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BIRD TISSUES FROM MUSEUM COLLECTIONS ARE RELIABLE FOR ASSESSING AVIAN HAEMOSPORIDIAN DIVERSITY

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ABSTRACT

Birds harbor a diverse group of haemosporidian parasites that reproduce and develop in the host blood cells, muscle tissue, and various organs, which can cause negative effects on the survival and reproduction of their avian hosts. Characterization of the diversity, distribution, host specificity, prevalence patterns, and phylogenetic relationships of these parasites is critical to the study of avian host–parasite ecology and evolution and for understanding and preventing epidemics in wild bird populations. Here, we tested whether muscle and liver samples collected as part of standard ornithological museum expeditions can be examined to study the diversity and distributions of haemosporidians in the same way as blood collected from individual birds that are typically banded and released. We used a standard molecular diagnostic screening method for mitochondrial DNA (cytochrome *b*) of the parasites and found that blood, muscle, and liver collected from the same host individual provide similar estimates of prevalence and diversity of haemosporidians from the genera *Parahaemoproteus* and *Leucocytozoon*. Although we found higher prevalence for the genus *Plasmodium* when we screened blood vs. liver and muscle samples, the estimates of the diversity of *Plasmodium* from different tissue types are not affected at the community level. Given these results, we conclude that for several reasons existing museum genetic resources collections are valuable data sources for the study of haemosporidians. First, ornithological museum collections around the world house tens of thousands of vouchered tissue samples collected from remote regions of the world. Second, the host specimens are vouchered and thus host identification and phenotype are permanently documented in databased archives with a diversity of associated ancillary data. Thus, not only can identifications be confirmed but also a diversity of morphological measurements and data can be measured and accessed for these host specimens in perpetuity.

Avian malaria parasites and related haemosporidians (Apicomplexa: Haemosporida) are protozoan parasites that infect vertebrate blood cells and are transmitted by hematophagous dipteran vectors (Valkiūnas, 2005; Santiago-Alarcon et al., 2012). Birds possess the highest diversity of haemosporidian parasites, historically encompassing 3 genera: *Haemoproteus* (containing the 2 sub-genera *Haemoproteus* and *Parahaemoproteus*), *Leucocytozoon*, and *Plasmodium* (Valkiūnas, 2005). However, growing evidence from phylogenetic analyses support *Haemoproteus* and *Parahaemoproteus* as distinct genera (Martinsen et al., 2008; Borner et al., 2016; Galen et al., 2018a). The highly diverse genus *Parahaemoproteus* primarily infects Passeriformes, whereas the markedly less diverse *Haemoproteus* is restricted to only a few groups of non-Passeriformes. Recent work on these parasites from non-Passeriformes has shown that even this more restrictive view of *Haemoproteus* includes a group of parasites that are non-monophyletic with novel genus-level clades infecting cranes (Bertram et al., 2017) and vultures (Yabsley et al., 2018). These recent results clearly demonstrate that the diversity of avian haemosporidians is far greater than we have historically

considered and their phylogeny is still largely unresolved (Martinsen et al., 2008; Outlaw and Ricklefs, 2011; Perkins, 2014; Borner et al., 2016; Galen et al., 2018a, 2018b), in part due to insufficient sampling from many host groups and across highly diverse regions, such as the Neotropics.

In the past 2 decades, with the development of molecular methods to detect and identify avian haemosporidian parasites (Bensch et al., 2000; Fallon et al., 2003; Hellgren et al., 2004; Waldenström et al., 2004; Bell et al., 2015), more than 3,000 unique genetic lineages have been recovered from all avian clades and zoogeographical regions except for Antarctica (see MalAvi database at http://mbio-serv2.mbioekol.lu.se/Malavi/, Bensch et al., 2009). These discoveries not only revealed an astonishing diversity of haemosporidian parasites but also have altered our understanding of their host use and specialization. Avian malaria parasites are a widely used model for understanding parasite–host interactions (Lauron et al., 2015; Fecchio et al., 2017, 2018a, 2018b, 2019), including the costs of these parasites on endemic host populations (Atkinson and Samuel, 2010), feather growth (Marzal et al., 2013; Coon et al., 2016), lekking behavior (Bosholn et al., 2016), ornamentation (Henschen et al., 2017), extra-pair paternity (Podmokła et al., 2015), lifespan, and reproductive output (Asghar et al., 2015). The growing interest in the ecology and evolution of this diverse group of parasites has led researchers to use avian tissues, other than blood, to assess the diversity of mitochondrial DNA sequences of *Haemoproteus*, *Leucocytozoon*, *Parahaemoproteus*, and *Plasmodium* (Galen and Witt, 2014; Marroquin-Flores et al., 2017), with some authors analyzing samples derived from both blood and muscle in the same study (Drovetski et al., 2014; Fecchio et al., 2017, 2018a, 2018b, 2018c, 2019; Clark, 2018; Galen et al., 2018a, 2018b). This practice has allowed researchers to increase sampling in macroecological studies but could have implications for the interpretation of results if the detectability of haemosporidians differs according to tissue type.

Although haemosporidian parasites infect and develop within bird tissues (including blood cells), the diversity, distribution, and specialization of each genus differ substantially and cannot be determined solely by microscopic examination of blood films. Microscopic identification of avian haemosporidians requires great expertise and few researchers possess the knowledge to identify morphological species (Valkiūnas et al., 2008a). To date, few parasitologists have linked morphology (known species) to genetic lineages (see Valkiūnas et al., 2008a, 2008b; Matta et al., 2014; Walther et al., 2016), preventing studies that rely on microscopic examination from contributing broadly to our understanding of the diversity and distribution of these parasites. Furthermore, large-scale bird sampling is constrained since the microscopic screening of blood slides is much more time consuming than molecular screening (Bell et al., 2015).

To improve our understanding of haemosporidian diversity and distributions, we sought to determine whether different avian tissue samples (blood, muscle, and liver) collected from the same host individual provide equal detection of haemosporidian parasites using molecular techniques. At present, ornithological museum collections around the world house large vouchered genetic resource collections (Edwards et al., 2005) including a variety of tissues (muscle, heart, liver, and blood) and might provide valuable sources of data on the prevalence, distribution, and diversity of haemosporidians infecting birds. However, a critical comparison among the results garnered from screening a variety of tissues types from the same host individuals is needed to determine whether these tissue types yield equally accurate assessments of the diversity and distribution of haemosporidian parasites. Therefore, we used a collection of blood, muscle, and liver tissues collected during a recent joint expedition in Central Mexico to assess whether parasite genera (*Leucocytozoon*, *Parahaemoproteus*, and *Plasmodium*) are preferentially detected among these 3 different tissue types. Finally, we determine whether differential amplification of a cytochrome *b* (*cyt b*) gene fragment from these different tissue types results in different measures of parasite diversity at the community level.

MATERIALS AND METHODS

Avian tissue sampling

Our sampling included a total of 83 individuals of 23 bird species collected with mist-nets in the Parque Ejidal San Nicolas Totolapan, Ciudad de México, Mexico (19°14′45.74″N, 99°15′5.47″W; 3,025 m above sea level) during the breeding season (2015 July 8–13). Three tissue samples were collected from each individual: blood samples were extracted from the brachial vein and stored on FTA cards; muscle and liver samples were taken during specimen preparation and stored in 95% ethanol until DNA extraction. All birds and tissue samples were collected under appropriate permits (see Acknowledgments) in Mexico, and vouchers are available at Academy of Natural Sciences of Drexel University and Museo de Zoología Alfonso L. Herrera, Facultad de Ciencias, Universidad Nacional Autónoma de México. Voucher numbers and associated host specimen information can be found in Suppl. Table S1.

Parasite detection and lineage delimitation

DNA was extracted from the 3 tissue types using the DNeasy 96 Blood and Tissue kit (Qiagen, Valencia, California). DNA samples were screened for the presence of haemosporidian parasites (*Haemoproteus*, *Leucocytozoon*, *Parahaemoproteus*, and *Plasmodium*) using nested polymerase chain reaction (PCR) to amplify a 477-bp fragment of the cyt *b* gene. Two separate nested PCRs were used: one that screens for *Haemoproteus*, *Parahaemoproteus*, and *Plasmodium* together using initial primers H332F (Bell et al., 2015) and HaemNR2 (Waldenström et al., 2004), and then the nested set of primers H350F (Bell et al., 2015) and HaemR2 (Bensch et al., 2000), and another that screens for *Leucocytozoon* by using initial primers HaemNFI and HaemNR3 (Hellgren et al., 2004) and then the nested set of primers L350F and L890R (Lutz et al., 2015). All nested PCRs were run using OneTaq Quick-Load 2X Master Mix with standard buffer (New England Biolabs, Ipswich, Massachusetts), with 1 positive and 1 negative control in each 96-well plate reaction. Specific details on PCR setup, template concentrations used, and PCR cycling conditions can be found in Bell et al. (2015).

Positive PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, California), sequenced using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California), and run on an ABI 3100 DNA sequencer (Applied Biosystems). Purified PCR products were sequenced using the primers FIFI and R2 (Ishtiaq et al., 2007) for *Haemoproteus*, *Parahaemoproteus*, and *Plasmodium* amplifica-

Table I. Number of samples of each tissue type that tested positive via PCR screening and sequencing for *Plasmodium* (*Plas*), *Parahaemoproteus* (*Para*), *Haemoproteus* (*Haem*), or *Leucocytozoon* (*Leuc*).

	No. PCR positive			No. identified by sequencing			
Tissue type	*Plas*/*Para*/*Haem*	*Leuc*	Total	*Plas*	*Para*	*Leuc*	Total
Blood	32	16	48	12	8	8	28
Liver	22	17	39	0	8	16	24
Muscle	35	10	45	1	15	12	28

tions and the primers L545R and L825R (Lutz et al., 2015) were used for *Leucocytozoon* amplifications. Contigs of forward and reverse sequences were assembled and reconciled using Geneious v.8.1.9 (http://www.geneious.com; Kearse et al., 2012).

Assembled sequences were aligned using BioEdit v7.2.0 (Hall, 1999) and collapsed to unique haplotypes using the FaBox haplotype collapser and converter tool (Villesen, 2007). Sequence identities were verified with a local BLAST against the MalAvi database (Bensch et al., 2009) by using BioEdit v7.2.0 (Hall, 1999). New lineages were named after the host of origin following the standard protocol of the MalAvi database (Bensch et al., 2009). All sequences are deposited in MalAvi and GenBank. MalAvi sequence codes and GenBank accession numbers can be found in Table S2.

Statistical analyses

To assess whether parasite genera are preferentially detected from different tissues (blood, muscle, and liver), we compared the number of positive screens from each tissue type, based on whether nested PCR amplified DNA, with a chi-square test of independence. We examined *Plasmodium* and *Parahaemoproteus* together as they are sequenced by the same nested primer set, whereas *Leucocytozoon* was examined separately as it is amplified by a different nested primer set. For all chi-square tests, we obtained the *P*-value by simulation with 10,000 random samples. We used chi-square tests of independence, for each parasite genus separately (*Plasmodium*, *Parahaemoproteus*, and *Leucocytozoon*), to determine whether the number of positive amplifications differs across tissue types. We also used chi-square tests of independence to determine whether the number of parasite lineages detected for each genus are differentially amplified across tissues. To identify whether lineages were differentially amplified, we compared the number of amplified samples in each tissue type for each lineage to a Poisson distribution. We did not use a Bonferroni correction to avoid inflating our Type II error and incorrectly concluding that haemosporidian lineages are amplified equally from all assayed tissue types.

To determine whether differential amplification results in different measures of parasite diversity, we compared the number of lineages obtained for each haemosporidian genus from each tissue type with chi-square tests of independence. Because the number of lineages found might be influenced by the number of amplified samples, we used rarefaction to measure the diversity of the parasite assemblage for each haemosporidian genus from each tissue type. All analyses were conducted in R v.3.3.2, and we used the "rarefy" function in the vegan package (Oksanen et al., 2017) to calculate rarefaction estimates. Post hoc power analyses were conducted with the pwr package in R (Champely, 2017), which follows the recommendations of Cohen (1988).

RESULTS

We screened blood, muscle, and liver samples for *Haemoproteus*/*Parahaemoproteus*/*Plasmodium* and *Leucocytozoon* from 83 individuals. The number of samples that screened positive for *Haemoproteus*/*Parahaemoproteus*/*Plasmodium* or for *Leucocytozoon* did not differ across tissue types (*Haemoproteus*/*Parahaemoproteus*/*Plasmodium*, $\chi^2 = 4.86$, $P = 0.09$; *Leucocytozoon*, $\chi^2 = 2.42$, $P = 0.35$; Table I). The number of samples for which we successfully amplified and sequenced haemosporidian DNA also did not vary with tissue type for *Leucocytozoon* ($\chi^2 = 3.12$, $P = 0.23$) or for *Parahaemoproteus* ($\chi^2 = 3.61$, $P = 0.20$). However, we detected more positive *Plasmodium* amplifications from blood samples than from liver or muscle samples ($\chi^2 = 21.59$, $p < 0.001$; Table I). The genus *Haemoproteus* was not identified in any tissue sample.

Parahaemoproteus ($\chi^2 = 19.89$, $P = 0.79$; Table II) and *Leucocytozoon* ($\chi^2 = 7.74$, $P = 0.99$; Table III) lineages did not exhibit differential amplification across tissue types. We could not assess differential amplification among *Plasmodium* lineages because we found low numbers of amplified *Plasmodium* infections among liver and muscle samples (0 and 1, respectively), which did not permit a chi-square test. However, 1 *Plasmodium* lineage, ALEDIA01, exhibited differential amplification across tissue types ($\chi^2 = 22.0$, $P < 0.001$; Table IV).

Table II. Successful amplification and sequencing of *Parahaemoproteus* by lineage from each of 3 tissue types.

	Found in		
Lineage name	Blood	Liver	Muscle
PSAMIN01	1	0	0
SETAUD21	1	1	2
ATLPIL01	1	0	0
JUNPHA01	3	4	6
JUNPHA02	1	0	0
PTICIN01	1	1	2
POESCL01	0	1	0
VIRHUT03	0	1	0
ATLPIL02	0	0	1
HYLLEU01	0	0	1
PIPMACO1	0	0	1
VIRHUT02	0	0	2
Total	8	8	15

Table III. Successful amplification and sequencing of *Leucocytozoon* by lineage from each of 3 tissue types.

	Found in		
Lineage name	Blood	Liver	Muscle
CATOCC01	4	3	3
MYAOCC01	1	2	1
TURASS01	1	2	2
CNEORN01	2	2	3
TROAED25	0	1	1
JUNPHA03	0	1	0
VIRHUT04	0	1	0
CATOCC02	0	1	1
CATOCC03	0	1	1
TURASS02	0	1	0
SPIPAS07	0	1	0
Total	8	16	12

Table IV. Successful amplification and sequencing of *Plasmodium* by lineage from each of 3 tissue types.

	Found in		
Lineage name	Blood	Liver	Muscle
REGSAT01	1	0	0
ALEDIA01	11	0	0
VIRHUT01	0	0	1
Total	12	0	1

The number of amplified lineages of *Plasmodium* did not differ with tissue type ($\chi^2 = 2.0$, $P = 0.78$), and neither did the number of amplified lineages of *Parahaemoproteus* ($\chi^2 = 0.33$, $P = 0.96$) or of *Leucocytozoon* ($\chi^2 = 3.36$, $P = 0.20$; Table V). Rarefaction showed that the diversity of *Plasmodium*, *Parahaemoproteus*, and *Leucocytozoon* assemblages did not differ across tissue types (Table V). Power analyses indicated that we had sufficient power to detect medium and large effect sizes, but our power to detect small effects was not high (Table VI).

DISCUSSION

Among the 3 avian haemosporidian parasite genera studied from this sample of Mexican bird specimens, only *Plasmodium* exhibited differential detectability when screened from different tissue types and was more prevalent in blood than in muscle and liver tissues collected from the same host individuals. However, this differential detectability did not affect estimates of parasite diversity at the community level for *Plasmodium*. For *Parahaemoproteus* and *Leucocytozoon*, both detectability and diversity were similar across the 3 screened tissues types collected from the same host individuals. Although there was a slight difference in detectability, the parasite diversity index did not differ among screened tissue types, which supports the potential of using tissue types other than blood to assess diversity and distribution of an assemblage of haemospordian parasites in a local bird community.

Parasite detectability

Our results agree with those of Ramey et al. (2013), who reported no difference between blood and wing muscle in detection probability and prevalence of *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* from northern pintail (*Anas acuta*). However, our finding differs from Svensson-Coelho et al. (2016), who reported low probability of *Plasmodium* detection from blood in comparison with liver, heart, and pectoral muscle from white-shouldered fire-eye (*Pyriglena leucoptera*). At the community level, Harvey and Voelker (2017) identified more *Haemoproteus* infections from blood as compared to pectoral muscle. By contrast, they identified more *Plasmodium* infections in pectoral muscle than in blood samples. The inconsistency in detectability presented in these 4 comparison studies (including the present study) might be related to a variety of factors that ultimately would affect prevalence (the percentage of infected individuals in a host population). For example, Valkiūnas (2005) documented that a relapse of parasitemia in blood cells occurs during the host breeding season, which could lead to higher prevalence in blood tissue compared with muscle or liver tissue. This may explain why we found *Plasmodium* in higher prevalence in blood compared with muscle and liver during the breeding season in this Mexican bird community. Haemosporidian infections apparently persist within infected individuals (see Valkiūnas, 2005), and perhaps older individuals could have higher abundance of parasites in muscle or liver tissue (chronic infection) acquired and accumulated during their lifespan. In contrast, younger hosts may experience an acute phase of infection with a higher abundance of the parasite found in the bloodstream. These age-related effects would have implications for studies aiming to detect the effect of haemosporidian parasites at the individual level (e.g., survivorship or fecundity, immune or behavioral response to a parasite) since a few missing infections in a host population could drastically affect the parameters used in the analyses. Ideally, for that type of study, it would be advantageous to screen as many tissue types as possible for parasites since during chronic infections the parasites can also be present in a variety of tissues other than muscle, liver, and blood (e.g., brain, lung, spleen, and intestine; see Valkiūnas, 2005). Furthermore, analysis of blood films would confirm whether the parasite has reached the gametocyte stage in the hosts and can be transmitted to vectors, or whether it may only be an abortive infection.

Parasite diversity

Our results contrast with 2 previous studies of single host species comparing diversity of *Leucocytozoon* (Ramey et al., 2013) and *Plasmodium* (Svensson-Coelho et al., 2016) parasites recovered from a variety of tissue types. The molecular detection of 11 haemosporidian *cyt b* lineages from blood samples compared to only 6 haemosporidian *cyt b* lineages recovered from wing muscle collected from the same host individuals (Ramey et al., 2013) might suggest that using blood samples for investigations of genetic diversity of *Leucocytozoon* is advantageous.

However, we show that both prevalence and estimates of lineage diversity for *Leucocytozoon* are similar at the community level regardless of the tissue type used (Tables I, III, V). Despite similar estimates of lineage diversity for *Leucocytozoon*, our raw data indicate a higher number of lineages in muscle compared to blood. This highlights the importance of muscle tissue deposited

Table V. Number of infections, number of lineages, and diversity of *Plasmodium*, *Parahaemoproteus*, and *Leucocytozoon* detected from each tissue type. Diversity is measured with rarefaction and represented as mean estimated number of species ± SE. Rarefy2 subsamples 2 infections from each assemblage. The mean is equal to 1 plus the probability of interspecific encounter (Hurlbert, 1971). Rarefy8 rarefies parasite assemblages to 8 infections. Rarefaction estimates of the *Plasmodium* assemblages detected in liver and muscle could not be calculated because the number of infections was too small.

	Tissue type		
Parasite genus	Blood	Liver	Muscle
Plasmodium			
No. of infections	12	0	1
No. of lineages	2	0	1
Rarefy2	1.17 ± 0.37	NA*	NA
Rarefy8	1.67 ± 0.47	NA	NA
Parahaemoproteus			
No. of infections	8	8	15
No. of lineages	6	5	7
Rarefy2	1.89 ± 0.31	1.79 ± 0.41	1.82 ± 0.38
Rarefy8	6.00 ± 0.0	5.0 ± 0.0	5.00 ± 0.77
Leucocytozoon			
No. of infections	8	16	12
No. of lineages	4	11	7
Rarefy2	1.75 ± 0.43	1.95 ± 0.22	1.89 ± 0.31
Rarefy8	4.0 ± 0.0	6.70 ± 0.81	5.54 ± 0.77

* NA, not applicable.

in museums throughout the world to access parasite diversity to reconstruct the phylogenetic relationships of this diverse group of avian parasites. For *Plasmodium*, Svensson-Coelho et al. (2016) recovered all lineages from 4 tissue types, but they argued that screening only 1 tissue per individual bird might overlook *Plasmodium* lineages and underestimate their diversity. Harvey and Voelker et al. (2017) found higher genetic diversity among *Haemoproteus* and *Plasmodium* from blood samples compared with pectoral muscle. We have found 3 *Plasmodium* lineages of which 1, lineage ALEDIA01, was in high abundance and exclusively recovered from blood samples. This abundant lineage may drive the lack of significant difference for *Plasmodium* diversity across different tissue types from this Mexican community. However, we must be cautious about this conclusion, because we did not find many infections in muscle and liver tissue to confirm whether blood, muscle, and liver yield similar genetic diversity for *Plasmodium*. One potential explanation is that the individual birds from which the ALEDIA01 lineage was detected are not suitable hosts for this particular lineage, and thus the lineage was unable to infect host red blood cells (abortive infection). Molecular studies on avian haemosporidian parasites have shown several cases of spillover infections, yet we still do not understand whether blood or other tissue is more likely to capture these spillover events. Although blood might be more likely to capture spillovers if "dead-end" sporozoites are readily detected in the blood, other tissues and organs might be more likely to retain haemosporidian cells from "abortive" infections. Without visually inspecting blood films, it is impossible to determine whether these birds contained disseminate infections with parasites infecting red blood cells. Furthermore, a combined methodology using traditional histological techniques in association with the molecular diagnostic is essential to determine the virulence of such pathogens in avian hosts (Valkiūnas and Iezhova, 2017). Nevertheless, the presence of *Plasmodium* should be taken into consideration when assessing the local haemosporidian diversity, because this lineage is present within the avian community, even if the lineage is unable to reach the disseminate infection stage in all hosts. The ability to infect host blood cells is not a requisite to include an individual haemosporidian lineage in a diversity index. Many studies conducted in the past decades lack confirmation of sampled haemosporidian taxa reaching a reproductive stage. Thus, during analysis of parasite community diversity, haemosporidian parasites should not be considered differently from other organisms by including only those lineages/species that are able to infect a specific host species.

Large-scale sampling studies using molecular markers have shown that both diversity and distributions of haemosporidian parasites are determined by climate, geography, and host relationships (e.g., Ellis et al., 2015; Clark et al., 2017; Fecchio et al., 2017, 2018a, 2018b). The results of these 4 tissue

Table VI. Post hoc power analyses indicate that we can detect medium and large effect sizes but that we have limited power to detect small effect sizes. W is effect size, and values of 0.1, 0.3, and 0.5 indicate small, medium, and large effect sizes, respectively (Cohen 1988). N is the total number of observations, sig. level is the significance level (Type I error probability), and power is the power of the test (1 – Type II error probability). See Champely (2017) for details.

W	N	Sig. level	Power
0.1	249	0.05	0.272
0.3	249	0.05	0.993
0.5	249	0.05	1
0.1	249	0.1	0.389
0.3	249	0.1	0.997
0.5	249	0.1	1

comparison studies (Ramey et al., 2013; Svensson-Coelho et al., 2016; Harvey and Voelker, 2017; this study), although all relatively small in scale, demonstrate inconsistency in estimating haemosporidian lineage diversity recovered from screening different host tissue types. Therefore, we should take tissue type into account and incorporate it into analyses of haemosporidian diversity and distribution. Given the biology of parasite reproduction and development within avian hosts, tissues could be treated as different developmental habitats, which should be considered in future studies aiming to measure haemosporidian diversity, distribution, and phylogeny.

Conclusion and directions

Our sample size (83 individuals) resulted in a smaller number of parasite detections compared with larger communities surveyed extensively across multiple years (e.g., Fecchio et al., 2013; Svensson-Coelho et al., 2013). However, post hoc analyses indicated that we had sufficient power to detect medium and large effect sizes (power > 0.99; see Cohen, 1988), but our power to detect small effects was not high (power $= 0.272$). Therefore, we must conservatively state that we currently do not have strong evidence that parasite detectability differs across tissue types for all 3 haemosporidian genera infecting birds. This finding reinforces the reliability of previous macroecological studies that assessed the haemosporidian diversity by mixing tissues types (Drovetski et al., 2014; Fecchio et al., 2017, 2018a, 2018b, 2019; Clark, 2018) and suggests that future research can freely make comparisons between studies using different avian tissue types. Some trends were apparent, and a larger study incorporating sampling of birds across years with associated blood films and a variety of tissue samples would be beneficial to further explore the relationship between tissue type and parasite detectability. Visual examination of blood films would confidently confirm whether a bird harbored a haemosporidian infection capable of reaching the gametocyte stage, which is capable of transmission to dipteran vectors. Also, slides are imperative in understanding the morphology and taxonomy of this diverse group of parasites (Valkiūnas, 2005). Certainly, the ability to screen the tens of thousands of avian tissue samples deposited in museum collections would boost the diversity of genetic lineages recovered from hosts across megadiverse tropical bird communities, such as those found in Amazonia and the Andes, and perhaps resolve their phylogenetic relationships. Accessing these remote places is logistically difficult, time demanding, and financially expensive; thus, the ability to screen material housed in museums worldwide would provide data from regions unlikely to be sampled again. In addition to broadening the availability of host tissues, haemosporidian researchers would benefit by screening tissue samples collected with vouchered host specimens because both the host genotype and phenotype are documented and archived; thus, the recorded haemosporidian parasite–host associations could be reassessed at any time. Essentially, vouchered hosts which can be identified and studied by specialists at any time provide the gold standard of host association data for parasite studies, whereas this kind of documentation is often lacking in studies that solely collect blood and release the host individuals. Last, additional host metadata associated with host voucher specimens can be used to assess the effects of host condition on parasite prevalence and diversity.

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LITERATURE CITED

Asghar, M., D. Hasselquist, B. Hansson, P. Zehtindjiev, H. Westerdahl, and S. Bensch. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. Science 347: 436–438.

Atkinson, C. T., and M. D. Samuel. 2010. Avian malaria *Plasmodium relictum* in native Hawaiian forest birds: Epizootiology and demographic impacts on ‘apapane *Himatione sanguinea*. Journal of Avian Biology 41: 357–366.

Bell, J. A., J. D. Weckstein, A. Fecchio, and V. V. Tkach. 2015. A new real-time PCR protocol for detection of avian haemosporidians. Parasites & Vectors 8: 383. doi:10.1186/s13071-015-0993-0.

Bensch, S., O. Hellgren, and J. Pérez-Tris. 2009. MalAvi: A public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Molecular Ecology Resources 9: 1353–1358.

Bensch, S., M. Stjernman, D. Hasselquist, Ö. Östman, B. Hansson, H. Westerdahl, and R. T. Pinheiro. 2000. Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proceedings of the Royal Society of London B 267: 1583–1589.

Bertram, M. R., S. A. Hamer, B. K. Hartup, K. F. Snowden, M. C. Medeiros, D. C. Outlaw, and G. L. Hamer. 2017. A novel Haemosporida clade at the rank of genus in North American cranes (Aves: Gruiformes). Molecular Phylogenetics and Evolution 109: 73–79.

Borner, J., C. Pick, J. Theide, O. M. Kolawole, M. T. Kinglsey, J. Schulze, V. M. Cottontail, N. Wellinghausen, J. Schmidt-Chanasit, I. Bruchhaus, and T. Burmester. 2016. Phylogeny of haemosporidian blood parasites revealed by a multi-gene approach. Molecular Phylogenetics and Evolution 94: 221–231.

Bosholn, M., A. Fecchio, P. Silveira, E. M. Braga, and M. Anciães. 2016. Effects of avian malaria on male behavior and female visitation in lekking blue-crowned manakins. Journal of Avian Biology 47: 1–9.

Champely, S. 2017. Pwr: Basic functions of power analysis. R package version 1.2-2. Available at: https://CRAN.R-project.org/package=pwr. Accessed 4 January 2018.

Clark, N. J. 2018. Phylogenetic uniqueness, not latitude, explains the diversity of avian blood parasite communities worldwide. Global Ecology and Biogeography 27: 744–755.

Clark, N. J., S. M. Clegg, K. Sam, W. Goulding, B. Koane, and K. Wells. 2017. Climate, host phylogeny and the connectivity of host communities govern regional parasite assembly. Diversity and Distributions 24: 13–23.

Cohen, J. 1988. Statistical power analysis for the behavioral sciences, 2nd ed. Lawrence Erlbaum Associates, Hillsdale, New Jersey, 567 p.

Coon, C. A. C., L. Garcia-Longoria, L. B. Martin, S. Magallanes, F. de Lope, and A. Marzal. 2016. Malaria infection negatively affects feather growth rate in the house sparrow *Passer domesticus*. Journal of Avian Biology 47: 779–787.

Drovetski, S. V., S. A. Aghayan, V. A. Mata, R. J. Lopes, N. A. Mode, J. A. Harvey, and G. Voelker. 2014. Does the niche breadth or trade-off hypothesis explain the abundance–occupancy relationship in avian Haemosporidia? Molecular Ecology 23: 3322–3329.

Edwards, S. V., S. Birks, R. T. Brumfield, and R. Hanner. 2005. Future of avian genetic resources collections: Archives of evolutionary and environmental history. Auk 122: 979–984.

Ellis, V. A., M. D. Collins, M. C. I. Medeiros, E. H. R. Sari, E. D. Coffey, R. C. Dickerson, C. Lugarini, J. A. Stratford, D. R. Henry, L. Merrill, et al. 2015. Local host specialization, host-switching, and dispersal shape the regional distributions of avian haemosporidian parasites. Proceedings of the National Academy of Sciences of the United States of America 112: 11294–11299.

Fallon, S. M., R. E. Ricklefs, B. L. Swanson, and E. Bermingham. 2003. Detecting avian malaria: An improved polymerase chain reaction diagnostic. Journal of Parasitology 85: 1044–1047.

Fecchio, A., J. A. Bell, M. D. Collins, I. P. Farias, C. H. Trisos, J. A. Tobias, V. V. Tkach, J. D. Weckstein, R. E. Ricklefs, and H. Batalha-Filho. 2018a. Diversification by host switching and dispersal shaped the diversity and distribution of avian malaria parasites in Amazonia. Oikos 127: 1233–1242.

Fecchio, A., M. R. Lima, M. Svensson-Coelho, M. Â. Marini, and R. E. Ricklefs. 2013. Structure and organization of an avian haemosporidian assemblage in a Neotropical savanna in Brazil. Parasitology 140: 181–192.

Fecchio, A., R. Pinheiro, G. Felix, I. P. Faria, J. B. Pinho, G. Lacorte, E. M. Braga, I. P. Farias, A. Aleixo, V. V. Tkach, et al. 2018b. Host community similarity and geography shape the diversity and distribution of haemosporidian parasites in Amazonian birds. Ecography 41: 505–515.

Fecchio, A., P. Silveira, J. D. Weckstein, J. H. Dispoto, M. Anciães, M. Bosholn, V. V. Tkach, and J. A. Bell. 2018c. First record of *Leucocytozoon* (Haemosporida: Leucocytozoidae) in Amazonia: Evidence for rarity in Neotropical lowlands or lack of sampling for this parasite genus? Journal of Parasitology 104: 168–172.

Fecchio, A., M. Svensson-Coelho, J. Bell, V. A. Ellis, M. C. Medeiros, C. H. Trisos, J. G. Blake, B. A. Loiselle, J. A. Tobias, R. Fanti, et al. 2017. Host associations and turnover of haemosporidian parasites in manakins (Aves: Pipridae). Parasitology 144: 984–993.

Fecchio, A., K. Wells, J. A. Bell, V. V. Tkach, H. L. Lutz, J. D. Weckstein, S. M. Clegg, and N. J. Clark. 2019. Climate variation influences host specificity in avian malaria parasites. Ecology Letters 22: 547–557.

Galen, S. C., and C. C. Witt. 2014. Diverse avian malaria and other haemosporidian parasites in Andean house wrens: Evidence for regional co-diversification by host-switching. Journal of Avian Biology 45: 374–386.

Galen, S. C., J. Borner, E. S. Martinsen, J. Schaer, C. C. Austin, C. J. West, and S. L. Perkins. 2018a. The polyphyly of *Plasmodium*: Comprehensive phylogenetic analyses of the malaria parasites (order Haemosporida) reveal widespread taxonomic conflict. Royal Society Open Science 5: 171780. doi:10.1098/rsos.171780.

Galen, S. C., R. Nunes, P. R. Sweet, and S. L. Perkins. 2018b. Integrating coalescent species delimitation with analysis of host specificity reveals extensive cryptic diversity despite minimal mitochondrial divergence in the malaria parasite genus *Leucocytozoon*. BMC Evolutionary Biology 18:128. doi:10.1186/s12862-018-1242-x.

Hall, T. A. 1999. BIOEDIT: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Harvey, J. A., and G. Voelker. 2017. Avian haemosporidian detection across source materials: Prevalence and genetic diversity. Parasitology Research 116: 3361–3371.

Hellgren, O., J. Waldenström, and S. Bensch. 2004. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium* and *Haemoproteus* from avian blood. Journal of Parasitology 90: 797–802.

Henschen, A. E., L. A. Whittingham, and P. O. Dunn. 2017. The relationship between blood parasites and ornamentation depends on the level of analysis in the common yellowthroat. Journal of Avian Biology 48: 1263–1272.

Hurlbert, S. H. 1971. The nonconcept of species diversity: A critique and alternative parameters. Ecology 52: 577–586.

Ishtiaq, F., E. Gering, J. H. Rappole, A. R. Rahmani, Y. V. Jhala, C. J. Dove, C. Milensky, S. L. Olson, M. A. Peirce, and R. C. Fleischer. 2007. Prevalence and diversity of avian hematozoan parasites in Asia: A regional survey. Journal of Wildlife Disease 43: 382–398.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, et al. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649.

Lauron, E. J., C. Loiseau, R. C. Bowie, G. S. Spicer, T. B. Smith, M. Melo, and R. N. Sehgal. 2015. Coevolutionary patterns and diversification of avian malaria parasites in African sunbirds (family Nectariniidae). Parasitology 142: 635–647.

Lutz, H. L., W. M. Hochachka, J. I. Engel, J. A. Bell, V. V. Tkach, J. M. Bates, S. J. Hackett, and J. D. Weckstein. 2015. Parasite prevalence corresponds to host life history in a diverse assemblage of Afrotropical bids and haemosporidian parasites. PLoS One 10: e0128851. https://doi.org/10.1371/journal.pone.0121254

Marroquin-Flores, R. A., J. L. Williamson, A. N. Chavez, S. M. Bauernfeind, M. J. Baumann, C. R. Gadek, A. B.

Johnson, J. M. McCullough, C. C. Witt, and L. N. Barrow. 2017. Diversity, abundance, and host relationships of avian malaria and related haemosporidians in New Mexico pine forests. PeerJ 5: e3700. doi:10.7717/peerj.3700.

Martinsen, E. S., S. L. Perkins, and J. J. Schall. 2008. A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): Evolution of life history traits and host switches. Molecular Phylogenetics and Evolution 47: 261–273.

Marzal, A., M. Asghar, L. Rodríguez, M. Reviriego, I. G. Hermosell, J. Balbontín, L. Garcia-Longoria, F. de Lope, and S. Bensch. 2013. Co-infections by malaria parasites decrease feather growth but not feather quality in house martin. Journal of Avian Biology 44: 437–444.

Matta, N. E., I. A. Lotta, G. Valkiūnas, A. D. González, M. Pacheco, A. A. Escalante, L. I. Moncada, and O. A Rodríguez-Fandiño. 2014. Description of *Leucocytozoon quynzae* sp. nov. (Haemosporida, Leucocytozoidae) from hummingbirds, with remarks on distribution and possible vectors of leucocytozoids in South America. Parasitology Research 113: 457–468.

Oksanen, J., F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, et al. 2017. Vegan: Community ecology package. R package version 2.4-3. Available at: https://CRAN.R-project.org/package=vegan. Accessed 2 December 2017.

Outlaw, D. C., and R. E. Ricklefs. 2011. Rerooting the evolutionary tree of malaria parasites. Proceedings of the National Academy of Sciences of the United States of America 108: 12183–1387.

Perkins, S. L. 2014. Malaria's many mates: Past, present, and future of the systematics of the order Haemosporida. Journal of Parasitology 100: 11–25.

Podmokła, E., A. Dubiec, A. Arct, S. M. Drobniak, L. Gustafsson, and M. Cichoń. 2015. Malaria infection status predicts extra-pair paternity in the blue tit. Journal of Avian Biology 46: 303–306.

Ramey, A. M., J. P. Fleskes, J. A. Schmutz, and M. J. Yabsley. 2013. Evaluation of blood and wing pectoral muscle tissues for molecular detection and characterization of hematozoa infections in northern pintails (*Anas acuta*) wintering in California. International Journal for Parasitology: Parasites and Wildlife 2: 102–109.

Santiago-Alarcon, D., V. Palinauskas, and H. M. Schaefer. 2012. Diptera vectors of avian Haemosporidian parasites: Untangling parasite life cycles and their taxonomy. Biological Reviews 87: 928–964.

Svensson-Coelho, M., J. G. Blake, B. A. Loiselle, A. S. Penrose, P. G. Parker, and R. E. Ricklefs. 2013. Diversity, prevalence, and host specificity of avian *Plasmodium* and *Haemoproteus* in a Western Amazon assemblage. Ornithological Monographs 76: 1–47.

Svensson-Coelho, M., G. T. Silva, S. S. Santos, L. S. Miranda, R. E. Ricklefs, C. Y. Miyaki, and M. Maldonado-Coelho. 2016. Lower detection probability of avian *Plasmodium* in blood compared to other tissues. Journal of Parasitology 102: 559–561.

Valkiūnas, G. 2005. Avian malaria parasites and other Haemosporidia. CRC Press, Boca Raton, Florida, 946 p.

Valkiūnas, G., C. T. Atkinson, S. Bensch, R. N. M. Sehgal, and R. E. Ricklefs. 2008a. Parasite misidentification in GenBank: How to minimize their number? Trends in Parasitology 24: 247–248.

Valkiūnas, G., and T. A. Iezhova. 2017. Exo-erythrocytic development of avian malaria and related haemosporidian parasites. Malaria Journal 16: 101. doi:10.1186/s12936-017-1746-7.

Valkiūnas, G., P. Zehtindjiev, D. Dimitrov, A. Križanauskienė, T. A. Iezhova, and S. Bensch. 2008b. Polymerase chain reaction-based identification of *Plasmodium* (*Huffia*) *elongatum*, with remarks on species lineages deposited in GenBank. Parasitology Research 102: 1185–1193.

Villesen, P. 2007. FaBox: An online toolbox for fasta sequences. Molecular Ecology Notes 7: 965–968.

Waldenström, J., S. Bensch, D. Hasselquist, and Ö. Östman. 2004. A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. Journal of Parasitology 90: 191–194.

Walther, E., G. Valkiūnas, E. A. Wommack, R. C. Bowie, T. A. Iezhova, and R. N. Sehgal. 2016. Description and molecular characterization of a new *Leucocytozoon* parasite (Haemosporidia: Leucocytozoidae), *Leucocytozoon californicus* sp. nov., found in American kestresl (*Falco sparverius sparverius*). Parasitology Research 115: 1853–1862.

Yabsley, M. J., R. E. T. Vanstreels, E. S. Martinsen, A. G. Wickson, A. E. Holland, S. M. Hernandez, A. T. Thompson, S. L. Perkins, C. J. West, A. L. Bryan, et al. 2018. Parasitaemia data and molecular characterization of *Haemoproteus catharti* from New World vultures (Cathartidae) reveals a novel clade of Haemosporida. Malaria Journal 17: 12. doi:10.1186/s12936-017-2165-5.