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RESEARCH ARTICLE

Haemosporidian prevalence and community composition vary little across a chickadee hybrid zone

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ABSTRACT

Within animal hybrid zones, parasites may determine competitive outcomes between host species and thus affect hybridization dynamics. We addressed this hypothesis by evaluating haemosporidian prevalence and community composition in a rapidly moving hybrid zone between Black-capped Chickadees (*Poecile atricapillus*) and Carolina Chickadees (*P. carolinensis*). Using molecular methods, we screened for haemosporidians in multiple chickadee populations across the hybrid zone and investigated whether parasite prevalence varied as a function of admixture among these birds. We identified 36 parasite lineages from 3 haemosporidian genera (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) but found no genera or lineages more likely to infect any particular chickadee taxon. Instead, haemosporidian prevalence varied across sites and seasons: *Leucocytozoon* was more prevalent during chickadees' breeding season, whereas *Haemoproteus* prevalence peaked during nonbreeding periods. *Leucocytozoon* infected proportionally fewer birds at the leading edge of the hybrid zone than near its center. However, haemosporidian communities were similar among chickadee populations, and evidence for parasite exchanges between chickadee taxa was lacking. These results underscore the complexity of bird–parasite relationships and suggest that haemosporidians are unlikely to play a major role in the ongoing movement of this hybrid zone.

Keywords: avian malaria, chickadees, haemosporidians, hybrid zone, Leucocytozoonosis, parasite-mediated selection, *Poecile atricapillus*, *Poecile carolinensis*

LAY SUMMARY

- We tested whether patterns of parasite diversity and prevalence support the hypothesis that parasite communities may determine competitive outcomes within an iconic avian hybrid zone.
- We used molecular methods to detect and identify haemosporidian lineages infecting Black-capped, Carolina, and hybrid chickadees across their hybrid zone in southeastern Pennsylvania.
- Analyses revealed 36 unique haemosporidian lineages, the prevalence and richness of which varied little across chickadee taxa.
- For some parasites, locality and season predicted haemosporidian prevalence.
- Haemosporidians are likely not key drivers underlying movement of this chickadee hybrid zone.

La prevalencia de hemosporidios y la composición de la comunidad varían poco en una zona híbrida del género *Poecile*

RESUMEN

En las zonas híbridas de los animales, los parásitos pueden determinar los resultados de la competencia entre las especies hospederas y de este modo afectar las dinámicas de las hibridaciones. Analizamos esta hipótesis evaluando la prevalencia de hemosporidios y la composición de la comunidad en una zona híbrida que se mueve rápidamente entre *Poecile atricapillus* y *P. carolinensis*. Usando modelos moleculares, realizamos pruebas de detección de hemosporidios en múltiples poblaciones de *Poecile* a través de la zona híbrida e investigamos si la prevalencia de parásitos varió como una función de la mezcla entre estas aves. Identificamos 36 linajes de parásitos pertenecientes a 3 géneros de hemosporidios (*Plasmodium*, *Haemoproteus*, y *Leucocytozoon*), pero no encontramos que ningún género o linaje presente mayor probabilidad de infectar a algún taxón de *Poecile* en particular. En cambio, la prevalencia de hemosporidios varío a través de los sitios y las estaciones: *Leucocytozoon* fue más prevalente durante la estación reproductiva de *Poecile*

mientras que la prevalencia de *Haemoproteus* tuvo un máximo durante los períodos no reproductivos. *Leucocytozoon* infectó proporcionalmente menos aves en el borde delantero de la zona híbrida que cerca del centro. Sin embargo, las comunidades de hemosporidios fueron similares entre las poblaciones de *Poecile* y no hubo evidencia de intercambios de parásitos entre los taxones de *Poecile*. Estos resultados subrayan la complejidad de las relaciones entre aves y parásitos y sugiere que los hemosporidios no juegan probablemente un rol importante en el movimiento en curso de esta zona híbrida.

Palabras clave: hemosporidios, Leucocitozoonosis, malaria aviar, *Poecile atricapillus*, *Poecile carolinensis*, selección mediada por parásitos, zona híbrida

INTRODUCTION

When parasites are capable of reducing host fitness, they can act as powerful selective forces in host populations and ultimately affect how host species fare against competitors (Haldane 1948, Price et al. 1986, Grosholz 1992, Bonsall and Hassell 1997, Sainsbury et al. 2000, Tompkins et al. 2001). This is especially true of generalist parasites that infect multiple host species with varying consequences. Sometimes, the presence of a parasite-tolerant host may cause the decline of a more vulnerable one within its range (Tompkins et al. 2001), even if these hosts do not directly compete for resources (Hudson and Greenman 1998). However, competing organisms commonly share parasites through relatedness, sympatry, or life history similarities that expose them to infection. For example, introduced populations of eastern gray squirrels (*Sciurus carolinensis*) have rapidly replaced native red squirrels (*S. vulgaris*) across much of Great Britain due to the presence of a poxvirus that is deadly to red but not gray squirrels (Sainsbury et al. 2000).

Parasite-mediated competition plays a key role in biological invasions, in which the parasites themselves can serve as bridges (Torchin and Mitchell 2004) or barriers (Ricklefs 2010, Olsson-Pons et al. 2015) to expanding host populations. Moreover, parasites can affect the dynamics of animal contact zones. When species undergo secondary contact, they may exchange parasites that have otherwise evolved in isolation, and such exchanges can lead to contact-zone movement (Reullier et al. 2006). For instance, in a variant of the novel weapons hypothesis (Callaway and Ridenour 2004), a species expanding its range may transfer parasites to a naïve congener that would otherwise outcompete the invader and prevent it from becoming established in certain areas (Schmitz and Nudds 1994, Kulma et al. 2013). This congener may experience more frequent or virulent infections, thereby become an inferior competitor, and allow the advancing host species to determine the limits of the resulting contact zone. Alternatively, according to the enemy release hypothesis, an advancing host species may gain a competitive advantage against others by escaping from coevolved parasites that otherwise plague it within its core range (Marzal et al. 2018).

Hybridization between hosts can make the outcome of these exchanges more interesting, but also more complex. In some cases, hybrid individuals are more resistant to infection than parental species because of increased major histocompatibility complex (MHC) variation and exchanges of alleles that confer better immune function (Eastwood et al. 2017, Cozzarolo et al. 2018). However, genetic incompatibilities may also translate to metabolic problems or maladaptive behavior (Wagner et al. 2020) that make hybrids less capable of resisting parasites. Hybrids may also inherit parasites that are otherwise specific to each of their parental species and experience deadly coinfections (Theodosopoulos et al. 2019). Thus, parasites can contribute to hybrid zone expansion, shrinkage, or stability by selecting for or against hosts with hybrid phenotypes (Sage et al. 1986, Theodosopoulos et al. 2019) and by affecting the relative fitness of parental vs. hybrid individuals (Guttel and Ben-Ami 2014).

Among birds that compete or hybridize, haemosporidians make ideal subjects for studying parasite-mediated competition. This is because avian haemosporidians are both widespread and diverse, occurring in a majority of bird families and comprising nearly 4,000 genetically distinct lineages (Bensch et al. 2009). Moreover, haemosporidians cause avian malaria and leucocytozoonosis (Valkiūnas 2005), and can reduce host fitness through direct mortality (Atkinson et al. 2000), decreased reproductive potential (Marzal et al. 2005, Knowles et al. 2010a, Asghar et al. 2011), telomere shortening (Asghar et al. 2015), or decreased mass gain during migration (Garvin et al. 2006).

Although knowledge of haemosporidian distributions, host associations, and prevalence among birds has vastly improved within the last two decades (Marzal 2012), only a few studies have examined the ecological roles of these parasites in avian contact and hybrid zones. In one study, two *Hippolais* warbler species exchanged otherwise host-specific haemosporidians across a contact zone in Western Europe (Reullier et al. 2006). Among two *Ficedula* flycatchers on a Swedish island, the species with higher haemosporidian diversity and lower rate of infection appeared to be a stronger competitor (Kulma et al. 2013, Jones et al. 2018). In North America, two studies have attempted to test the hypothesis that haemosporidians can select for or against hybrid parulids, in the Blue-winged × Golden-winged warbler (*Vermivora cyanoptera* and *V. chrysoptera*) hybrid zone (Vallender et al. 2012) and in

a hybrid zone between Yellow-rumped Warbler subspecies (*Setophaga coronata coronata* and *S. c. audubonii*) (Cozzarolo et al. 2018). However, hybrid warblers and parental species were not differentially predisposed to infection in either of these studies.

We investigated the distribution, diversity, and prevalence of haemosporidians in Black-capped Chickadees (*Poecile atricapillus*, hereafter BCCH) and Carolina Chickadees (*P. carolinensis*, CACH), two sister passerine species (family Paridae) that compete and hybridize across a narrow tension zone extending from Kansas to New Jersey (Reudink et al. 2007). Within this zone, BCCH and CACH freely hybridize (Curry 2005, Reudink et al. 2007), and although hybrids are fertile, their low hatching success (Bronson et al. 2003a, Driver 2017) and decreased cognitive abilities (McQuillan et al. 2018) likely select against them (Rice and McQuillan 2018). Thus, hybrid zone width for these chickadees is likely limited by post-zygotic selection against hybrid offspring (Bronson et al. 2005).

Currently, the position of the BCCH × CACH hybrid zone is rapidly shifting in regions such as southeastern Pennsylvania, where CACH are undergoing a northward range expansion that is likely aided by climate change (Taylor et al. 2014b). Areas that supported "pure" BCCH populations as recently as the late 1990s now contain hybrid populations and are poised to become CACH-dominated in the near future (Reudink et al. 2007, Taylor et al. 2014a, Wagner et al. 2020). However, increases in minimum winter temperatures only explain northward CACH movement and not the mechanism by which they displace BCCH (McQuillan and Rice 2015). One possible mechanism is the social dominance of male CACH over BCCH (Bronson et al. 2003b), which is further supported by CACH siring more extra-pair offspring with females of both species (Reudink et al. 2006). However, disease and parasites may also play a crucial role in shaping these birds' distributions and their relative abilities to compete with one another—a possibility suggested by Cornell (1974), but never tested in the chickadee system.

In this study, we measured the prevalence and community structure of haemosporidian lineages across the BCCH × CACH hybrid zone to test whether certain haemosporidians may provide CACH with a competitive edge against more infection-prone BCCH. We screened nearly 1,000 chickadees for haemosporidians along a hybrid zone transect in southeastern Pennsylvania using standard molecular techniques to detect and identify haemosporidian lineages from blood samples. We asked (1) whether different chickadee taxa and their hybrids possessed similar haemosporidian communities and infection rates with certain lineages; (2) whether expanding CACH populations were transferring parasites to BCCH; and (3) whether chickadee taxa, age, locality, or seasonality were the strongest predictors of infection. We expected CACH to harbor greater lineage richness than BCCH (having evolved in warmer climates), and hybrids to possess higher infection rates than parental species due to potential compatibility with both BCCH- and CACH-specific parasites. Furthermore, we expected BCCH populations to experience more frequent infections in the hybrid zone due to the presence of CACH and hybrids there.

METHODS

Chickadee Study Populations

We collected blood samples from 4 chickadee study populations in southeastern Pennsylvania across a latitudinal transect spanning the BCCH × CACH hybrid zone. The southernmost site, Great Marsh (40.14°N, 75.73°W; hereafter GM) harbors only CACH as a breeding species. The next site to the north, Nolde Forest Environmental Education Center (40.27°N, 75.95°W; NF) hosted a mixed population in 1998 (Reudink et al. 2007), but has since become CACH-dominated and no longer contains "pure" BCCH (Taylor et al. 2014b, Wagner et al. 2020). Hawk Mountain Sanctuary (40.65°N, 76.00°W; HM) is at the hybrid zone's current center, with BCCH, CACH, and hybrid backcrosses, though it had a much larger BCCH presence in the early 2000s (Reudink et al. 2007). The northernmost site, Tuscarora State Park (40.80°N, 76.03°W; TU) sits at the northern edge of the hybrid zone; this site exclusively harbored BCCH until 2 F1 hybrids and a single CACH occurred there in 2016 (this study).

We screened for haemosporidians using blood samples collected at two distinct times of year: May–June (breeding), and October–March (nonbreeding). During the breeding season, we captured birds by placing mist nets in front of active artificial nest cavities; during nonbreeding periods, we captured chickadees via nets placed in front of feeders. We aged newly captured individuals based on the shape of their outer rectrices (Pyle 1997) and fitted each with a numbered aluminum USGS band and a unique combination of 2 or 3 plastic color bands. We obtained ~30 µL blood samples by brachial venipuncture with samples subsequently stored in ~300 µL of lysis buffer at 4°C.

Our sample of 960 screened chickadees included 169 BCCH, 490 CACH, and 301 hybrids identified by genotype (see below). We sampled more birds during breeding (n = 707) than nonbreeding periods (n = 253). Samples from CACH-dominated populations (GM and NF) came from 2012 to 2019, whereas those from mixed or BCCH-dominated populations with fewer nesting pairs (HM and TU) came from 2006 to 2019. All samples comprised first winter to adult-aged birds that likely hatched within or near study sites, and we excluded overwintering BCCH that may have been irruptive migrants.

Molecular Methods

We extracted DNA from blood samples with the DNeasy Blood and Tissue Kit (Qiagen, Germantown, Maryland, USA), using the manufacturer's protocol, with some earlier extractions conducted with a modified version (Driver 2017) of the Puregene Core A extraction kit (Qiagen, Germantown, Maryland, USA). We then ran a nested polymerase chain reaction (PCR) protocol (Bell et al. 2015) to screen extracts for haemosporidian mtDNA, using separate assays to detect *Plasmodium*/*Haemoproteus* and *Leucocytozoon*. For the *Plasmodium*/*Haemoproteus* assay, we amplified a 477 base pair (bp) cytochrome-*b* (cyt *b*) gene fragment using primers H332F/HaemNR2 for the initial reaction and primers H350F/HaemR2 for the nested PCR. For *Leucocytozoon*, we amplified a similar 525-bp cyt *b* fragment using HaemNFi/HaemNR3 and L350F/L890R as the respective initial and nested primer sets. Both assays included purified sterile water as a negative control and previously screened samples with known infections as positive controls. We ran PCR products on a 1% agarose gel stained with SYBR Safe (Life Technologies, Carlsbad, California, USA) and viewed them under UV light to identify samples that were positive for infections. For a detailed list of reagents, steps, and thermocycler settings, see Bell et al. (2015).

We sent positive nested PCR products to Functional Biosciences, Inc. (Madison, Wisconsin, USA) for Sanger sequencing and used *Geneious* v8.1.8 (Kearse et al. 2012) to trim and reconcile forward and reverse strands to produce a consensus sequence. We then performed a BLAST search of these consensus sequences within the MalAvi database (Bensch et al. 2009) to identify them as specific haemosporidian lineages. For clean sequences that did not match any previously deposited lineages in MalAvi (see Acknowledgments), we named them with the prefix POECAR, POEATR, or POEHYB, based on the chickadee taxon in which they were initially found. Positive PCR products that did not display clean sequences were re-sequenced once and then excluded from further analyses if they failed a second time.

Outside of GM, we assigned species or hybrid status to sampled chickadees based on a PCR-RFLP approach developed by McQuillan et al. (2017) and identified birds based on BCCH or CACH-specific nuclear single-nucleotide polymorphism (SNP) genotypes. We used all but 2 (cop171 and cop283) of the 10 marker loci developed by McQuillan et al. (2017), following their PCR and digestion protocols for the other 8. We then visualized digested PCR products through gel electrophoresis and determined each bird's identity based on the appearances of gel bands. For controls, we used water and genetically pure CACH and BCCH extracts from previously identified individuals.

For each SNP assay, we assigned chickadees numerical values based on how many CACH alleles they possessed. We then used these values from all 8 assays to establish an index where birds with values of 0 were pure BCCH, and those with a 1 were pure CACH. For analyses that required nominal categories (e.g., identifying infection rates in BCCH vs. CACH), we assigned hybrid status to birds scoring 0.1–0.9 on this scale. For analyses that compared hybrids against both parental species, we transformed this index ($|\ 0.5 - \%\text{CACH alleles}\ | \times 2$) such that parental individuals had values of 0 and the most heavily admixed individuals (such as F1 hybrids) had values close to or equaling 1.

Statistical Analyses

To quantify haemosporidian community composition among chickadees, we divided study sites into BCCH, CACH, or hybrid populations and created a non-metric multidimensional scaling (NMDS) plot that visualized community structure of all parasite lineages across sites and chickadee ancestry categories (BCCH, CACH, or hybrid). We performed this analysis through the *metaMDS* function from the *vegan* package (Dixon 2003) in R 3.6.1 (R Core Team 2019).

To assess whether chickadee age, genotype, site, and annual timing of sample collection (season) best predicted probability of infection, we used generalized linear mixed models (GLMMs) with binomial error structure. For our first set of GLMMs, we only tested infection probabilities between BCCH and CACH, excluding birds identified as hybrids. We independently fitted models on 8 response variables, which were *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* infections (1–3); infections caused by the 4 most common lineages (4–7); and instances of mixed infections (8). For each response, we treated infection status as a binary dependent variable (infected vs. uninfected), year of each sample as a random factor, and combinations of 3 fixed effects as explanatory variables. These fixed effects were chickadee species (categorical: BCCH vs. CACH), study site (categorical: GM, NF, HM, TU), age (categorical: greater or less than a year old), and season (categorical: breeding vs. nonbreeding). We also considered interaction effects between age and site, age and season, and species and site (which one would expect if parasite transfer occurs in the hybrid zone). Then, to determine whether hybrids were more likely to be infected than *either* parental species, we included them in a second set of models and replaced the categorical "species" variable with our transformed hybrid index of admixture in individuals (continuous: scale of 0–1, 1 being the most admixed).

For both sets of models, we relied on two separate selection approaches: a "traditional" one used by González et al. (2014) and Lutz et al. (2015), and a stepwise approach by Cozzarolo et al. (2018). With the traditional approach, we ran sets of 101 models with all possible combinations of fixed effects and interactions and used a corrected Akaike

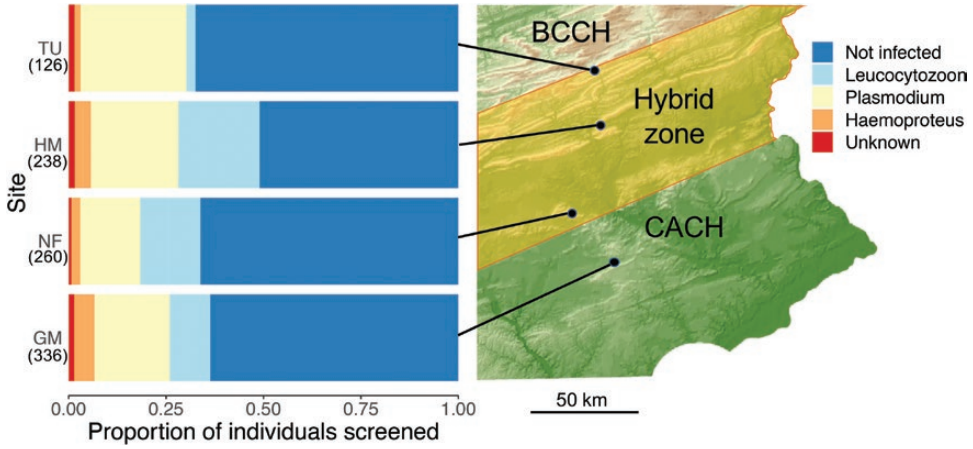

FIGURE 1. Haemosporidian prevalence across chickadee study sites in southeastern Pennsylvania. Orange band depicts the current extent of the BCCH × CACH hybrid zone; eastern and southern margins trace the state boundaries. Numbers in parentheses represent sample sizes.

information criterion (AIC_c) to rank models from best to worst fit. Models with $\Delta AIC_c < 2$ were considered equally good fits (Burnham and Anderson 2002). We used the combined weights of variables from each set of models to rank the importance of site, season, and chickadee genotype in predicting infection status.

For the stepwise approach, we created a maximal model for each response variable and then reduced to a best-fitting minimal model by removing nonsignificant interactions and fixed effects through likelihood ratio tests. We used AIC scores to assess each model's goodness of fit and identified key predictors based on how much their removal increased ΔAIC. This approach allowed us to single out minimal models for plotting and to make pairwise comparisons of infection probabilities among predictor variables.

We fitted all models using the *glmer* function from the R package *lme4* (Bates et al. 2015). We used the package *MuMin* (Barton 2009) for ranking models and the package *multcomp* (Hothorn et al. 2008) for Tukey's pairwise comparisons.

RESULTS

Prevalence and Diversity of Chickadee Haemosporidians

Of the 960 chickadees screened, 351 (36.6%) were infected with haemosporidians, including 32.5% of BCCH, 34.5% of CACH, and 42.2% of hybrids ($\chi^2 = 6.2$, df = 2, $P = 0.04$). Proportions of infected birds were highest (47.5%) at hybrid-dominated HM, followed by the CACH-dominated sites GM (34.2%) and NF (32.3%), and BCCH-dominated TU (31%; $\chi^2 = 16.8$, df = 3, $P < 0.001$; Figure 1). At HM, birds in different ancestry categories (BCCH, CACH, and hybrids) showed similar rates of infection ($\chi^2 = 2.8$, df = 2, $P = 0.24$).

Of 351 infections, sequencing was unsuccessful for 13, and another 19 birds were infected by at least one *Plasmodium* or *Haemoproteus* sequence that exhibited sequence ambiguities and could not be identified as a specific lineage (possibly due to infection with multiple parasites). In the remaining samples, 33 unique haemosporidian lineages occurred, including 11 *Plasmodium*, 9 *Haemoproteus*, and 13 *Leucocytozoon* (Table 1). Fourteen lineages were novel, not matching any sequences previously recorded in MalAvi. These novel lineages occurred in <6% of infected birds. The most common lineages (accounting for >75% of infections) were BAEBIC04 (a *Plasmodium*), CB1, COLBF21, and DUMCAR02 (all *Leucoyctozoon*) (Table 1). Mixed infections occurred in only 8.3% of infected birds (Supplementary Material Table S1); most included BAEBIC04 or CB1. Only one mixed infection occurred in a BCCH.

Plasmodium ($n = 194$) was the most prevalent haemosporidian genus across all chickadee ancestry categories, age groups, seasons, and sites (except at NF where *Leucocytozoon* was equally common) (Figures 1–3). Predominance of *Plasmodium* was largely driven by BAEBIC04 infections (Table 1). Neither *Plasmodium* nor *Haemoproteus* prevalence varied among sites ($\chi^2 = 7.4$, df = 3, $P = 0.06$ for *Plasmodium*; $\chi^2 = 2.9$, df = 3, $P = 0.4$ for *Haemoproteus*) or chickadee ancestry categories ($\chi^2 = 0.9$, df = 2, $P = 0.62$ for *Plasmodium*; $\chi^2 = 1.3$, df = 2, $P = 0.52$ for *Haemoproteus*). However, *Leucocytozoon* ($n = 125$) was more prevalent in hybrids than either parental species ($\chi^2 = 14.1$, df = 2, $P < 0.001$; Figure 2) and was dramatically less common at the northernmost TU site relative to other sites ($\chi^2 = 27.2$, df = 3, $P < 0.001$). In BCCH hosts alone, *Leucocytozoon* was more prevalent at HM than TU ($\chi^2 = 8.6$, df = 1, $P = 0.003$). Finally, both *Plasmodium* ($\chi^2 = 5.8$, df = 1, $P = 0.016$) and *Leucocytozoon* ($\chi^2 = 13.7$, df = 1, $P < 0.001$)

TABLE 1. List of haemosporidian cyt *b* lineages detected in chickadees, with the numbers of birds infected by each lineage. Last 2 columns list the localities and seasons in which lineages occurred. Asterisk (*) indicates novel lineages.

Lineage name[a]	Total	BCCH	Hybrids	CACH	Localities[b]	Seasonality[c]
L_CB1	51	3	23	25	All	Both
L_COLBF21	31	4	12	15	All	Both
L_DUMCAR02	30	3	10	17	All	B
L_BAEBIC07	4	0	4	0	NF	B
L_CARCAR09	3	1	0	2	GM, HM	B
L_DUMCAR04	3	0	2	1	GM, HM	B
L_SETCOR07	3	0	2	1	GM, NF	Both
L_ZONALB38	3	0	3	0	HM, NF	B
L_POEHYB01*	1	0	1	0	HM	B
L_POEHYB02*	1	0	1	0	HM	B
L_POEHYB03*	1	0	1	0	HM	N
L_SETCOR14	1	0	1	0	HM	B
L_VIGIL02	1	0	1	0	NF	B
P_BAEBIC04	158	26	51	81	All	Both
P_PADOM11	13	2	5	6	All	Mostly B
P_CORPIL02	3	1	2	0	TU	B
P_SEIAUR01	3	0	2	1	GM, HM	B
P_CATUS05	2	0	0	2	NF	B
P_CATUS06	1	0	1	0	NF	B
P_GEOTRI09	1	1	0	0	HM	B
P_PADOM09	1	0	0	1	GM	B
P_POEATR02*	1	1	0	0	TU	B
P_POEATR03*	1	0	1	0	NF	B
P_POEATR05*	1	1	0	0	HM	B
H_MAFUS02	19	1	4	14	GM, NF, HM	Mostly N
H_POEATR07*	1	0	1	0	NF	N
H_POEATR08*	1	1	0	0	HM	B
H_POEATR09*	1	1	0	0	HM	B
H_POECAR03*	1	0	0	1	GM	N
H_POECAR04*	1	0	0	1	GM	N
H_POECAR05*	1	0	0	1	GM	N
H_POEHYB04*	1	0	1	0	HM	B
H_POEHYB05*	1	0	1	0	HM	B
	351	55	127	169	Total birds infected[d]	
	960	169	301	490	Total birds sampled	

[a] L = *Leucocytozoon*, P = *Plasmodium*, H = *Haemoproteus*.
[b] GM = Great Marsh, NF = Nolde Forest, HM = Hawk Mountain, and TU = Tuscarora.
[c] B = breeding season (May–June) and N = nonbreeding season (October–March).
[d] Includes low-quality sequences and samples containing multiple lineages.

were more prevalent during the breeding season, whereas *Haemoproteus* ($n = 33$) was largely absent from May and June samples ($\chi^2 = 42.9$, df = 1, $P < 0.001$; Figure 3).

Predictors of Infection

GLMMs excluded the 13 birds with positive amplifications that were not successfully sequenced, as well as 81 individuals whose age we could not determine. Chickadee genotype did not predict infections with any particular lineages, parasite genera, or haemosporidians as a whole, regardless of how this variable was addressed in models. Though BCCH may have harbored proportionally fewer CB1 infections than CACH ($\chi^2 = 3.39$, df = 1, $P = 0.065$) and only one mixed infection, CACH's predisposition toward these types of infections received weak model support (Supplementary Material Tables S2 and S3), and heavily admixed chickadees appeared no more likely to harbor infections than parental species or backcrosses (Tables 2 and 3).

Haemoproteus infections were predicted by season alone (Table 2), with detection more likely during nonbreeding periods (Figure 4D). For *Plasmodium* (and especially BAEBIC04), GLMMs revealed a strong interactive effect between site and age (Tables 2 and 3) on the likelihood of infection: age groups at TU and GM showed trends opposite to those at HM and NF, with older chickadees at TU

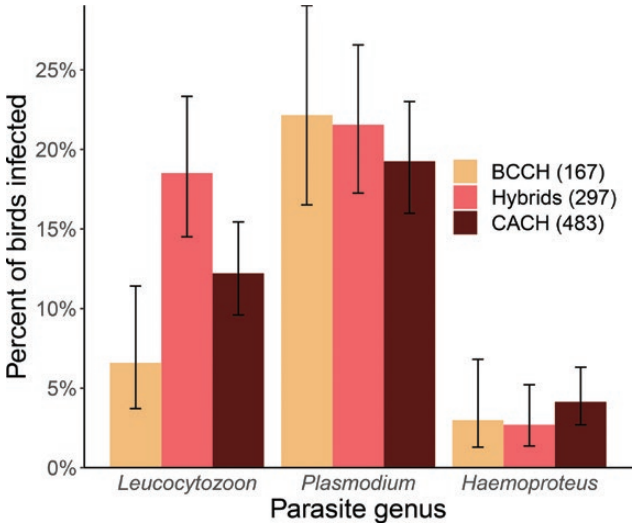

FIGURE 2. Prevalence of haemosporidians among chickadee taxa (95% CI). *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* infections pooled across all sites and seasons. BCCH = Black-capped Chickadee and CACH = Carolina Chickadee. Numbers in parentheses indicate sample sizes.

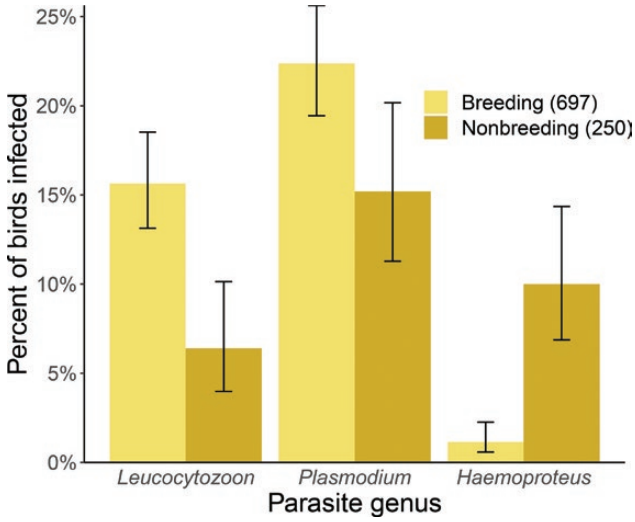

FIGURE 3. Prevalence of haemosporidian infections across seasons (95% CI). Parasites are organized by genus: *Leucocytozoon*, *Plasmodium*, and *Haemoproteus*. Sampling during breeding season occurred from May to June and during the nonbreeding season from October to March. Numbers in parentheses indicate sample sizes.

and especially at GM being much more likely to be infected than younger birds (Figure 4C). However, *Plasmodium* and BAEBIC04 otherwise showed no other tendencies to infect particular groups of birds (Supplementary Material Tables S4 and S5). Finally, season and site predicted *Leucocytozoon* infections through both model-selection approaches (Table 2): *Leucocytozoon* as a whole was least likely to occur during nonbreeding periods and at TU (Figure 4A,B). However, predictors were not consistent among each of the 3 most common *Leucocytozoon* lineages. Site weakly predicted CB1 infections; season best predicted COLBF21 infections; and the combination of site, season, and age predicted DUMCAR02 infections (Tables 2 and 3). Specifically, CB1 and DUMCAR02 were less detectable at TU than other sites; COLBF21 and DUMCAR02 were most likely to occur in May and June; and DUMCAR02 was most likely to occur in older birds. Because age did not predict CB1 and many unknown-age birds coincidentally possessed this lineage, we ran models again without the age effect. With the sample size accordingly increased, site had greater predictive power (Supplementary Material Tables S4 and S5).

Parasite Communities

No haemosporidian lineage with more than 10 occurrences was limited to a single chickadee ancestry category or site (Table 1); those with fewer occurrences were too rare to assess meaningful patterns. Chickadee ancestry categories hosted similar levels of haemosporidian lineage diversity, with BCCH carrying 13 lineages, CACH carrying 15, and hybrids carrying 18. Based on non-metric multidimensional scaling analyses (NMDS), parasite communities were similar across all ancestry categories, but differed slightly between TU and all other sites (Figure 5). This trend reflects the occurrence of fewer lineages (7) at TU than at HM (20), GM (14), or NF (14).

DISCUSSION

This study evaluated the prevalence and lineage richness of haemosporidians across 2 chickadee species that naturally hybridize and is the first to specifically compare haemosporidian communities between BCCH, CACH, and their hybrids. Contrary to studies conducted in other avian contact zones (Reullier et al. 2006, Jones et al. 2018), we found little evidence of chickadee taxa exchanging parasites in a unidirectional manner.

Few Signs of Parasite Exchanges

Though BCCH possessed lower parasite lineage richness than CACH and experienced more *Leucocytozoon* infections in the hybrid zone's center (HM) than its largely CACH-free leading edge (TU), the possibility of *Leucocytozoon* and other parasites "jumping" from CACH to BCCH remains unlikely for several reasons. First, chickadee genotype played no role in predicting haemosporidian prevalence, and if even if it did, models that predicted *Leucocytozoon* showed no signs of an interaction effect between birds' identities (BCCH or CACH) and the sites where they were sampled. Second, the bulk of infections were caused by lineages (BAEBIC04, CB1, COLBF21, DUMCAR02, MAFUS02) that occurred across all sites

TABLE 2. Generalized linear mixed models with $\Delta AIC_c < 2$. *k* is the number of variables and w_i is Akaike weight.

Response variable	Model and interactions[a]	*k*	Dev	ΔAIC_c[b]	w_i
Leucocytozoon	Season: Age + Site	9	596.3	0	0.247
	Season + Site + Age	8	598.53	0.19	0.224
	Season + Site[3]	**7**	**600.7**	**0.33**	**0.21**
	Season: Age + Hybrid index + Site	10	596.17	1.91	0.095
Plasmodium	Site: Age + Season	11	843.99	0	0.249
	Site: Age	**10**	**846.21**	**0.18**	**0.228**
	Site: Age + Season + Hybrid index	12	842.66	0.73	0.173
	Site: Age + Hybrid index	11	844.9	0.91	0.158
	Site: Age + Season:Age	12	843.27	1.33	0.128
Haemoproteus	**Season**	**4**	**242.86**	**0**	**0.359**
	Season + Hybrid index	5	242.44	1.6	0.161
	Season + Age	5	242.58	1.73	0.151
BAEBIC04	Site: Age + Season	11	754.46	0	0.228
	Site: Age	**10**	**756.54**	**0.03**	**0.224**
	Site: Age + Season:Age	12	753.37	0.96	0.141
	Site: Age + Season + Hybrid index	12	753.7	1.29	0.119
	Site: Age + Hybrid index	11	755.79	1.33	0.117
CB1	Site	6	338.84	0	0.224
	Hybrid index	4	344.46	1.58	0.102
	Site + Hybrid index	7	338.5	1.69	0.096
	Site + Season	7	338.62	1.8	0.091
	Site + Age	7	338.79	1.98	0.083
COLBF21	**Season**	**4**	**243.58**	**0**	**0.258**
	Season + Site	7	238.5	0.99	0.157
	Season + Hybrid index	5	243.4	1.84	0.103
	Season + Age	5	243.52	1.96	0.097
DUMCAR02	**Season + Site + Age**	**8**	**182.38**	**0**	**0.335**
	Season + Site + Age + Hybrid index	9	180.95	0.61	0.262
Multiple lineages	**Random effect only**	**3**	**240.42**	**0**	0.268
	Season	4	239.51	1.1	0.155
	Age	4	239.75	1.34	0.137
	Hybrid index	4	240.3	1.89	0.104

[a] Lowest AIC_c for each response variable are as follows: 612.5 (*Leucocytozoon*), 864.2 (*Plasmodium*), 248.9 (*Haemoproteus*), 774.7 (BAEBIC04), 348.9 (CB1), 249.6 (COLBF21), 196.5 (DUMCAR02), and 244.4 (Multiple lineages).
[b] Models shown in bold were also obtained through stepwise model selection as in Cozzarolo et al. (2018).

TABLE 3. Support for fixed effects and interactions in generalized linear mixed models. Numbers are sums of model weight values (see Table 2).

Response variable	Season	H. index	Site	Age	H. index: Site	Age: Season	Age: Site
Leucocytozoon	1	0.29	1	0.7	0.02	0.37	0.04
Plasmodium	0.58	0.37	0.98	0.98	0.03	0.14	0.96
Haemoproteus	1	0.35	0.15	0.4	<0.01	0.13	0.01
BAEBIC04	0.56	0.29	0.87	0.89	0.01	0.15	0.84
CB1	0.33	0.42	0.69	0.33	0.01	0.06	0.03
COLBF21	0.96	0.33	0.42	0.35	0.02	0.1	0.01
DUMCAR02	1	0.43	0.98	0.95	0.03	0.25	0.06
Mixed lineages	0.39	0.29	0.07	0.36	<0.01	0.04	<0.01

and categorical chickadee taxa. Third, chickadee parasite communities showed extensive overlap between BCCH, CACH, and all forms of hybrid individuals. Together, these results suggest that CACH-to-BCCH transfer of *Leucocytozoon* is probably inconsequential.

One likely explanation for the lack of directional exchanges and overall similarity among birds' haemosporidian communities is that these haemosporidians' distributions are shaped by hosts other than chickadees. This may be especially true for widespread,

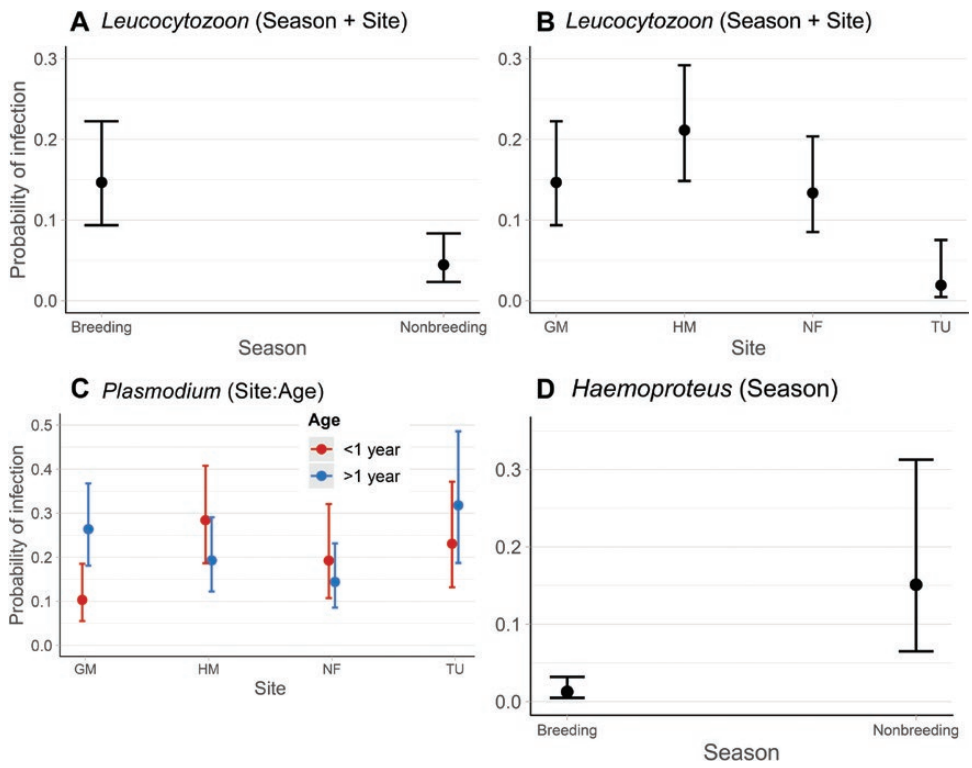

FIGURE 4. Probabilities of infections among haemosporidian genera according to predictor variables from generalized linear mixed models of best fit. Models are shown in the title of each plot, with ":" indicating an interaction between predictors. Predictions were based on least-square means from models ± 95% confidence intervals.

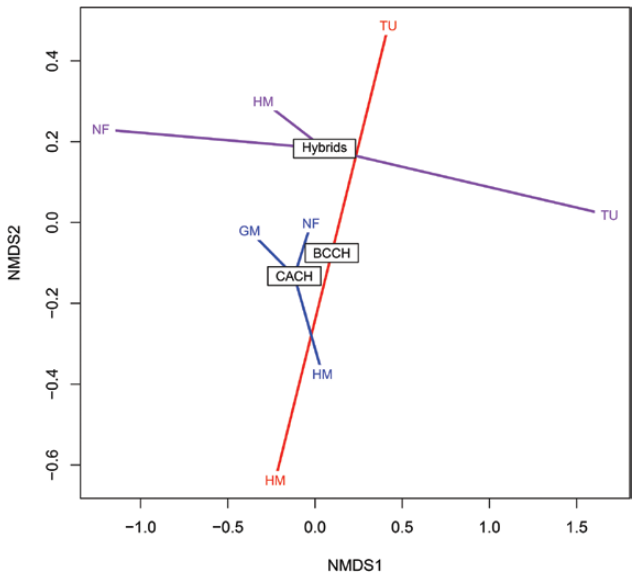

FIGURE 5. Non-metric multidimensional scaling plot depicting haemosporidian community similarity in chickadees. CACH populations are represented in blue, BCCH in red, hybrids in purple.

generalist parasites such as CB1, which is reported in over 40 passerine species (Bensch et al. 2009). However, even BAEBIC04 (which may specialize on Paridae) also infects Tufted Titmice (*Baeolophus bicolor*) (Smith et al. 2018, S. Ray personal communication); titmice may serve as a reservoir within the BCCH × CACH hybrid zone and obscure haemosporidian exchange between chickadee taxa. For future studies, sampling of other passerines across the BCCH × CACH hybrid zone will be crucial for addressing this missing host information and for discerning which lineages (if any) are affected by the presence of BCCH or CACH.

Overall, birds identified as hybrids carried a larger proportion of *Leucocytozoon* infections than either parental species, with BCCH seldom being infected with this genus. However, models suggested that these trends were much more likely a function of where chickadees were sampled and not their genotypic identities: BCCH, CACH, and hybrids showed similar *Leucocytozoon* prevalence at HM. Thus, *Leucocytozoon* in this system may simply be predisposed to occur at certain sites, and its prevalence may reflect vector rather than chickadee communities. For instance, *Leucocytozoon* tends to be more common at high altitudes (Haas et al. 2012, Imura et al. 2012, González et al. 2014, Sehgal 2015, Svoboda et al. 2015), where clean, fast-flowing streams provide ideal habitat for its blackfly vectors (Carlsson 1967). HM contains many such streams, which may ultimately provide better blackfly habitat and translate to locally higher *Leucocytozoon* abundance than at anthropogenically disturbed sites such as TU.

Parasite-Mediated Selection in the Hybrid Zone

Because BCCH and CACH displayed similar rates of infection and did not appear to exchange common haemosporidian lineages, we conclude that haemosporidians as a whole are unlikely to select against certain chickadee taxa and contribute to ongoing hybrid zone movement. Moreover, we found no evidence of hybrid chickadees being any more prone to infection than parental species. However, it remains possible that certain lineages, regardless of prevalence, could confer differential consequences among chickadees. For instance, a "rare" CACH-associated lineage may cause direct mortality in BCCH but elude detection by killing BCCH hosts before they can be sampled. Likewise, even widespread lineages may be associated with subtle, yet differential costs to chickadee survival and reproductive fitness.

Though our study did not investigate the intensity of haemosporidian infections, we believe that measuring haemosporidian parasitemia would be the next logical step in confirming or denying whether these organisms select against certain taxa within the BCCH × CACH system. Like many parasites, haemosporidian lineages can have density-dependent effects within their hosts (Crofton 1971, Valkiūnas 2005). Simply knowing whether BCCH, CACH, or hybrids experience different parasite loads would therefore fill in a crucial knowledge gap that this study was unable to address with presence/absence data.

The costs of haemosporidian infections are well documented in 2 European parids, Great Tit (*Parus major*) (van Rooyen et al. 2013, Dubiec et al. 2017) and Blue Tit (*Cyanistes caeruleus*) (Knowles et al. 2010b, Martínez-De La Puente et al. 2010, Podmokła et al. 2014). These studies have examined breeding, survivorship, and other demographic information for infected birds; have incorporated parasitemia data; and have even involved experimental anti-malarial treatments on birds to quantify the effects of these parasites on avian hosts. For future studies, we therefore encourage similar types of work for understanding the ecological roles of haemosporidians among BCCH, CACH, and hybrid chickadee populations.

Insights on Haemosporidian Phenology

Despite our investigation's primary focus on hybrid zone dynamics, our screening of chickadees at different times of year revealed divergent phenological patterns among haemosporidian genera. Such patterns are worth discussing if sampling birds at a single point in their annual cycle introduces bias and prevents studies from gaining full understanding of their haemosporidian communities. For instance, our study would have vastly underestimated *Haemoproteus* prevalence had we limited our sampling to May–June.

A myriad of outside factors may explain why we rarely detected *Leucocytozoon* from fall/winter samples while *Plasmodium* remained common and *Haemoproteus* detections increased during these periods. In temperate regions where transmission and vector activity are seasonal, haemosporidians such as *Leucocytozoon* may disappear from hosts' bloodstreams (Hasselquist et al. 2007, Szöllősi et al. 2016) and migrate to fixed tissues (Valkiūnas 2005) as transmission periods wind down. Furthermore, *Leucocytozoon* prevalence may peak from May to June because of chronically infected birds undergoing a relapse or "recrudescence" in blood parasitemia that typically coincides with peak vector activity (Beaudoin et al. 1971, Valkiūnas et al. 2004). For the blackflies that transmit *Leucocytozoon* in this region, such activity may also occur in early summer (Adler et al. 1982).

Though *Plasmodium* also displayed the highest prevalence from May to June, members of this genus are known to infect many migratory passerines and experience transmission on breeding and tropical wintering grounds (Hellgren et al. 2007). This could explain why many chickadees carried *Plasmodium* infections through winter. Likewise, the winter presence of *Haemoproteus* may be driven by adaptations geared toward migratory hosts. MAFUS02, which had only been recorded in mimids prior to this study, experiences transmission in the Caribbean (Ricklefs and Fallon 2002) as well as Pennsylvania. However, this does not explain its near absence in chickadees from May to June. One possible cause of this absence may be vectors not peaking until late summer and recrudescence occurring after our May–June sampling window. However, ceratopogonid (biting midge) phenology remains understudied in North America, and data from other Pennsylvania bird communities suggest that MAFUS02 and other *Haemoproteus* are generally more prevalent in spring vs. fall (S. Ray personal communication).

CONCLUSION

Though haemosporidians in this case revealed little about chickadee hybrid zone dynamics, our findings offer several interesting comparisons to studies conducted within other hybrid zones and parid systems. With only 36.6% of birds infected, haemosporidians were not nearly as prevalent as in other Paridae (Martínez-De La Puente et al. 2010, van Rooyen et al. 2013, Dubiec et al. 2017), but were far more lineage-rich than in most parid-focused studies of similar scale (Wood et al. 2007, Cosgrove et al. 2008, Podmokła et al. 2014, Wilkinson et al. 2016). Our findings were similar to those of Cozzarolo et al. (2018) to the extent that local environmental factors appeared to play a larger role in parasite prevalence than birds' genotypic identities. Such results may be a reflection of these North American haemosporidians being unspecialized, which was not the case for the more host-specific haemosporidian lineages found in *Ficedula* and *Hippolais* contact zones (Reullier et al. 2006, Jones et al. 2018). Finally,

our study highlighted the importance of sampling birds for parasites throughout their annual cycles and accounting for parasite lineages that may be absent or undetectable at certain times of the year. Further work on chickadees and other North American hybrid zones should therefore emphasize sampling across seasons, evaluating haemosporidian prevalence in larger bird communities, and investigating the means by which these parasites potentially alter the fitness of their hybridizing hosts.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Ornithology* online.

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Ethics statement: Research was conducted in compliance with IACUC.

Conflicts of interest: The authors declare no conflicts of interest.

Author contributions: All authors conceived the initial project; R.L.C. and A.A.R. conducted fieldwork; A.A.R. collected, compiled, and analyzed parasite data; J.D.W. and R.L.C. supervised lab work and statistical analyses; A.A.R. wrote the manuscript; R.L.C. and J.D.W. edited the manuscript.

Data depository: All haemosporidian cyt *b* DNA sequences reported in this article have been deposited in both GenBank (MW876523–MW876860; MW892840–MW892842) and Malavi (http://130.235.244.92/Malavi/).

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