the islands of Kaua'i, Oahu, Maui, and Hawai'i (Hawai'i, USA) between 2015 and 2022. We also took blood samples from Hawai'i amakihi, Chlorodrepanis virens, and domestic canaries, Serinus canaria forma domestica in the first 45 days after experimental infection with P. relictum. Canaries were infected between 2020 and 2022 by intramuscular injection with lineage GRW4 of P. relictum, using infected whole blood from wild birds caught on Hawai'i Island or P. relictum passaged one to four times in canaries. Chlorodrepanis virens, which were captured from Upper Waīakea Forest Reserve (19 38′N, 155 21′W, 1,635 m), were infected by mosquito bite or intramuscular injection using a thawed, deglycerolized, aliquot of P. relictum GRW4 (KV115) passaged four times in canaries in 2015 (Paxton et al., 2023). Thin smears were prepared on glass slides from a drop of whole blood for some wild-caught bird samples and all laboratory-infected bird samples. Slides were air dried for 30 min., fixed for 1 min in 100% methanol (Spectrum Chemical Mfg. Corp, New Brunswick, NJ, USA) and stained for 1 h, within 24 h of preparation, with a 10% working solution of Giemsa stain stock in buffered solution (Ricca Chemical Company, Arlington, TX, USA). The remaining blood was placed immediately in 1 mL of prepared Queen's Lysis Buffer (Seutin et al., 1991) and stored at room temperature for 1–90 days or frozen at 20 C before qPCR analysis. All fieldwork was conducted under Hawai'i Division of Forestry and Wildlife Protected Wildlife Permit WL19-23 and United States Geological Survey Bird Banding Laboratory permit 23600. Laboratory work was performed under animal care and use protocols approved by the Institutional Animal Care and Use Committee at the University of California in Santa Cruz, USA (Kilpm2003) and by the Institutional Animal Care and Use Committee at the Smithsonian National Zoological Park (NZP-IACUC Proposal #15–18). Other permits included the US Fish and Wildlife Service Migratory Bird Scientific Collection Permit (MB67895B), United States Department of the Interior Bird Banding Laboratory (permit #21144), Hawai'i State Protected Wildlife Research Permit (WL 17–08), and Hawai'i State Access and Forest Reserve Special Use Permit.

2.2. Examination of blood smears

One of the authors (C.M. Seidl) examined blood smears on slides for malaria and estimated parasitemias for 299 samples from 141 wild-caught birds within 14 species and 158 samples from 33 experimentally infected birds (two species) by scanning 50 microscope fields at 1000x magnification using oil immersion and a DM1000 (Leica, Wetzlar, Germany) light microscope and counting the number of red blood cells infected with any stage of P. relictum. We estimated the total number of cells examined by counting the number of red blood cells in three randomly selected fields and multiplying the average of these three numbers by the total fields viewed. We examined an average of 5,509 cells per slide (median 5,000; range 800–20,000). Examining more red blood cells would have increased the probability of detecting infection, and decreased the uncertainty of parasitemia estimates, but would have required additional time, and would not have altered the relationship between parasitemia estimated by qPCR and microscopy.

2.3. qPCR quantification

We extracted DNA from avian blood samples with a Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for the Purification of Total DNA from Animal Blood and nucleated red blood cells. We quantified the concentration of genomic DNA with a Qubit fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and normalized samples to a starting concentration of 2 ng/lL. The infection status of blood samples was quantified using a qPCR assay with a hydrolysis probe optimized for the GRW4 lineage of P. relictum found in Hawai'i (Beadell et al., 2006) as previously described (Videvall et al., 2021; Neddermeyer et al., 2023; Paxton et al., 2023). The primers used for the assay are adapted to target the cytochrome b region (Zehtindjiev et al., 2008), and to date, only one lineage of P. relictum has been documented in Hawai'i (Beadell et al., 2006). We tested each sample in duplicate or triplicate and averaged the cycle threshold (Ct) scores across runs. We considered the result to be a positive detection for Plasmodium if a run crossed the threshold baseline within 40 cycles. Samples with one positive run were considered positive and assigned the single run value.

2.4. Data analysis

We performed all analyses using R 4.2.0 (R Core Team, 2022). A generalized linear mixed effects model with a binomial distribution and a logit link was used to examine the relationship between parasitemia using microscopy (fraction of cells infected) as the response variable and the qPCR Ct score as the predictor variable. In this model each blood cell examined was an individual data point, and sample ID (a blood sample from a bird on a given day) was included as a random effect to account for the grouping of multiple cells in each sample. We used the glmer() function to fit the model and the bootMER() function to estimate standard errors for new predictions; both are in the lme4 package (Bates et al., 2015).

Our goal was to estimate the fraction of infections that may have been missed in a large previous study that examined infection in 2,206 bird samples from eight species using only microscopy (van Riper et al., 1986). We had intended to use the fitted model described above (Fig. 1) to estimate the probability of detecting infection (yes/no) by microscopy as a function of the qPCR Ct score. However, the predictions from this model showed some deviations from the detection (yes/no) of malaria on our slides (Fig. 2; see below). So, we fitted a second model with the detection of infection (yes/no) by microscopy on an entire smear (rather than an individual cell) as the response variable, and the qPCR Ct score as the predictor using logistic regression (a generalized linear model with a binomial distribution and a logit link) for the same set of samples (Fig. 2). We compared the fit of this Smear model and the Cell model by Akaike Information Criteria (AIC). Because we found (see Section 3) that the model fit to the presence/absence of infection on smears (the Smear model, 5.5 K cells) was a much better fit than the model fit to infections in individual cells (i.e., the parasitemia), we used the Smear model to estimate the fraction of infections missed by microscopy. To re-examine patterns of infections in the historical study that used microscopy (van Riper et al., 1986), we had to account for differences in the average number of cells examined in our study (5,500 cells per slide) and the previous study (25,000 cells per slide). We adjusted the likelihood of detecting infection by calculating the difference in the likelihood of detection when viewing 5,500 cells versus 25,000 cells using the Cell model. The higher number of cells examined was equivalent to a shift in the detectable Ct scores of 1.8 cycles (see Fig. 2, Cell model 5.5 K and Cell model 25 K), and accounts for the increased detection probability with the number of cells viewed (Valkiunas et al., 2008). We used this to create an adjusted Smear model (Fig. 2) that estimates the probability that an infection would be detected in each species by microscopy when examining 25,000 cells, and used the qPCR Ct scores from our more recent sampling of wild-caught individuals from that species.

For each species, we estimated the fraction of infections that would be detected by microscopy by drawing from a Bernoulli/binomial distribution with a probability of detection based on the Smear model, 25 K cells, for each qPCR-positive sample and divided

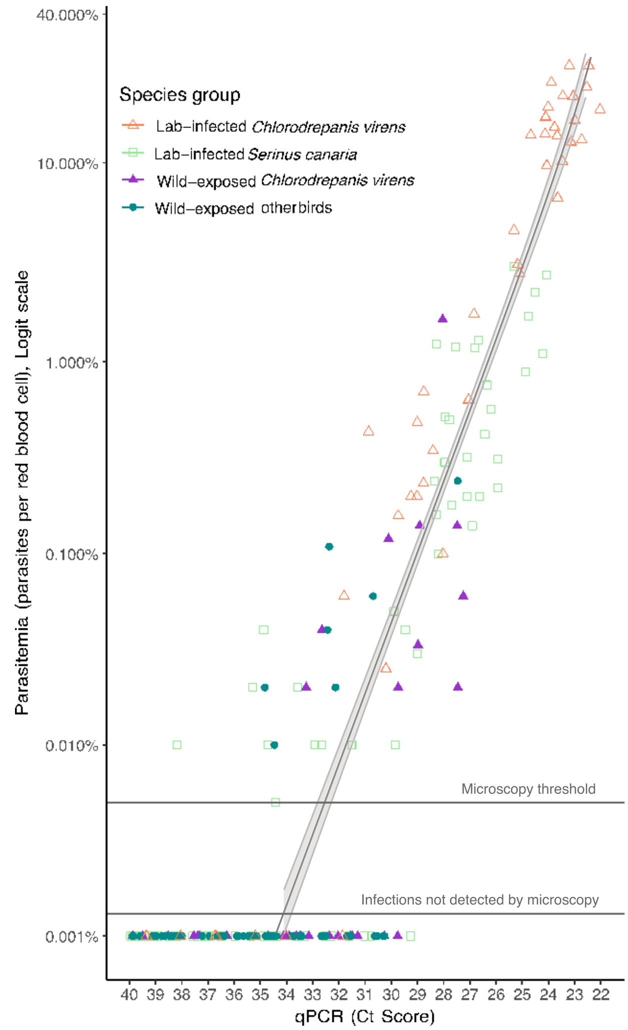

Fig. 1. Relationship between Plasmodium relictum parasitemia, quantified by microscopic examination of blood smears and quantitative PCR (qPCR) cycle threshold (Ct) scores for 210 blood samples. Point shape and fill indicates the bird species (USA) or species group and the source of P. relictum infection (i.e., laboratory infection or wild-exposure). The line shows the fitted model and 95% confidence intervals (Logit (Parasitemia) = 17.78 0.85 (S.E. = 0.034) * Ct score, N = 210, Z = 25.2, P < 2 10^{16}; random effect standard error: 1.11). Samples with a Ct score 40 were considered negative and are not shown. The dotted line indicates the lowest microscopy detection threshold for our study (one parasite in 20,000 red blood cells). qPCR-positive samples for which infection was not detected by microscopy are shown below the dashed line and given a parasitemia of 0.001% to facilitate visualization. Lab, laboratory.

the sum of these values by the total number of qPCR-positive samples to calculate the fraction of infections detected by microscopy. We repeated this set of binomial draws for each species 1,000 times to estimate the uncertainty in the fraction of qPCR-positive samples that would have tested positive by microscopy in our analyses. The inverse of this fraction was the ratio of missed infections when using only microscopy. We used this ratio to estimate the likely infection prevalence for eight species that would have been obtained in the previous study (van Riper et al., 1986) had qPCR been available at that time. We note that this assumes that the parasitemias (but not prevalences) of infected birds from each species are similar between our sample of infected birds from 2015–2022 and the birds captured in the previous study (van Riper et al., 1986).

Finally, we used our parasitemia data from wild-caught birds to test the hypothesis that mist net sampling techniques undersam-

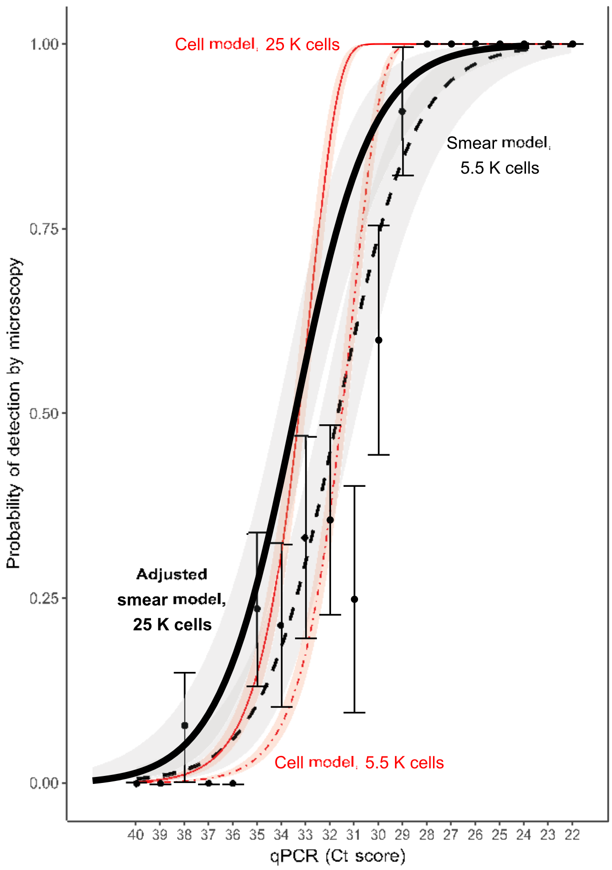

Fig. 2. The probability of detecting a Plasmodium relictum infection by microscopy plotted against the quantitative PCR (qPCR) cycle threshold (Ct) score for that sample with four models. Points show the fractions of smears (with a mean of 5,500 cells examined) where at least one infected cell was seen, grouped by rounding the Ct score, and error bars show the binomial standard error of this fraction. The dot and dash line (cell model, 5.5 K) shows the probability of detecting at least one infected cell when viewing 5,500 red blood cells by microscopy using the fitted model in Fig. 1. The solid thin line (cell model, 25 K) shows the probability of detecting at least one parasite cell in a smear by microscopy when viewing 25,000 red blood cells, also based on the fitted model from Fig. 1. The black dashed line, (smear model, 5.5 K cells), shows a logistic regression model fitted to the points in this graph where a blood sample is a point (logit (Fraction of smears posi-tive) = 19.9 0.63 (S.E. = 0.085) * Ct score; N = 210, Z = 7.4, P = 1.57 10^{13}). The solid thick black line (smear model, 25 K cells) is the same as the dashed black line but is shifted 1.8 Ct scores to the left to estimate the probability of detecting infection when 25,000 cells are viewed, rather than 5,500 (see Section 2 for details).

ple individuals with high parasitemias. We did this for C. virens which we sampled in the wild and for which experimental infection studies have been published (van Riper et al., 1986; Atkinson et al., 2000, 2001). Chlorodrepanis virens are frequently infected in their first year, have lower likelihood of infection as adults, and then remain chronically infected for life (Jarvi et al., 2002; Atkinson and Samuel, 2010; McClure et al., 2020). We classified wild-caught birds in our dataset into ''high parasitemia'' and ''low parasitemia'' using parasitemia thresholds drawn from the experimental infection studies in which the parasitemias of newly infected birds were tracked over time and encompassed the initial period when parasitemias are highest, peak, and decline (high parasitemia) to below a threshold level that they do not rise above for the remainder of the study (low parasitemia). We found two parasitemia thresholds that could be used to separate the high parasitemia and low parasitemia periods of infection: 1% and 0.5%, which occurred 47 and 67 days p.i, respectively, for C. virens (Supplementary Fig. S1). The high parasitemia periods are 5 days less than these values because parasites are usually not detectable in these species until day five p.i. We determined the Ct score corresponding to 1% and 0.5% parasitemias using our fitted model which predicts parasitemia by microscopy from qPCR Ct score (see Section 2). We then estimated whether the fraction of qPCR-positive birds caught with high parasitemias was less than what we would expect. The expected fraction was the ratio of the length of the high parasitemia period, H, to the lifespan of an infected bird, L, both in days: H/(L + H). We compared the expected and observed fractions using a binomial distribution with the binom.test() function in R. The average life expectancy, L, of infected adult birds was calculated, assuming constant type II survival as adults, as 365/(1 adult annual survival) or 961 days for C. virens (Kilpatrick et al., 2006).

3. Results

Of the 299 samples we tested for malaria by both microscopy and qPCR, we detected P. relictum in 36% (109/299) of samples by microscopy and 72% (216/299) of samples by qPCR. Seven samples were positive by microscopy (parasitemias 0.005%-0.1%; six of seven had one infected cell in 5,000–20,000 cells) but negative by qPCR; three in wild-caught and four in experimentally infected birds.

There was a strong and nearly linear relationship between parasitemia measured using microscopy and Ct score on a logit scale between Ct scores of 29 and 22 (Fig. 1). For higher Ct scores, an increasing fraction of samples was negative by microscopy, but infections were occasionally detected by microscopy for Ct scores as high as 38.2 (Figs. 1 and 2). We used the fitted model to predict the probability of detecting at least one infected cell in 5,500 cells (the average number of cells we examined) by microscopy for a range of Ct scores (Fig. 2). We also fitted a model to the presence/absence of malaria on slides (detecting at least one infected cell regardless of parasitemia; Fig. 2). Both relationships suggested that detecting infection by microscopy was very high for Ct scores between 22 and 26 and fell below 50% for Ct scores higher than 32 Cts (Fig. 2). However, the Cell model (Fig. 2) fitted to parasitemias had a much steeper slope than the Smear model fitted to the presence/absence of infection (Fig. 2). Although the Smear model would be expected to fit slightly better than the Cell model, since it was fitted directly to presence/absence data (rather than fitted to the parasitemia data with red blood cells as data points), it was, in fact, a much better fit to the data (delta-AIC = 941.5).

We then used the Smear model, adjusted to reflect examination of 25,000 cells, to quantify the probability of detecting infection by microscopy (Fig. 2,; see Section 2) for eight species using the mean parasitemias for each species from our qPCR-positive wild bird samples (N = 656). The probability of detecting a qPCR-positive infection by microscopy ranged from 20–23% for three introduced species with low parasitemias, Zosterops japonicus (N = 284), Leiothrix lutea (N = 101), and Lonchura punctulate (N = 12), to 44.0% and 68.7% for two native species, C. virens (N = 132) and Himatione sanguinea (N = 58), with higher parasitemias (Fig. 3A).

We used these estimates of the fractions of infection detected by smear to re-examine the infection prevalence of P. relictum in the same eight species from a large-scale study conducted four decades earlier when only microscopy was possible (van Riper et al., 1986). In the previous study (van Riper et al., 1986), the prevalence of infection in Z. japonicus, C. virens and H. sanguinea using microscopy were 0.90%, 7.3%, and 29.2%, respectively (Fig. 3B). These historical prevalence estimates suggest that C. virens and H. sanguinea had 8–32-fold higher infection prevalence than Z. japonicus (Fig. 3B). However, if the samples had been tested by qPCR, the ratio of infection prevalence of these species would have been much smaller (4–10-fold), because the low parasitemias of most Z. japonicus infections resulted in only 21.9% being detect-

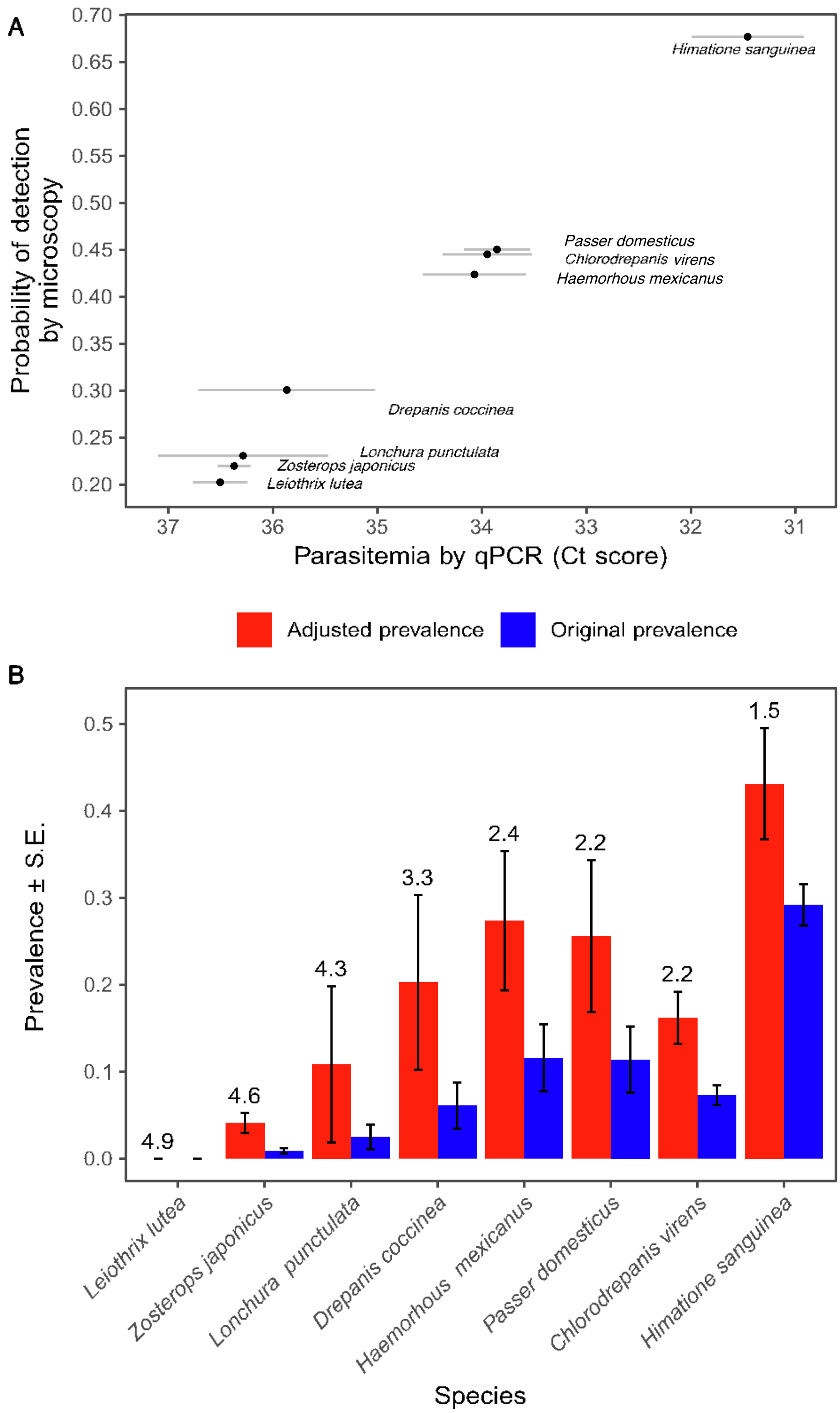

Fig. 3. Prevalence of Plasmodium relictum infection in eight species of birds in Hawai'i (USA). (A) Average parasitemia by quantitative PCR (qPCR) cycle threshold (Ct) score (±S.E.) and detection probability by microscopy when viewing 25,000 cells for each species. Note that higher Ct scores indicate less P. relictum DNA. (B) Infection prevalence measured using microscopy in the same eight species (van Riper et al. 1986) (blue (light grey) bars) and estimates for prevalence if blood samples had been tested by qPCR (red (dark grey) bars). Numbers above red (dark grey) bars are the ratios of adjusted prevalence by qPCR to the measured prevalence by microscopy fit with the adjusted smear model with 25,000 cells (bold black line) from Fig. 2.

able by microscopy, whereas the fractions of infections detected by microscopy in C. virens and H. sanguinea were much higher (Fig. 3A).

Next, we examined whether heavily infected C. virens would be undersampled by mist netting. The expected fraction of infected birds with high parasitemias, if the probability of capture did not

Table 1
Expected and observed probability of capturing birds with high parasitemia Plasmodium relictum (lineage GRW4) infections using passive mist net capture techniques. N is the sample size of infected birds and H is the length of the high parasitemia period. Acute parasitemia thresholds and the length of the high parasitemia period are derived from an experimental infection study conducted by Atkinson et al. (2001). The expected fraction is the ratio of H to the lifespan of an infected bird, L, both in days: H/(L + H). L is derived from Kilpatrick et al. (2006). The expected and observed fractions are compared using a binomial test.

Species	Acute parasitemia threshold (%)	H (days)	N	Expected fraction	Observed fraction (95% CI)	Observed/expected (95% CI)	P-value
Chlorodrepanis virens	1	42	132	0.044	0.030 (0.0083, 0.076)	0.69 (0.19, 1.7)	0.67
C. virens	0.5	62	132	0.065	0.038 (0.012, 0.086)	0.59 (0.18, 1.3)	0.29

CI, confidence interval.
P-value 0.05 considered statistically significant to reject the null hypothesis.

vary between high parasitemia and low parasitemia stages, was 0.044 and 0.065 for the two thresholds (Table 1). The fraction of wild birds sampled with high parasitemias was slightly lower than expected, but these fractions were moderately uncertain and the ratio of the observed fraction of birds with high parasitemias caught in mist nets to the expected fraction overlapped one with both thresholds (Table 1). Our power to detect a bias in sampling was low, due to a low expected fraction of birds with high parasitemias (5%) and only a moderate sample size (132 qPCR-positive birds).

4. Discussion

The ability to accurately measure infection patterns depends on the sensitivity of the detection method. Innovations in molecular detection and quantification techniques have improved the ability of researchers to detect and quantify parasite loads, and have broadened our understanding of parasites as drivers of many ecological and evolutionary patterns (Ricklefs and Fallon, 2002; Asghar et al., 2015; Valkiunas and Atkinson, 2020). Previous studies have shown that molecular techniques are more sensitive screening and quantification tools for Plasmodium spp. infections than microscopy (Jarvi et al., 2002; Valkiunas et al., 2008; Braga et al., 2011; Ishtiaq et al., 2017). Our study aimed to provide a quantitative framework for integrating historical and contemporary datasets that use qPCR and/or microscopy. We developed a statistical relationship to relate qPCR values to parasitemia values measured by microscopy, using data across three orders of magnitude of infection intensity. We found that the probability of detecting a parasite by microscopy when examining 5,500 cells dropped below 50% for infected individuals with Ct scores higher than 32 or parasitemias below approximately 0.01%.

Most wild-caught qPCR-positive birds in Hawai'i had low parasitemias (<0.001%, 35 Ct), resulting in microscopy missing a substantial fraction of infections. However, the fraction missed depended on parasitemia, which varied among species (Fallon and Ricklefs, 2008; Ricklefs et al., 2018), and given the large increase in the probability of detecting malaria parasitemia (Fig. 1), any factors which may alter parasitemia (e.g., age, location, season) will change the probability of detecting infection. Infection in several abundant non-native species was underestimated by 4–5-fold, while infection in native birds was only underestimated by 1.5–2.3-fold. The higher detection rates of infection for native species with higher parasitemias have contributed to assertions that Hawaiian honeycreepers are the dominant reservoirs for avian malaria transmission (van Riper et al., 1986; Atkinson et al., 1995; Woodworth et al., 2005; Atkinson and Samuel, 2010; McClure et al., 2020). Yet, we found that infection prevalence in Z. japonicus was much higher than suggested by historical studies done with microscopy; our estimates were similar to those observed in recent studies that used PCR or serology to detect infection (Atkinson et al., 2014; McClure et al., 2020; Neddermeyer et al., 2023). Zosterops japonicus is the most abundant forest bird in Hawai'i (Kendall et al., 2022), but their role in transmission is unknown because it is unclear how infectious its low-level parasitemias are to biting mosquitoes. While higher parasitemias infect a higher fraction of biting mosquitoes, the relationship between host parasitemia and the fraction of feeding mosquitoes that become infectious is unknown. More generally, abundant species with mid-level to lower parasitemias may be important reservoirs, especially in avian communities where native species are absent (McClure et al., 2020).

Our study has several limitations. First, our relationships between Ct scores and parasitemias estimated using microscopy are specific to the laboratory and equipment where the qPCRs were performed and are dependent on template concentrations in the qPCRs (i.e., we used a concentration of 2 ng/lL per reaction), primarily because we lack a standard curve for normalizing Ct scores with other laboratories. Second, we do not yet know the relationship between parasitemia and infectiousness to biting mosquitoes, so we cannot translate parasitemia into infectiousness and determine the relative contributions of avian species to P. relictum transmission. Third, the difference between the slopes of our Cell model fitted to parasitemia data and our Smear model fitted to the presence/absence of infection suggested the distribution of parasitized red blood cells on smears was aggregated (Davidson, 1958; Godfrey et al., 1987). This made it difficult to accurately estimate parasitemias by microscopy for highly parasitized smears. Fourth, our sample size of qPCR-positive C. virens individuals was too low to precisely estimate the fraction of birds caught in mist nets with high parasitemias, thus we could not conclusively determine if parasitemias measured using birds caught in mist nets are a biased subset of infected birds. Undersampling high parasitemia birds can result in underestimates of infection prevalence within a species and has the potential to underestimate transmission if high parasitemia individuals are responsible for creating the majority of infectious mosquitoes. Given the low expected fraction of high parasitemia birds, we would need several hundred qPCR-positive individuals of each species to address this question conclusively. Finally, our revised estimates for infection rates from the historical study (van Riper et al., 1986) use parasitemia data collected between 2015 and 2022 and assume similar parasitemia levels in each species over time. Average parasitemias have the potential to change through the evolution of resistance in avian hosts or through changes in P. relictum infection strategies.

Our samples came from a system with a single haemosporidian parasite and lineage (GRW4 of P. relictum), but in many systems hosts are frequently infected with multiple species (e.g., Haemoproteus and Leucocytozoon) and lineages (Beadell et al., 2004; Valkiũnas et al., 2006; Loiseau et al., 2010; Chaisi et al., 2019). Measuring lineage-specific parasitemias would require a multiplex qPCR, or other approaches that target individual parasite lineages.

PCR-based methods are now commonly used to investigate infection prevalence of Plasmodium spp. parasites (Ricklefs and Fallon, 2002; Fallon et al., 2003; Ishtiaq et al., 2017; Chaisi et al., 2019; Ciloglu et al., 2019). We have created a tool for integrating data from qPCR and microscopy, and show that molecular methods are critical to accurately compare infection prevalence among host species. Several species of introduced birds in Hawai'i had much higher infection prevalence than historical studies suggested, and might play an important role in transmission. Microscopy is useful for characterizing parasite life stages and host blood cell counts, and is an inexpensive and quick method for determining parasitemias in high-intensity infections (Godfrey et al., 1987; Valkiunas et al., 2008). Microscopy is especially valuable for determining whether mosquito-infecting gametocytes are present in a host species, or that host is non-infectious. However, microscopy is insensitive for low parasitemias and produces misleading patterns of infection prevalence. Accurately quantifying host-parasite interactions requires either a combination of techniques such as qPCR and microscopy, or multiple qPCR and genetic assays that detect different malaria life stages, and in other systems, mixed parasite infections (Wang et al., 2015; Karadjian et al., 2016; Ciloglu et al., 2019; Gadalla et al., 2021).

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.ijpara.2023.10.001.

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