AVIAN MALARIA AND BODY CONDITION

IN FOUR SPECIES OF SONGBIRDS

by

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Introduction

Protozoan blood parasites are common in songbirds throughout the world (Garvin et al. 2004). Recent attention has been brought to those causing avian malaria, as they are excellent research models for host-parasite interactions (Ricklefs and Sheldon 2007) due to their relatively easy screening methods involving microscopy and polymerase chain reaction (PCR) (Valkiunas 1993; Bensch et al. 2009). The primary genus associated with avian malaria is *Plasmodium*; however, two closely related genera, *Haemoproteus* and *Leucocytozoon,* have also been associated with this disease (Hellgren et al. 2004). Even though phylogenetically similar, these genera differ in their life history, and especially in their transmission vectors. Parasites in the genus *Plasmodium* are transmitted through mosquitoes in the family Culicidae, Leucocytozooans are transmitted through black flies from the family Simulidae, and Haemoprotozoans are transmitted by sand and louse flies belonging to the families Ceratopogonidae and Hippoboscidae, respectively (Atkinson and van Riper III 1991).

Avian malaria infections tend to be mild and at low-levels; however, studies have shown that some infections can negatively impact bird survival (Sol et al. 2003), reproductive success (Merino et al. 2000), and body condition (Dawson and Bortolotti 2000; Hatchwell et al. 2001). The avian form of malaria is the most diverse among all animals, with approximately 40 species of *Plasmodium*, 130 of *Haemoproteus,* and 35 of *Leucocytozoon* that can infect birds (Valkiunas 2005). Studies have found that these parasites can affect bird species differently, causing varying degrees of severity, with *Plasmodium* and

Leucocytozoon being more pathogenic than *Haemoproteus* (Bennett et al. 1993; Palinauskas et al. 2008). There is also evidence of certain bird hosts being more susceptible to malaria than others, and even within a species, intrinsic factors such as age and sex can influence the success of an infection (Ots and Horak 1998). Songbirds become infected during the breeding season, as these are also optimal conditions for biting flies and mosquitoes, but can carry infections into the winter and even experience recurring infections over multiple years (Garvin et al. 2004).

The breeding season is known to be a period of increased physical demand due to the various activities associated with it, and birds may be facing a trade-off between their own survival and well-being, and that of their offspring (Sheldon and Verhulst 1996; Zuk and Stoehr 2002). This stressful time can cause body condition to be compromised, which could then result in the suppression of immune function (Gonzalez et al. 1999; Norris and Evans 2000). This occurrence has been studied in barn swallows (*Hirundo rustica*) and great tits (*Parus major*), in which brood size was experimentally increased, causing reduced cellular immunity and a drop in white blood cell counts (Horak et al. 1998a; Pap and Markus 2003). Immuno-compromised birds could then be at a higher risk of getting infected, and not being able to fight off the infection (Gustafsson et al. 1994).

Even within the breeding season, certain groups may be at a higher risk of parasite infection due to increased stress. Various studies have shown that male and female birds can differ in stress levels during the reproductive season. This could be due to inherent variation caused by physiology and behavior (Horak et al. 1998b; Ots et al. 1998), or

because one sex invests more time and energy in reproduction than the other (Sanz et al. 2000; Visser and Lessells 2001). In most species of songbirds, females tend to have a higher work load than males during the breeding season, creating higher stress levels and ultimately making them more vulnerable to parasite infections during this critical time. These differences in stress may also exist between adults and juvenile birds, making juveniles more vulnerable to infection due to their inexperience at foraging and escaping predators (Woodrey and Moore 1997).

In this study, four species of songbirds were examined for blood parasites during the reproductive season; the American redstart (*Setophaga ruticilla*), the gray catbird (*Dumetella carolinensis*), the cedar waxwing (*Bombycilla cedrorum*), and the red-eyed vireo (*Vireo olivaceus*). In all four species, the female builds the nest and incubates the eggs by herself, while the male only shares the responsibility of feeding the offspring after they hatch (Putnam 1949; Bent 1953; Lawrence 1953; Zimmerman 1963). Nevertheless, waxwings and catbirds are known to breed twice every season, and the male takes full responsibility of feeding fledglings from the first clutch while the female builds the second nest and incubates the next clutch (Putnam 1949; Zimmerman 1963). These differences in work load during the breeding season may result in gender specific differences in stress and vulnerability to infection.

The first objective of this study was to determine parasite prevalence in these four species, and whether a particular genus of parasite was more or less prevalent in each. We predicted these birds would be infected based on previous studies done around the study

area showing prevalence of all three genera of parasites in passerines as well as falcons and hawks (Powers et al. 1994; Tarof et al. 1997; Castelluci et al. 1998). In addition, Pennsylvania has such a large problem with black flies every year (which transmit *Leucocytozooans*) that they have a "Black Fly Suppression Program" (Bureau of Point and Non-Point Source Management 2015).

The second objective was to evaluate whether parasite infection was related to variation in body condition, measured by body mass (corrected for body size), hematocrit levels, and heterophil to lymphocyte ratio (H/L). We expected to find infected birds in poor body condition (lower body mass, lower hematocrit, and higher H/L ratio), while uninfected birds would be in a better health state (higher mass, higher hematocrit, and lower H/L ratio).

The final objective was to determine if intrinsic factors, such as age and gender, were related to parasite infection, and whether a particular group within a species was more vulnerable than others. We expected that female redstarts and vireos would be more stressed than males during the breeding season, and thus in poor body condition and more prone to infection. We also expected gender not to affect body condition or parasite infection in waxwings and catbirds. Juveniles from all species were expected to be in worse body condition than adults due to their inexperience.

Methods

Study Location. – Samples were collected at Powdermill Avian Research Center (PARC) in southwestern Pennsylvania (40° 09' N, 79° 16' W), a banding station running year-long since 1961. The area is surrounded by mixed deciduous forest to the east and low intensity agricultural areas to the west, with nets being placed along old fields and marshy ponds. Birds were captured from May 23 to July 10, 2014 on 26 sampling occasions, for a total of 406 birds from the four targeted species (including 16 recaptures).

Bird species. – Species were chosen based on their abundance at the study site, as well as due to similarities and differences in life history. The focal species are Neotropical migrants, breeding throughout the northern US and Canada, mostly from mid-May through July (Cimprich et al. 2000; Smith et al. 2011), although redstarts tend to have a shorter breeding period in June (Sherry and Holmes 1997), and waxwings are known for being late breeders, not starting until mid-June and running through August (Witmer et al. 2014). However, these species differ in their breeding strategies.

The American redstart is a small warbler that exhibits sex-specific roles during the breeding season; females build the nest (Sturm 1945), incubate, and shade the chicks on their own, whereas the male only shares responsibility of feeding the chicks once they hatch (Bent 1953). The female is so committed to her offspring that studies have seen females shading an empty nest after experimental removal of chicks (Bent 1953).

In the red-eyed vireo, the female builds the nest on her own, but the male may assist by gathering materials or by feeding her during nest construction (Barlow and Rice

1977). Only the female has a vascularized brood patch, but the male may develop a partial one (Pyle 1997). Still, the female incubates the eggs and shades the chicks by herself (Lawrence 1953; Southern 1958). Even though both parents are known to feed the young, the female tends to feed more often (Lawrence 1953; Southern 1958).

Gray catbird males appear to be more involved in reproduction than males in the previous two species. The male may contribute to nest construction by bringing materials (Whittle 1923; Zimmerman 1963). He will also occasionally feed the female during incubation (Whittle 1923; Zimmerman 1963) and guard the nest during this period (Slack 1976). The male may also develop a partial brood patch (Putnam 1949; Pyle et al. 1987) and may help with shading the chicks (Putnam 1949), but most importantly, the male takes full responsibility for feeding the chicks after the female starts construction of the second nest (Zimmerman 1963).

Cedar waxwings seem to behave like most songbirds in that females do most of the nest construction, incubation, and brooding; however, the male may do more construction of the second nest while the female feeds the first brood, or he may take full responsibility for feeding the first clutch while the female builds the second nest (Putnam 1949).

Morphometrics. – All morphometric measurements were conducted by experienced banders at PARC. Birds were aged and sexed according to plumage variation, when possible, following Pyle (1997). Age was classified as local (L), hatch year (HY), after hatch year (AHY), second year (SY), and after second year (ASY). Birds born during the breeding season in 2014 were considered HY (unless the sheath was still visible around the wing feathers, in

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which case they were considered L). Birds born during the breeding season in 2013 were SY, and when precise aging was not possible, birds were classified as AHY or ASY. Measurements were taken for wing cord and mass.

Blood sampling. – Blood samples were collected by puncturing the brachial vein using a 27-1/2-gauge needle and a 50 µL heparinized capillary tube. Two thin blood smears were made immediately on glass slides and a drop of whole blood was stored in lysis buffer for parasite amplification and sexing purposes, as three of the four study species were sexually monochromatic.

Sexing. – Whole blood samples were extracted using a standard isopropanol/ammonium acetate precipitation (Williams et al. 2012). Males and females were discriminated by amplifying a specific intron in the CHD-W gene, located on the W chromosome, and thus only found in females, and an intron in the CHD-Z gene, located on the Z chromosome, and occurring in both sexes. The procedure used was a variation of Griffiths et al. (1998), which uses the P8 (5'-CTCCCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATCGCTAAATCCTTT-3') primers. PCR reactions were run in a total 10 µL volume containing 1 µL of template DNA, 0.5 µM of each primer, 1 µL buffer with 2.5 mM $MgCl_2$ pH 8.7, 1 µL dNTPs, and 0.2 U Taq DNA polymerase. Amplifications were run on an ABI 2720 thermal cycler using the following cycling conditions: an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, followed by a final elongation at 72 °C for 5 min. The samples were then run on a 2% agarose gel for 50

min. The presence of two bands was scored as female, whereas one band was scored as male.

For cedar waxwings, the size difference between the two introns was very small and could not be resolved well on agarose gels. Instead, a primer set that had P8 labelled with a fluorescent dye (6-FAM) was used for this species. PCR products were diluted with 100 μL dH2O, and 0.5 μL of each diluted PCR product was added to 10 μL of Hi-Di Formamide and 0.1 μL of LIZ 500 size standard. Samples were then resolved on an ABI 3130XL Genetic Analyzer (Applied Biosystems). The presence of two peaks was scored as female whereas one peak was scored as male.

Body condition. – Bird body condition was determined by measures of body mass (corrected for body size), hematocrit levels, and H/L ratio.

Hematocrit. – Blood samples in capillaries were centrifuged for 10 min at 6,000 r.p.m. after which hematocrit was measured to the nearest 0.5 mm using a metal ruler. The capillaries were then snapped and the plasma was transferred to a 1.5 mL microcentrifuge tube and stored at - 20 °C. Hematocrit measures the relative volume of red blood cells in whole blood, and reflects the oxygen carrying capacity of blood. Low levels (anemia) are associated with disease and parasite infection as well as mineral deficiencies (Svensson and Merila 1996), while high levels may reflect increased physical demands (Saino et al. 1997).

Leukocyte profile. – Two slides were prepared for each individual sample, but only the better quality one was chosen for leukocyte counts. Slides were air-dried and fixed with methanol on the same day of collection, and later stained with Giemsa (pH 7.2). The slides

were examined under 1000x oil immersion and 100 leukocytes were counted per slide, and classified as lymphocytes, heterophils, eosinophils, monocytes, and basophils following Campbell and Ellis' "Hematology of Birds" (2007). Three people were responsible for the leukocyte counts, but each focused on a different bird species. During the leukocyte counts, the presence or absence of hemoparasites was recorded.

In passerines, lymphocytes (60-73%) and heterophils (7–20%) are the most common leucocyte types, with monocytes, eosinophils, and basophils being rare (Davis et al. 2008). Lymphocytes are involved in adaptive immunity, heterophils and monocytes are phagocytic cells, eosinophils respond to parasite infections, and basophils are involved in response to inflammation (Davis et al. 2008).

Counts of heterophils and lymphocytes from blood smears were used to calculate the H/L ratio, which is a common measure of stress in birds, as changes in the proportion of heterophils to lymphocytes are believed to be caused by changes in baseline corticosterone levels, the main stress hormone in birds (Horak et al. 1998b). This H/L ratio is a more reliable measure of stress than measuring hormone levels, as the latter is known to change rapidly following a stressor, whereas leucocyte numbers remain constant for up to one hour after capture (Cirule et al. 2012). High H/L ratios have been associated with susceptibility to infection (Al-Murrani et al. 2006), slow growth rates (Moreno et al. 2002), and survival to the next breeding season (Lobato et al. 2005; Kilgas et al. 2006b). Furthermore, a high H/L ratio is typically caused by increased levels of heterophils, non-specific phagocytic cells, whose constant lysis may be harmful to host tissues (Davis et al. 2008).

Parasite amplification. – Parasite detection using PCR was conducted following the procedure by Fallon et al. (2003). Primers 343F (5'-GCTCACGCATCGCTTCT-3') and 496R (5'-GACCGGTCATTTTCTTTG-3') amplify a 286 base pair (bp) fragment of ribosomal RNA within the mitochondrial DNA malaria genome, allowing testing for the presence of parasite lineages in the genera *Plasmodium* and *Haemoproteus*. Reactions were run in a total 10 µL volume containing 1 µL of template DNA, 0.5 µM of each primer, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer with 3 mM MgCl$_2$ pH 8.7, and dNTPs. The cycling protocol used was the following: 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 90 s, and 72 °C for 60 s, with a final elongation step at 60 °C for 30 min. Samples were run on a 1% agarose gel for 25 min.

Parasite infection was confirmed by using a second set of primers: 213F (5'-GAGCTATGACGCTATCGA-3') and 372R (5'-GGAATGAGAGTTCACCGTTAT-3'), following the procedure by Beadell and Fleischer (2005). These primers amplify a 160 bp fragment of mitochondrial DNA present in all three parasite genera (*Plasmodium, Haemoproteus,* and *Leucocytozoon*). The PCR mix was the same as the one used for the previous set of primers, as well as the cycling conditions, except for the use of 40 cycles instead of 30, and an annealing temperature of 50 °C instead of 60 °C. After PCR, 5 µL of the product was digested with XmnI (unique to *Haemoproteus*) and XbaI (unique to *Leucocytozoon*). A total volume of 10 µL contained 1 U of restriction enzyme, 0.1 mg/mL of bovine serum albumin (BSA), and 1 X NEBuffer 2, and was incubated for 3 hours at 37 °C. The digested products were run on a 2% agarose gel for 30 min. A single 160 bp fragment was classified as

Plasmodium, a smaller 121 bp fragment (cut by XmnI) was classified as *Haemoproteus*, and an even smaller 109 bp fragment (cut by XbaI) was classified as *Leucocytozoon*.

We cloned the 213F/372R PCR products for six individuals, using the pGEM-T Easy Vector System (Promega) in order to confirm parasite lineages. We sequenced eight clones for each sample, using vector-specific primers pGEMF (5'-CGACTCACTATAGGGCGAATTG-3') and pGEMR (5'-CTCAAGCTATGCATCCAACG-3'). Conditions for PCR (20 µL) of vector inserts contained 0.5 µM primers , 2 µl dNTPs, 2 µL buffer with 15 mM MgCl$_2$, and 0.4 U Taq DNA polymerase. Cycling conditions were the following: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 15 s, and 72 °C for 60 s, with a final elongation step at 72 °C for 5 min. Samples were analyzed with ABI Big Dye Terminator Cycle Sequencing v3.1 Chemistry (Applied Biosystems) in an ABI 3130XL Genetic Analyzer (Applied Biosystems). Sequences were trimmed, edited and contiged using Sequencher v 4.8 (Genes Codes Corporation), then aligned with known sequences of the three genera of parasites downloaded from GenBank.

Statistical analysis – All data were analyzed in MINITAB 17. Fisher's exact test was used to detect differences in the proportions of single and mixed infections for each bird species, as well as to compare parasite infection between migrating and breeding waxwings. Pearson's chi-square (χ^2) was used to test for heterogeneity in the prevalence of single and mixed infections between species, as well as to compare parasite prevalence by each genus in every bird species. A two-sample t-test was used to examine H/L ratios within infected vireos, and to compare mass, hematocrit, and H/L ratios between migrating and

breeding waxwings. General linear models (GLMs) were used to determine if measures of body condition (mass, hematocrit, H/L ratios) were associated with age, sex, and infection status. Date was used as a covariate only when a significant correlation was found against a particular variable (hematocrit in all species, as well as H/L ratio in waxwings). Interaction terms were not included, as none of them were significant at $\alpha = 0.05$. Unless specified, all tests were run using infection by microscopy, as infection by PCR was too high to use for statistical tests.

Data were tested for normality and transformed when needed to meet the assumptions of the tests. Mass data for catbirds and H/L ratios for all species were log transformed. Data that failed to meet the assumptions even after transformation were still used, as departures from normality may only have a slight effect on the Type I error rate if samples sizes are large, following the central limit theorem (Zar 2010). To assess the repeatability of H/L ratios, 8% of the slides were counted twice. A paired T-test revealed no significant difference between counts (T = -0.06, P = 0.950). When recaptures were available, only one sample from each bird was used to avoid pseudoreplication: the first bird captured was used, unless the recapture had more body condition data, in which case the second sample was used.

Results

Blood samples were collected from 406 individuals belonging to the four focal species. Sample size was highest in waxwings, due to a great influx of migratory birds in May, whereas sample size was lowest for redstarts. No juvenile samples were available from vireos and waxwings (Table 1).

Table 1. Total sample sizes by bird species, sex, and age collected by mist-nets at Powdermill Avian Research Center from May to July 2014.

	Adults				Juveniles			
	Female	Male	Unknown	Total	Female	Male	Unknown	Total
Redstarts	15	31	0	46	5	8	3	16
Vireos	26	36	3	65	--	--	--	--
Catbirds	24	47	3	74	5	9	0	14
Waxwings	63	98	1	161	--	--	--	--

Parasite infection

Most individuals (94%) were infected by one or more of the three genera of parasites as indicated by PCR tests. The highest prevalence of infected individuals was found in vireos (100%), followed by waxwings (95%), redstarts (94%), and catbirds (88%; Table 2). Samples from these individuals were also examined for parasites using microscopy; however, this technique was only able to identify approximately one third of the total infected individuals (37% infection), and detection was found to vary by species. Microscopy was able to detect a large number of infections in vireos (79%), but low numbers in redstarts (6%), catbirds (30%), and waxwings (26%; Table 2).

Table 2. Number of infected individuals per species detected by PCR and microscopy.

	Infected	Not infected	Total	Percent infected by PCR	Percent infected by microscopy
Redstarts	15	1	16	94	6
Vireos	62	0	62	100	79
Catbirds	79	11	90	88	30
Waxwings	151	8	159	95	26
Total	307	20	327	94	37

Infections detected by PCR were then classified as single or mixed, single if the individual tested positive for only one parasite genus, and mixed if the individual tested positive for more than one parasite genera (Table 3). Two species, vireos and waxwings, had significantly higher numbers of mixed infections compared to single infections (Fisher's exact test; P < 0.001 for both). Redstarts tended to have more mixed than single infections, but sample size was low, and numbers for single and mixed infections in catbirds were similar (Table 3). When single/mixed infections were compared among all species, vireos showed fewer single infections than expected and higher mixed infections than expected, whereas catbirds showed the opposite trend, with fewer mixed and higher single infections than expected (χ^2 = 29.4, df = 3, P < 0.001). Redstarts and waxwings showed almost no deviation from expected values (parasite-free individuals were excluded from this test).

Table 3. Number of individuals per species with single or mixed infections detected by PCR.

	Single infection	Mixed infection	Total
Redstarts	5	10	15
Vireos	7*	55*	62
Catbirds	42	37	79
Waxwings	56*	95*	151
Total	110	197	307

* Significant at P < 0.05.

Within infected birds, detected by PCR, we then determined how often each

parasite genus was present in each species. Chi-square tests revealed that vireos and

catbirds had a greater proportion of *Haemoproteus* infections than expected, and a lower

proportion of *Leucocytozoon* infections than expected (Table 4). Waxwings showed the

opposite trend, with a lower proportion of *Haemoproteus* infections than expected, and a

higher proportion of *Leucocytozoon* infections than expected. Redstarts did not show much

deviation from expected values (χ^2 = 96.58, df = 6, P < 0.001; Table 4).

Table 4. Chi-square table comparing infections by each parasite genus in every bird species.

Genus	American redstart			Red-eyed vireo			Gray catbird			Cedar waxwing		
	O	E	χ^2	O	E	χ^2	O	E	χ^2	O	E	χ^2
Haemoproteus	7	6.89	0.00	53	30.3	17.0	50	28.9	15.3	14	57.9	33.3
Plasmodium	10	15.2	1.76	59	66.7	0.90	57	63.7	0.71	147	127	3.02
Leucocytozoon	13	7.94	3.22	20	35.0	6.40	19	33.4	6.19	91	66.7	8.82

O = observed, E = expected, χ^2 = contribution to the chi-square. Infections determined by
PCR.

Body condition

We checked for cross-correlations between measures of body condition for all species.

Only one significant correlation was found between hematocrit and mass in catbirds (r = -

0.312, P = 0.008), in which mass decreased with increasing hematocrit. General linear

models for different indices of body condition revealed that there was no significant

association between parasite infection and mass, hematocrit, or H/L ratios (P > 0.05; Table

5). This test was not performed in redstarts due to small sample size.

Table 5. GLM testing parasite infection in relation to body mass, hematocrit, and H/L ratio in vireos, catbirds, and waxwings.

	Red-eyed vireo			Gray catbird			Cedar waxwing		
Factor	DF	F	P	DF	F	P	DF	F	P
Mass	1,55	0.23	0.631	1,69	2.50	0.118	1,130	2.21	0.140
Hematocrit	1,47	0.98	0.326	1,69	0.41	0.525	1,125	0.06	0.802
H/L ratio	1,58	1.48	0.229	1,77	1.93	0.169	1,128	0.84	0.360

Sex and age differences in body condition

Infection. – Parasite infection did not differ by sex (Table 6) or age (Table 7) in any of the bird species (Fisher's exact test; P > 0.05).

Table 6. Infected individuals by sex in each of the bird species.

	Female				Male			
	Infected	Not infected	Total	Percent infected	Infected	Not infected	Total	Percent infected
Redstarts	0	7	7	0	1	7	8	13
Vireos	23	3	26	88	25	10	35	71
Catbirds	9	19	28	32	16	37	53	30
Waxwings	17	37	54	31	18	62	80	23

Table 7. Infected individuals by age in redstarts and catbirds.

	Adult				Juvenile			
	Infected	Not infected	Total	Percent infected	Infected	Not infected	Total	Percent infected
Redstarts	1	2	3	33	0	12	12	0
Catbirds	21	46	67	31	4	10	14	29

Mass. – Significant sexual dimorphism was found in three species, with females being heavier than males in vireos, catbirds, and waxwings, but not redstarts (Table 8). Juveniles were heavier than adults in redstarts, but there was no difference by age in catbirds (Table 8).

Table 8. GLM testing body mass by age, and sex in all four species of birds.

Factor	American redstart $F_{1,53}$	P	Red-eyed vireo $F_{1,55}$	P	Gray catbird $F_{1,69}$	P	Cedar waxwing $F_{1,130}$	P
Age	7.36	0.009*	--	--	0.01	0.927	--	--
Sex	0.74	0.394	8.55	0.005*	45.0	0.000*	28.5	0.000*

* Significant at $P < 0.05$.

Hematocrit. – No significant differences were found in hematocrit numbers by age or sex in any of the species; however, hematocrit levels significantly decreased over time in all species (Table 9).

Table 9. GLM testing hematocrit by date, age, and sex in all four species of birds.

Factor	American redstart $F_{1,39}$	P	Red-eyed vireo $F_{1,47}$	P	Gray catbird $F_{1,69}$	P	Cedar waxwing $F_{1,125}$	P
Date	5.49	0.024*	14.1	0.000*	12.4	0.001*	16.5	0.000*
Age	0.77	0.385	--	--	0.68	0.411	--	--
Sex	0.95	0.335	0.10	0.754	0.13	0.720	0.20	0.654

* Significant at $P < 0.05$.

H/L ratio. – No significant differences by sex or age were found in H/L ratios in any of the species (Table 10); however, within infected vireos, females had higher H/L ratios than males (T_{46} = 2.16, P = 0.036; Fig.1). In waxwings, H/L ratio decreased significantly with time (Table 10).

Table 10. GLM testing H/L ratio by date, age, and sex in all four species of birds.

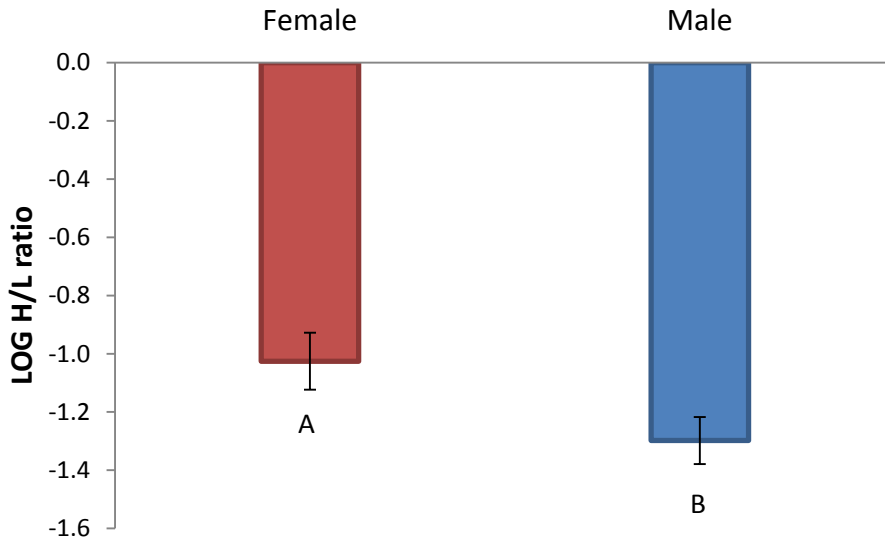| Factor	American redstart $F_{1,12}$	P	Red-eyed vireo $F_{1,58}$	P	Gray catbird $F_{1,77}$	P	Cedar waxwing $F_{1,128}$	P
Date	--	--	--	--	--	--	4.84	0.030*
Age	0.06	0.817	--	--	2.94	0.091	--	--
Sex	0.66	0.432	2.49	0.120	0.00	0.982	0.91	0.342

* Significant at $P < 0.05$.

Figure 1. Mean H/L ratios (log transformed) by sex for infected red-eyed vireos. Bars with different letters differ significantly at P < 0.05. Error bars represent the standard error of the mean.

Time. – A time component was found in waxwings, in which birds captured later in the season (breeding) had significantly higher infection prevalence (38%) than birds captured earlier in the season (migrating; 20%; Fisher's Exact Test, P = 0.031; Fig.2). Breeding birds also had significantly lower mass, lower hematocrit, and lower H/L ratio than migrating birds (Table 11; Fig.3).

Table 11. Comparison of mean mass, hematocrit, and H/L ratio between migrating (sampled in May) and breeding (sampled in June-July) waxwings.

| | Migrating | | Breeding | | | | |
	N	Mean ± SE	N	Mean ± SE	DF	T	P
Mass/Wing length	103	0.348 ± 0.003	58	0.3342 ± 0.004	159	2.98	0.003*
Hematocrit	101	54.38 ± 0.440	53	51.42 ± 0.520	152	4.13	0.000*
LOG H/L ratio	84	0.292 ± 0.046	48	0.438 ± 0.042	130	2.14	0.034*

SE = standard error of the mean. * Significant at P < 0.05.
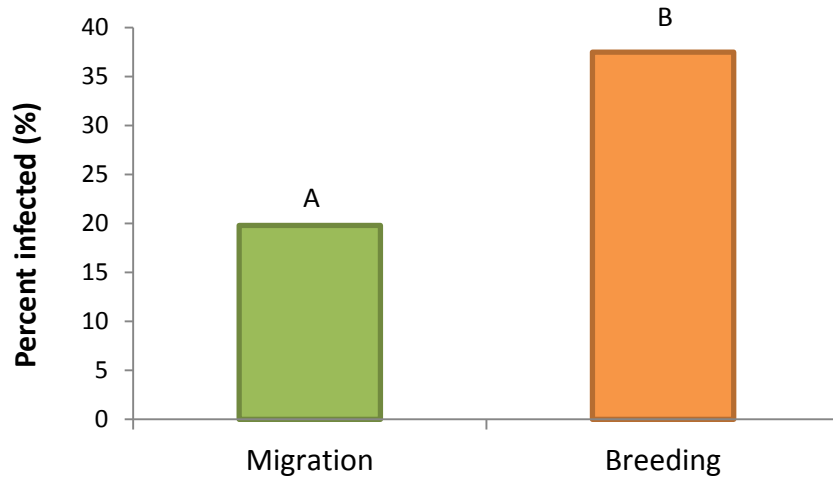
Figure 2. Percent of waxwings infected during migration and breeding. Bars with different letters differ significantly at P < 0.05.
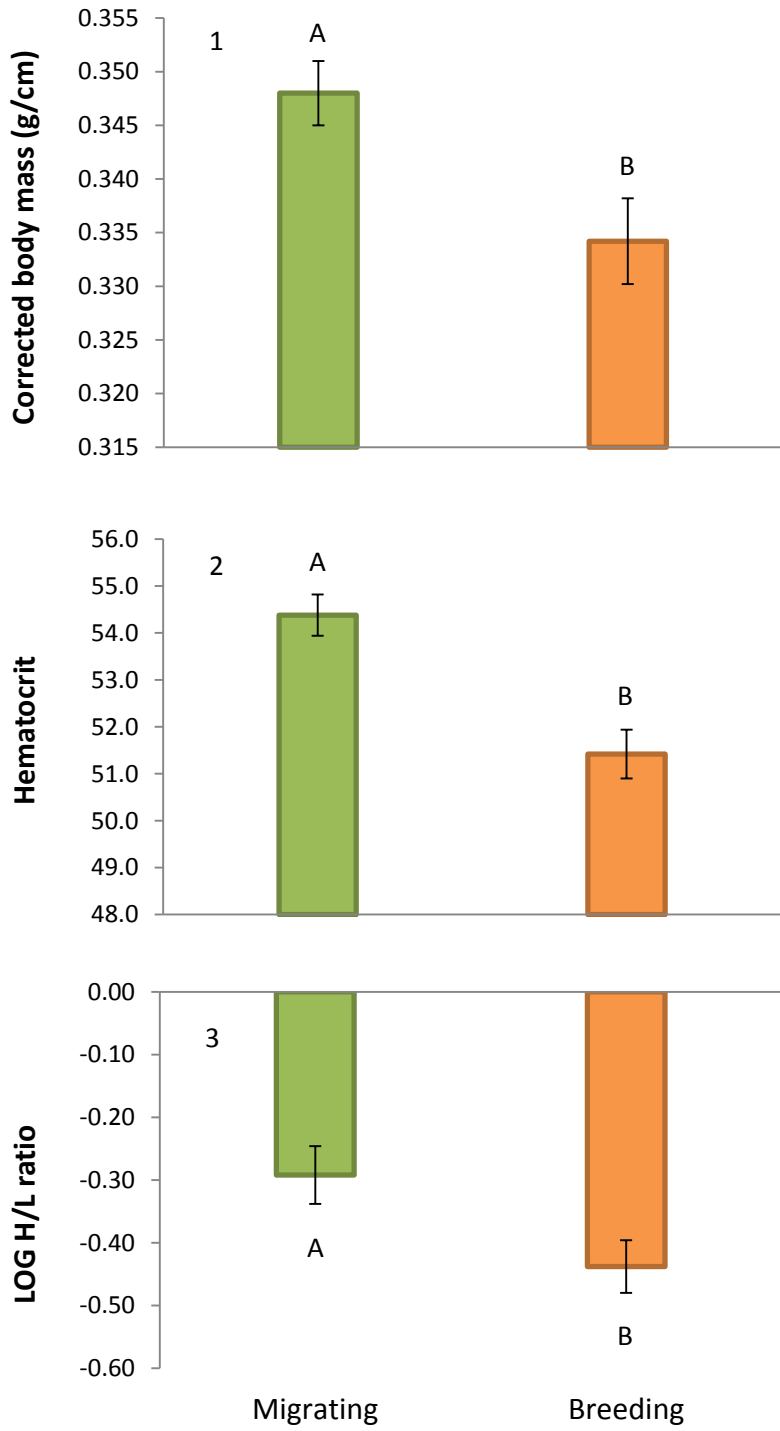
Figure 3. (1) Body mass corrected for wing length; (2) hematocrit; and (3) LOG H/L ratio in migrating and breeding cedar waxwings. Error bars represent standard error of the mean. Bars with different letters differ significantly at P < 0.05.

Discussion

Parasite infection

Most of the current studies on avian malaria have focused on optimizing parasite prevalence detection through the use of PCR techniques, rather than the less sensitive and time consuming microscopy (Fallon et al. 2003; Valkiunas et al. 2008). This PCR technique certainly allows for a more accurate estimate of parasite prevalence within a population; however, given the large incidence of malarial infection, it might be of greater biological importance to focus on parasitemia, the level of infection, rather than prevalence, the presence of infection (Bentz et al. 2006; Ricklefs and Sheldon 2007). Studies have shown that infections detected by microscopy have higher parasitemia than infections detected by PCR alone, thus emphasizing the importance of microscopy data (Bentz et al. 2006).

This study used both techniques, and found a 94% prevalence using PCR, ranging from 88 – 100%, and a 37% prevalence using microscopy, ranging from 6 – 79% across the four species. The high levels found by PCR are not surprising, as avian malaria is a common disease in songbirds, widespread and ubiquitous throughout the world. Previous studies have shown a high prevalence of these parasites in breeding Siberian tits (*Poecile cintus*) in which 80% were infected (Krams et al. 2010), and European blackbirds (*Turdus merula)* in which 97% were infected (Bentz et al. 2006). Microscopy detected approximately one third of total infections, which suggests that most birds are infected with avian malaria, but the majority of these infections are at low levels. This is consistent with a previous study of 11 species of birds over three continents, which found that 80% of the infections were at very low levels (less than 0.01% of infected erythrocytes) when counting the number of parasites

per 10,000 erythrocytes (Valkiunas et al. 2008). These low infections can be classified as chronic, whereas high level ones are considered to be acute infections (Cornelius et al. 2014). We also found differences in numbers of acute infections by species; microscopy only detected 6%, 26% and 30% of infections in redstarts, waxwings and catbirds, respectively, whereas it detected 79% of infections in vireos. This finding is not unusual, as we know that parasitemia can vary markedly within and between different bird species, even with infection by the same parasite lineage (Palinauskas et al. 2008).

We also found that vireos and waxwings had significantly more mixed than single infections, whereas catbirds showed the opposite trend, although not significant. Mixed infections are known to be common, with Valkiunas et al. (2003) recording over 80% of infections as mixed in some bird species. Studies have discussed the possibility that multiple parasite species could be interacting with each other causing different effects on host fitness, but more studies are needed on the interaction of different parasite genera (Richie 1988). Based on PCR data, these four species show some variability in their infection profiles, with vireos and catbirds being infected mostly by *Haemoproteus* and *Plasmodium*, redstarts showing similar numbers of all three genera, and waxwings being infected mostly by *Plasmodium* and *Leucocytozoon*. Since *Plasmodium* and *Leucocytozoon* are believed to be more pathogenic than *Haemoproteus* (Bennett et al. 1993; Palinauskas et al. 2008), we would predict redstarts and waxwings to be in worse body condition; however, redstart samples were composed mostly of juveniles, who may not have yet developed immunity against any of the parasite genera (Hunter et al. 1997; Sol et al. 2003).

Body Condition

Avian malaria is a serious condition which can cause changes in an organism, either at the molecular or the whole organism level. Infections can cause variations in body condition, which could be detrimental for the individual if the parasite is pathogenic, or which could go undetected in light of normal daily variations (Ots et al. 1998). Previous studies have come to different conclusions on the matter of avian malaria; some of them are able to find a significant effect of infection on measures of body condition (Norte et al. 2009; Cloutier et al. 2011; Table 12), whereas others fail to find an association (Krams et al. 2010, 2013; Knutie et al. 2013; Table 12). Most of these studies are conducted during the breeding season, when parasite abundance is expected to be at its highest level, and many of them include measures of body mass; however, fewer studies actually test hematological variables like hematocrit and H/L ratios (Ots et al. 1998; Krams et al. 2010, 2013; Table 12). Even within these studies, there does not appear to be a clear pattern, as a study by Norte et al. (2009) found high variation in H/L ratios within the same population of great tits (*Parus major*; Table 12).

In this study, we were not able to detect an association between infection and body condition due to three main reasons. First, chronic infections like the ones we found in this study may not be pathogenic enough to elicit a strong immune response by the host, and perhaps do not affect bird health at low levels (Cornelius et al. 2014). Second, this study classified parasite infections down to the genus level, but if pathogenicity is variable within a genus, then we may not have been able to detect these differences (Bennett et al. 1993). Finally, some host species may be more sensitive to infection than others, and the species

chosen for this study may have evolved along with these parasites, becoming resistant to them (Palinauskas et al. 2008). Furthermore, this study measured three hematological variables, but parasite infection can have detrimental effects on other blood variables such as hemoglobin (Krams et al. 2013) and leucocyte count (Cornelius et al. 2014; Dunn et al. 2013), as well as measures of reproductive success (Norte et al. 2009).

Sex and age differences in body condition

The last objective of this study aimed to test whether factors such as age or sex were associated with parasite infection and body condition, to determine whether a particular group was more vulnerable to infection than others. We predicted that female redstarts and vireos would be more stressed by the breeding season and thus have lower mass, lower hematocrit, higher H/L ratios, and higher parasite prevalence than males; whereas we believed all measures of body condition and parasite prevalence would be similar between male and female catbirds and waxwings. We also predicted that juveniles would be more stressed than adults due to their inexperience at foraging and escaping predators, and would thus have lower mass, lower hematocrit, higher H/L ratios and higher infection prevalence than adults. We found no difference in infection prevalence by age or sex in any of the species, perhaps because chronic infections like the ones we found are distributed equally throughout the population (Norte et al. 2009).

For body mass, we found that females were heavier than males in all species except redstarts. It is common for females to be heavier than males during the breeding season in catbirds (Lent 1990) and waxwings (Clench and Leberman 1978). Vireos usually show no

sexual difference in terms of mass, even during the breeding season, however most of the female vireos caught during this study had a very vascularized brood patch, meaning that they were ready to begin incubation, a process associated with mass gain due to egg development (Kullberg et al. 2001; Coulson et al. 2010). We did not see this trend in redstarts since most of the individuals sampled were juveniles and not involved in breeding; however, we did find that juveniles were heavier than adults in redstarts. This trend is common as fledglings have similar mass, but smaller wing measurements than adults at this time (Sherry et al. 1997), and we corrected mass by wing length, giving us higher values for juveniles.

We did not find an effect of sex or age on hematocrit levels; nevertheless, we did find that hematocrit decreased over time in all four species. A decrease in hematocrit with time is typically associated with parasite infection, as parasite vectors increase dramatically from spring to summer (Horak et al. 1998b); however, hematocrit was not associated with parasite infection in this study. Still, this finding is consistent with studies in which seasonal declines in hematocrit were associated with increases in ambient temperature, as birds do not need to spend as much energy thermoregulating as temperature increases (deGraw et al. 1979; Morton 1994).

For H/L ratios, we found no difference between males and females in catbirds and waxwings, as expected due to their similar work loads, but we found that within infected vireos, females had a higher H/L ratio than males. This difference was expected based on the higher work load of female vireos during the breeding season. Redstarts did not follow the expected trend, but this could have been due to a small sample size. It is interesting to

note that this sex-specific difference in H/L ratios only became apparent in the species which had the highest parasitemia, supporting the idea that low level infections may not affect body condition but high level ones might. In waxwings, H/L ratios significantly decreased with time, which revealed an interesting time component in this species.

Waxwings are among the latest-nesting birds in North America (Witmer et al. 2014), so we were able to separate collected samples into migratory (May) and breeding (June/July) periods. Higher parasite prevalence was found in breeding birds, which is consistent with elevated numbers of transmission vectors during this period, as well as the added stress of reproduction. A decrease in body mass from May to June/July may have been due to birds spending more time breeding and less time feeding (Horak et al. 1998b). A decrease in hematocrit with time was expected, and has been attributed to increases in ambient temperature (deGraw et al. 1979; Morton 1994). However, differences in H/L ratios found between these two periods were unexpected. Previous studies have determined that infection will increase H/L ratios; however, these studies tested infection by *Haemoproteus* (Dunn et al. 2013; Table 12), a parasite genus that was not common in waxwings. Our results are consistent with a study by Norte et al. (2009), in which infected great tits (*Parus major*) in Portugal also tended to have lower H/L ratios than uninfected birds. They attributed this finding to infection by *Leucocytozoon*, which is also consistent with our study, as waxwings had a higher than expected prevalence of this parasite genus, a trend which was not found in any of the other species. Norte et al. (2009) suggested that infection by *Leucocytozoon* may elicit a more specific immune response, involving antigenic stimulation of lymphocytes, instead of increasing heterophil numbers.

Future studies

An important problem in the near future is the constant increase in atmospheric temperatures worldwide. Studies have predicted that this will lead to increases in mosquito and fly populations, as well as expand their distribution (Zamora-Vilchis et al. 2012). As these insects are transmission vectors for malarial parasites, as well as other diseases, parasite abundance and distribution is also expected to increase with time. This poses a significant problem, as higher parasite abundance may lead to higher parasitemia in birds, but most importantly, an increase in parasite distribution, especially into higher elevations, could expose naïve hosts to malarial parasites. Studies have shown than naïve hosts are particularly sensitive to infection by these malarial parasites, causing high mortality (Atkinson et al. 1995, 2000).

Future studies should focus on determining pathogenicity of specific parasite species, as well as host sensitivity in various bird species, to allow for a better understanding of host-parasite interaction. This will help prioritize management techniques and prevent infection of highly sensitive hosts, particularly in light of increasing global temperatures. Experimental studies are also necessary to determine causation and provide evidence that parasites are actually detrimental for bird health, or if they preferentially infect unhealthy birds.

Table 12. Summary of recent studies on the effects of parasite infection on various aspects of bird physiology.

Species common name	Season	Parasites examined	Effect of parasite(s) on mass/hematocrit/H/L ratio	Other effects of parasite(s)	Detection method	Source
Rock pigeon	Breeding	H	No effect on body condition		Microscopy	Knutie et al. 2013
Siberian tit	Breeding	H, L, P	No effect on H/L ratio		Microscopy	Krams et al. 2010
Great tit	Breeding	H,L,P	No effect on H/L ratio	Uninfected nestlings had higher hemoglobin concentrations and higher survival	Microscopy	Krams et al. 2013
Great tit	Breeding	H	Females more infected than males: had a higher H/L ratio and higher hematocrit		Microscopy	Ots et al. 1998
Great tit	Breeding	H, L, P	Infection with L and P decreased body mass. Infection with H increased H/L ratio, but Infection with L decreased H/L ratios. Infected nestlings had a lower H/L ratio	Clutch size was negatively associated with infection by L and H. Infection with L and P decreased plasma protein. Infection with P and H increased leucocyte counts and decreased hemoglobin.	Microscopy	Norte et al. 2009
Red-billed gull	Breeding	P	Infected individuals were in poor body condition		PCR	Cloutier et al. 2011
House martin	Breeding	H, P	No effect on body condition	Double-infections led to lower growth rate of tail feathers and higher parasitemia	PCR	Marzal et al. 2013
Iberian bluethroat	Breeding	H, L P	No effect on body condition		PCR	Angeles Rojo et al. 2014
House martin	Breeding	H, P	Double infections led to lower body mass and higher hematocrit		PCR	Marzal et al. 2008
Scarlet rosefinch	Breeding	H, L, P	No effect on body condition		PCR	Synek et al. 2015
Seychelles warbler	Breeding	H	No effect on body condition	Infected birds had higher oxidative stress susceptibility during provisioning	PCR	van de Crommenacker et al. 2012
Brown noddy	Breeding	H, L, P	No effect on body condition		PCR & microscopy	Quillfeldt et al. 2013

Body condition in these studies was determined by correcting body mass by body size. H = Haemoproteus, P = Plasmodium, L = Leucocytozoon.

Table 12. Continued.

Species common name	Season	Parasites examined	Effect of parasite(s) on mass/hematocrit/H/L ratio	Other effects of parasite(s)	Detection method	Source
Eurasian blackcap	Non-breeding	H	No effect on body condition	H infection delayed arrival date of recaptured blackcaps	PCR & microscopy	Santiago-Alarcon et al. 2013
Gray catbird	Non-breeding	H, P	No effect on body condition or H/L ratio	Elevated leucocyte count in infected birds	PCR	Cornelius et al. 2014
Common yellowthroat	Non-breeding	H, P	No effect on body condition or H/L ratio		PCR	Cornelius et al. 2014
Western palm warbler	Non-breeding	H, P	No effect on body condition or H/L ratio		PCR	Cornelius et al. 2014
Yellowhammer	Non-breeding	H	Infection with Haemoproteus decreased H/L ratio	Infection with H increased leukocyte count	PCR	Dunn et al. 2013
Red-tailed hawk	Non-breeding	H, L	Males infected with Haemoproteus were in poorer body condition		PCR	Ishak et al. 2010
Berthelot's pipit	Non-breeding	L, P	Greater mass in infected individuals		PCR	Spurgin et al. 2011

Body condition in these studies was determined by correcting body mass by body size. H = Haemoproteus, P = Plasmodium, L = Leucocytozoon.

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VITA

Carolina Granthon was born August 13, 1989, in Lima, Peru. She is the daughter of Felix Oscar and Carla Martha. A 2007 graduate of San Silvestre School in Lima, Peru, she received a Bachelor of Science degree with majors in Biology and Chemistry from Texas Christian University, Fort Worth, in 2012.

After graduating college, she completed field work in the rainforest for a year, before returning to Texas Christian University, where she received her Master of Science degree in Biology in 2015. While working on her masters in biology, she held a Teaching Assistantship during the years 2013-2015.

ABSTRACT

AVIAN MALARIA AND BODY CONDITION
IN FOUR SPECIES OF SONGBIRDS

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Avian malaria is a common disease in songbirds, caused by protozoans in the genera

Plasmodium, *Haemoproteus,* and *Leucocytozoon*. These parasites can negatively impact bird

survival, reproductive success, and body condition. Four species of songbirds were sampled

during the reproductive season; the American redstart, the gray catbird, the cedar waxwing,

and the red-eyed vireo. The study aimed to determine parasite prevalence in these species,

as well as to evaluate a relationship between infection and body condition.

Results detected a high parasite prevalence using PCR (94%) but a much lower one

using microscopy (37%), suggesting that parasite prevalence is high while parasitemia is

low. Parasite infection did not seem to affect any measure of body condition. We found that

within infected vireos, females had a higher H/L ratio than males, and that breeding

waxwings had higher parasite prevalence and lower body condition than migrating birds,

consistent with infection by *Leucocytozoon*.