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Age-specific patterns of infection with haemosporidians and trypanosomes in a warbler: implications for sexual selection

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Abstract Although the selective loss of individuals susceptible to disease can favor the evolution of female preference for older males, the interrelationship between age, infection, longevity, and mating success remains poorly characterized in natural populations. In a longitudinal study of 61 male common yellowthroats (*Geothlypis trichas*), we found that the probability of infection with hematozoa increased as males aged from 1 to 5 years. Despite a significant, negative association between infection and longevity that partially masked age-effects, the odds that a male was infected with *Trypanosoma*, *Plasmodium*, or *Leucocytozoon* increased 71–212% per year. Nearly 75% of males in their first breeding season were either uninfected or infected with only a single parasite, while 50% of older males were infected with at least two parasites and 16% were infected with all three. No males escaped infection after their second breeding season. Older males were also more likely to sire extra-pair young (EPY) and, as a consequence, infection with multiple parasites was associated with a fourfold increase in the odds of producing EPY. Unlike younger males, 80% of the oldest males had a history of either surviving chronic infection or recovering. Combined with previous work showing higher diversity at the major histocompatibility complex among older males, our results suggest that the song and plumage traits that signal male age in common yellowthroats also, perforce, signal resistance to parasites. By preferring older males, females may obtain good genes for disease resistance even in the absence of any effect of infection on male ornamentation.

Keywords Hamilton–Zuk · Parasites · Good genes · Ornamentation · Common yellowthroat

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Introduction

A long-standing hypothesis explaining female preference for bright or elaborately ornamented males is that ornaments signal the genetically based resistance of males to parasites (Hamilton and Zuk 1982). This hypothesis proposes that a male's immune genotype influences the risk and/or intensity of infection and that infected males produce less elaborate ornaments or display behaviors (Møller 1990; but see Getty 2002, 2006). By allocating fertilizations to showy males, female obtain good genes for disease resistance for their young.

The link between a male's health and ornamentation can occur through multiple pathways, including the reduction in foraging efficiency and nutrient acquisition associated with morbidity (Hill and Montgomerie 1994); the loss of resources to immune function (Lozano 1994; Schull et al. 2016); parasite-induced changes to testosterone and glucocorticoid production (Folstad and Karter 1992; Mougeot et al. 2010); and/or the oxidative stress that can result from a prolonged immune response (von Schantz et al. 1999; Hasselquist and Nilsson 2012). Underlying these behavioral

and physiological pathways are changes to patterns of gene expression which, in turn, may be traced to a combination of genetic and environmental causes (Hill 2011). In recent experimental work, for example, Hill et al. demonstrate that plumage coloration in house finches (*Carpodactus mexicanus*) predicts the activation (differential expression) of genes linked to immune responsiveness and, ultimately, a male's ability to recover from infection (Hill and Farmer 2005; Balenger et al. 2015).

Although the proximate mechanisms linking health to ornamentation are increasingly well explored, evidence that infection in natural populations is revealed by sexually selected traits continues to be mixed (Møller et al. 1999; Griffith and Pryke 2006). While parasitized males produce relatively dull or less elaborate ornaments in some species (Hill and Farmer 2005; Lumpkin et al. 2014; Balenger et al. 2015), in others, ornamentation and display behaviors appear to be unrelated to a male's infection status (Dufva and Allander 1995) or even positively related to parasite burden (Trigo and Mota 2016) or current/prior immune activation (Velando et al. 2014).

One cause for null or counterintuitive relationships between male sexual displays and disease state may derive from the complexity of the underlying disease ecology. Tests of parasite-mediated sexual selection often focus on infection with single parasites, yet individual birds host multiple parasites (Møller et al. 1999; Balenger and Zuk 2014). Interactions among parasites themselves can be positive (facilitation) or negative (competition), yielding complex patterns of association across hosts with the potential for synergistic effects on the hosts' health and condition (Graham et al. 2005; Clark et al. 2016). Indeed, one-parasite/one-host models are increasingly recognized as insufficient to understand the dynamics of infection in natural populations (Bordes and Morand 2009; Knowles et al. 2013; Fenton et al. 2014; Hellard et al. 2015). Whether sexually selected traits and condition covary with specific pathogens may depend not so much on whether the "right" pathogen is selected for study, but on the additive and synergistic effects of concurrent infection by multiple pathogens (Davidar and Morton 2006; Marzal et al. 2008, 2013).

Second, the temporal dynamics of infection are often unknown, particularly as they relate longitudinally to male age (but see Bensch et al. 2007). In many species, the traits preferred by females become more elaborate or exaggerated as males age (e.g., Ballentine 2009), yet the cumulative risk of disease is likely greater for older than younger males since older males have been exposed to pathogens for a longer period of time. At the population level, age-related changes to the prevalence of disease may obscure the relationship between disease state, indices of male quality, and ornamentation (sensu Lifjeld et al. 2011). Further, and counter-intuitively, such patterns may generate the appearance of female preference for infected males, especially in species where male fertilization success increases with age, a common pattern in birds (Schmoll et al. 2007; Laskemoen et al. 2008).

Here, we explore age-specific patterns of infection and co-infection with three blood parasites in the common yellowthroat (*Geothlypis trichas*): *Plasmodium*, *Leucocytozoon*, and *Trypanosoma*. Variation at the MHC in common yellowthroats is related to malarial infection and also to ornaments known to be under selection via female choice, the melanin-based black mask (in Wisconsin, USA; Thusius et al. 2001; Dunn et al. 2013) and the carotenoid-based yellow bib (in New York, USA; Freeman-Gallant et al. 2010; Whittingham et al. 2015). Previous research has characterized geographic variation in prevalence of some hematozoa (Pagenkopp et al. 2008), but has not considered trypanosomes, patterns of co-infection, or how the probability of infection changes across a male's lifetime or relates to extra-pair mating success.

Materials and methods

We have studied common yellowthroats nesting in Saratoga County, NY, USA since 2005. Field and laboratory methods have been described in detail elsewhere (Freeman-Gallant et al. 2010). Briefly, we conducted daily censuses of the study site from early May through late July each year to determine male and female arrival dates, pairing status, and reproductive success. All males were captured in mist nets using song playback within several days of arrival and banded with a United States Fish and Wildlife Service aluminum band and 1–3 colored plastic bands. At the time of capture, we measured wing length (to nearest 0.5 mm), tarsometatarsus length (to nearest 0.1 mm), and weight (to nearest 0.1 g) and collected a small (20–100 μL) sample of blood from the brachial vein. Approximately 10–15 μL of blood was mixed immediately with an ice-cold buffer (10% DMSO, 90% newborn bovine serum) and cryopreserved at −80 °C in the laboratory. Remaining blood was lysed and stored at −80 °C in Queen's Lysis Buffer (Seutin et al. 1991). Whole genomic DNA was isolated from thawed samples using a DNeasy Blood and Tissue Kit (Qiagen) and stored at −20 or −80 °C until being screened for hematozoa. Blood samples were collected from nestlings 4 days after hatching and processed as described above.

Our dataset includes 61 males captured annually (if present) between 2008 and 2012, yielding a total sample of 120 observations. Nineteen males were sampled in a single year, 26 males were sampled in two consecutive years, 15 males were sampled in 3 years, and one male was sampled in 4 years. Because some males entered the population prior to 2008, years-in-site range from 1 to 5 years [mean 2.0 ± 1.0 (SD) years, $n = 120$ observations].

Males that returned to the site rarely moved more than 200 m between years (Taff et al. 2013), and among all 206 males in our study (2005–2012), only twice did a male disappear in 1 year only to reappear in a later year (2.3% of 86 observed transitions among males returning three or more years). We therefore assume that males in their first breeding season are yearling males and that years-in-site approximates male age. With respect to age-related trends in prevalence, this assumption is conservative since older (likely infected) males misidentified as first-time breeders would obscure rather than inflate the relationship between age and infection status.

Overall, 88 of the 120 observations (73.3%) that comprise our sample pertain to males that defended territories along a powerline corridor transecting mixed deciduous forest. The remaining observations are from males that defended territories 10 km away, along a riparian corridor formed by beaver (*Castor canadensis*) activity. Because the prevalence of parasites was nearly identical between sites, and because models with and without site as a predictor were qualitatively and quantitatively similar, we pooled males over sites for purposes of analysis. The Electronic Supplemental Materials (ESM) contains summary statistics for the two sites.

Extra-pair paternity

Common yellowthroats are socially monogamous, but adults in our population produce young outside the pairbond (Freeman-Gallant et al. 2010). As described previously, we used a suite of 3–4 microsatellite loci to identify extra-pair young and their sires. Because of incomplete sampling of young from some sites in some years, we determined extra-pair success (sired extra-pair young or not) for a subset of males, those breeding 2008–2010 and at one of the two study sites in 2011. This restriction yielded a final dataset of 105 observations from $n = 59$ males. Over the 4 years, we sampled 338 young from 101 broods, 35 of which contained extra-pair young (34.7%). Overall, 47 of 338 young (13.9%) were sired by males outside the pairbond, similar to the percentage reported previously for 2005–2007 (18% of young in 36.7% of broods; Freeman-Gallant et al. 2010). Sires for all but 2 extra-pair young (95.7%) could be identified, mostly from among the males occupying adjacent or nearby territories (see Taff et al. 2013).

Prevalence of hematozoa

We screened for the presence of haemosporidians using the nested, two-stage approach developed by Hellgren et al. (2004). First, we amplified a region of the parasites' mitochondrial DNA using the HaemNF1/HaemNR3 primers. Second, we used the products of the first reaction and the HaemF/HaemR2 and HaemFL/HaemR2L primers to detect sequences specific to *Plasmodium/Haemoproteus* and *Leucocytozoon*, respectively. All three reactions contained 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, and 0.1 units of hot-start Taq DNA polymerase. Cycling conditions follow Hellgren et al. (2004) except for a 50 °C annealing temperature. PCR products from the final two reactions were electrophoresed on a 2% agarose gel for 80 V-h and stained with SYBR Green. Each sample was run twice to minimize the occurrence of false negatives (see below), and all PCR runs contained both positive and negative controls.

We sequenced the PCR products from nine infected birds to assess the identity and diversity of hematozoa in our sample (cf. Szollosi et al. 2008). Among four males infected with *Leucocytozoon*, one sequence (found in two birds) was identical to a *Leucocytozoon* lineage isolated from black flies (*Simulium* sp.) in Colorado, USA (Murdock et al. 2015). The other two sequences show 98–99% similarity to *Leucocytozoon* mitochondrial sequences in GenBank. We detected two *Plasmodium* lineages among five birds infected with *Plasmodium* or *Haemoproteus*. Both sequences are among those previously identified by Pagenkopp et al. (2008) from common yellowthroats sampled in the northeastern USA (lineages P1 and P4-B). All five sequences have been deposited in GenBank (accession numbers KY662289-662293).

Because Pagenkopp et al. (2008) found a low prevalence of *Haemoproteus* across the North American range of common yellowthroats (~5% of 552 birds), and because we detected only *Plasmodium* lineages on our NY sample, we refer to positive PCR assays for *Plasmodium* or *Haemoproteus* as indicative of *Plasmodium* when summarizing results. We recognize, however, that our approach cannot distinguish between genera and that, as a consequence, our estimate of the occurrence of multiple infection is conservative.

We used a similar approach to detect infection by trypanosomes. Following Sehgal et al. (2001), we performed a nested PCR of the parasite's small subunit rRNA using 3.2 mM $MgCl_2$, 0.2 mM dNTPs, 1.3 µM of each primer, and 0.1 units of hot-start Taq DNA polymerase. Cycling conditions followed Sehgal et al. (2001), and analysis of PCR products was as described above for haemosporidians. We sequenced PCR products from seven infected birds; the seven products were identical to each other and to SSU rRNA sequences in GenBank identified as deriving from *Trypanosoma avium* (e.g., accession number TAU39578; Maslov et al. (1996)). The sequence amplified from common yellowthroats has been deposited in GenBank (accession number KY662288).

Over the three parasites, the occurrence of partial positives ranged from 19% for *Plasmodium* to 36–38% for

trypanosomes and *Leucocytozoon*, comparable to results reported for PCR-based detection methods in other studies (e.g., Hellgren et al. 2004). Although our replicate sampling reduced the number of false negatives, some infected birds are likely to have remained undetected and our estimates of prevalence are thus conservative (downwardly biased; McClintock et al. 2010). The occurrence of partial positives was independent of male age for all three parasites (Pearson's χ^2; $0.11 < \chi^2 < 3.4$, $df = 3$, $0.34 < P < 0.99$) and, therefore, undetected infections are unlikely to confound the relationship between age and infection in our study.

Statistics

Because 42 of 61 males were sampled in two or more years, we are able to explicitly model the influence of age on infection status within males (longitudinally) rather than rely on cross-sectional comparisons across males. First, we searched for associations between male age and infection (yes/no) with separate logistic regressions for each parasite that included year and sampling date as fixed effects and male ID as a random effect to account for non-independence among multiple observations from the same individual. We used a similar model to explore the effect of age on probability of infection with two or more parasites. For each logistic regression, we report exponentiated parameter estimates (odds ratios; e^{β}) to assist interpretation of the strength and direction of effects.

Following Marzal et al. (2016), we re-ran each of the models described above for a subset of 47 males where longevity (age at last reproduction; ALR) could be approximated. Including ALR allows us to control for the effect the selective loss of infected individuals may have on the relationship between age and prevalence of infection (see van de Pol and Verhulst 2006). If infected males have reduced longevity, they will not be present to be counted (as infected) among older males, thus masking the relationship between age and infection. We entered ALR as an additional fixed effect and present the results of models with and without ALR.

By necessity, for analyses involving ALR, we excluded all observations pertaining to males present during the final year of the study ($n = 12$) and two males who were present in 2005, the first year we captured and banded all males observed at our sites. These latter two males were in at least their 5th breeding seasons when they were last observed; although it is unlikely that they were, in fact, 6 years old or older, we nonetheless excluded them to be conservative. The other 12 males we excluded include 7 males who were in their second breeding season in 2012, 4 males who were in their third breeding season, and one male who was in his fourth breeding season. The parasite status of these males at the time of last observation mirrored the age-specific patterns described below. Their exclusion is therefore unlikely to have biased our results. Indeed, rerunning each baseline model (without ALR) with the subset of 47 males generated results that were qualitatively and quantitatively similar to those obtained with the full data set of 61 males (see ESM for full results).

To test for age-specific patterns of co-infection, we include presence/absence of one parasite and its interaction with age as predictors of infection by a second parasite. We focused these analyses on males in the first and second breeding season due to the small number of males surviving to their third year and beyond.

All mixed models were run using the generalized linear mixed analysis platform in SPSS 24.0 (IBM 2016).

Results

Pooling over years and males, most individuals (71%) were infected with *Plasmodium* and nearly half (45%) were infected with *Trypanosoma*. Infection with *Leucocytozoon* was least frequent, occurring in only 18% of samples (Table 1). *Plasmodium* and *Trypanosoma* were equally frequent in 1 year (2012), but at no other time did the relative ranking of the three parasites' prevalence differ. There is no evidence that between-year changes in prevalence were correlated across parasites (pairwise correlations; $-0.66 < r < -0.23$; $P > 0.23$).

Overall, 11% of males were infected with all three parasites simultaneously and another 29% were infected with two parasites, most commonly *Plasmodium* and *Trypanosoma* (23% of all samples). Only 17% of samples showed no evidence of infection by any of the three parasites (Table 1).

Age and infection

The prevalence of all three parasites increased with age in cross-sectional comparisons among males (Fig. 1). For trypanosomes, prevalence ranged from 32% among yearling males to >90% among males in the oldest age class. *Plasmodium* reached 100% prevalence among the oldest males, while *Leucocytozoon* could be detected in nearly half of them (46%). Not surprisingly, changes in the incidence of multiple infections tracked age-related changes in the prevalence of the individual parasites (Fig. 2). By age 3, no male was without one of the three parasites and 48% were infected simultaneously by two of them. By age 4, <10% of males harbored only a single infection and 45% were infected with all three parasites.

Multivariate logistic regressions accounting for multiple observations from the same male provide strong support for these general patterns (Table 2). Before accounting for age at last reproduction (ALR), the odds that a

Table 1 Prevalence of *Trypanosoma*, *Plasmodium*, and *Leucocytozoon* in common yellowthroats

	2008	2009	2010	2011	2012	Pooled
n (number of males)	20	34	28	26	12	120
Overall prevalence (%)						
Trypanosoma	55.0	41.2	50.0	26.9	66.7	45.0
Plasmodium	75.0	61.8	67.9	84.6	66.7	70.8
Leucocytozoon	10.0	26.5	17.9	15.4	16.7	18.3
Single infection (%)						
Trypanosoma	5.0	5.9	10.7	0.0	33.3	8.3
Plasmodium	25.0	23.5	32.1	57.7	25.0	33.3
Leucocytozoon	0.0	2.9	0.0	3.8	0.0	1.7
Double infection (%)						
Trypanosoma + *Plasmodium*	40.0	17.6	25.0	15.4	25.0	23.3
Trypanosoma + *Leucocytozoon*	0.0	2.9	7.1	0.0	0.0	2.5
Plasmodium + *Leucocytozoon*	0.0	5.9	3.6	0.0	8.3	3.3
Triple infection (%)						
Trypanosoma + *Plasmodium* + *Leucocytozoon*	10.0	14.7	7.1	11.5	8.3	10.8

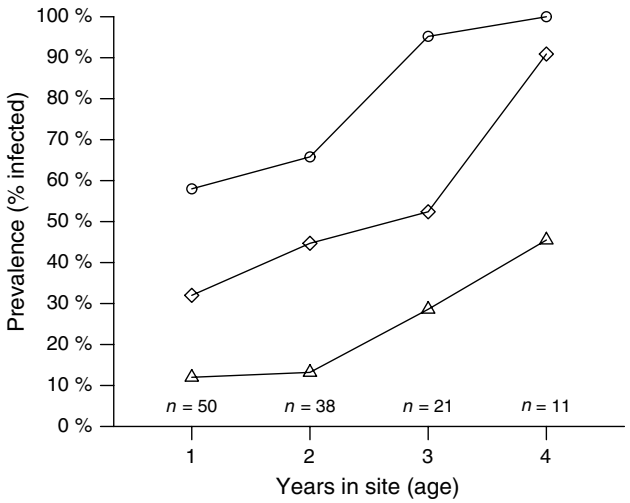

Fig. 1 Prevalence of *Plasmodium* (*circle*), *Trypanosoma* (*diamond*), and *Leucocytozoon* (*triangle*) among $n = 61$ common yellowthroat males, 2008–2012, according to years-in-site, an index of male age. Males in the oldest age group include $n = 8$ males in their fourth breeding season and $n = 3$ males in their fifth breeding season

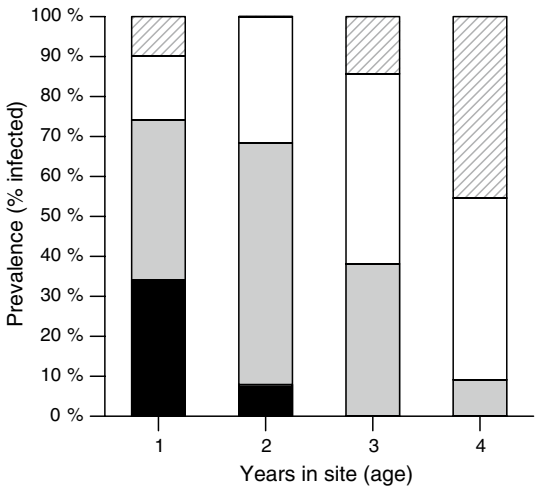

Fig. 2 Proportion of males uninfected (*dark fill*) or infected with one (*light fill*), two (*no fill*) or all three parasites (*hatched fill*). Males in the oldest age group include $n = 8$ males in their fourth breeding season and $n = 3$ males in their fifth breeding season

male was infected with trypanosomes increased 93% per year ($F_{1,113} = 9.62$, $P = 0.002$; Table 2) while the odds that a male was infected with *Plasmodium* more than doubled each year ($F_{1,113} = 10.4$, $P = 0.002$). The effect of age on *Leucocytozoon* infection was positive but marginally non-significant ($e^{\beta} = 1.72$; age effect: $F_{1,113} = 3.77$, $P = 0.055$). Overall, the odds that a male was infected with two or more parasites increased by a factor of 2.5 each year ($F_{1,113} = 14.1$, $P < 0.001$).

Age at last reproduction (ALR) was a significant predictor of infection in the case of trypanosomes and *Leucocytozoon* (Table 2). Among same-aged males, the odds that a male was infected with trypanosomes or *Leucocytozoon* decreased by 44 and 28%, respectively, for each additional year the male ultimately lived. Among males in their first breeding season, no male that ultimately lived to 3 years (ALR = 3) was infected with *Leucocytozoon* or trypanosomes, compared to infection rates of 20 and 35% among yearlings that failed to return (ALR = 1). Since males infected with these parasites had reduced longevity, models that fail to account for ALR are likely to underestimate age-related increases in prevalence. Indeed, the effect of age on prevalence was substantially higher when

Table 2 Relationship between age and probability of infection with and without controlling for longevity (age at last reproduction; ALR) using mixed models that include year and sampling date as fixed effects and male ID as a random effect. Odds ratios (e^{β}) and confidence intervals (CI) are shown for age and ALR only; see ESM for all other effects

	e^{β}	F^{a}	P	95% CI lower, upper
(1) *Trypanosoma*				
Age	1.93	9.62	0.002	1.27, 2.93
Age	6.03	10.05	0.002	1.95, 18.67
ALR	0.44	3.98	0.05	0.19, 1.00
(2) *Plasmodium*				
Age	3.12	10.37	0.002	1.55, 6.29
Age	2.72	3.34	0.072	0.91, 8.08
ALR	1.15	0.11	0.74	0.50, 2.66
(3) *Leucocytozoon*				
Age	1.72	3.77	0.055	0.99, 2.98
Age	5.55	7.32	0.008	1.57, 19.62
ALR	0.28	5.23	0.025	0.09, 0.85
(4) Multiple infections				
Age	2.48	14.15	0.001	1.54, 4.00
Age	5.95	9.82	0.002	1.92, 18.47
ALR	0.49	2.92	0.092	0.21, 1.13

[a] *df* for baseline models with 61 unique males (1, 113); *df* for models with ALR and 47 unique males (1, 76)

accounting for ALR for both parasites; in the case of *Leucocytozoon*, the odds ratio triples compared to the model without ALR and the age effect becomes highly significant ($F_{1,76} = 7.32$, $P = 0.008$; Table 2).

Interactions among parasites

In a multivariate regression of *Plasmodium* infection (yes/no) with age, year, sampling date, male ID, and the presence/absence of infection with trypanosomes as predictors, the interaction term between male age and trypanosome infection was significant ($F_{1,79} = 7.31$, $P = 0.008$). We thus examined the co-occurrence of trypanosome and *Plasmodium* infection among males in their first and second breeding seasons separately (Fig. 3). Among yearlings, the two parasites co-occurred more frequently than expected by chance given their individual prevalence; 81% of yearling males with trypanosomes were also infected with *Plasmodium*, compared to only 47% of yearling males without trypanosomes (Pearson's $\chi^2 = 5.2$, $df = 1$, $P = 0.02$). By contrast, the two parasites negatively covaried among males in their second breeding season (Fig. 3; Pearson's $\chi^2 = 8.3$, $df = 1$, $P = 0.004$).

Trypanosomes were also associated with infection with *Leucocytozoon*. After controlling for age, year, sampling

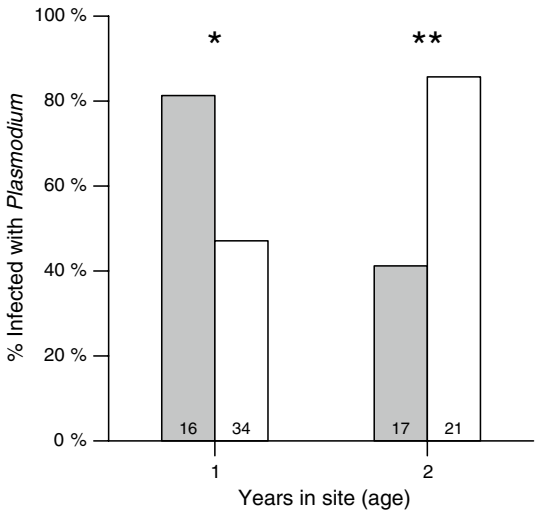

Fig. 3 Prevalence of infection with *Plasmodium* among males infected with trypanosomes (*shaded*) or not infected with trypanosomes (*unshaded*) during the males' first ($n = 50$) and second ($n = 38$) breeding seasons. *$P < 0.05$, **$P < 0.01$. Sample sizes are shown inside *bars*

date and male ID, infection with trypanosomes appeared to increase the odds of infection with *Leucocytozoon* fivefold ($e^{\beta} = 5.4$; effect of trypanosomes: $F_{1,80} = 5.63$; $P = 0.02$). The converse was also true—*Leucocytozoon* appeared to increase the odds of infection with trypanosomes ($e^{\beta} = 6.9$; effect of *Leucocytozoon*: $F_{1,80} = 6.50$; $P = 0.013$). In neither model were interactions with age significant so the interaction terms were dropped from both models.

In contrast to their interaction with trypanosomes, the two haemosporidians did not themselves covary. *Plasmodium* had no effect on the odds of infection with *Leucocytozoon* ($F_{1,80} = 0.137$; $P = 0.71$) nor did *Leucocytozoon* have any effect on *Plasmodium* ($F_{1,80} = 0.12$; $P = 0.73$).

Infection, age, and extra-pair paternity

Between 2008 and 2011, we determined extra-pair mating success for 59 males across 105 breeding attempts. Overall, 18 of 59 males (30.5%) sired at least one offspring outside the pairbond. Pooling over years and treating each breeding attempt as an independent event, success at becoming an extra-pair sire appears to increase with age (Fig. 4). Within males, the probability that an individual sired one or more extra-pair offspring increased significantly with age (multiple logistic regression including male identity as a random effect; $e^{\beta} = 1.69$, age effect: $F_{1,100} = 5.02$; $P = 0.027$).

The likelihood that males sired EPY was also predicted by infection with hematozoa. Although multiple logistic regressions of EP success (yes/no) on the presence or absence of individual parasites only approached marginal significance after controlling for male ID and year

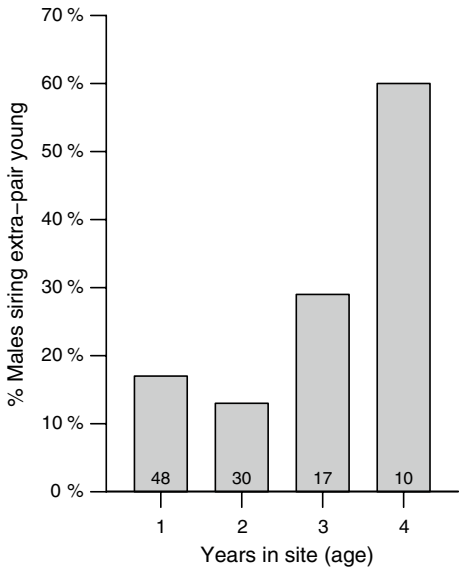

Fig. 4 Proportion of males producing one or more extra-pair young (EPY) according to age. Sample sizes are shown inside *bars*. Males in the oldest age group include $n = 7$ males in their fourth breeding season and $n = 3$ males in their fifth breeding season

Table 3 Incidence of new infections among previously uninfected males and recovery rates for common yellowthroat males in their first and second breeding seasons, n = number of unique males

	Incidence of new infection		% lose parasite (recover)
	Year 1	Year 2	Year 1 → Year 2
Trypanosoma	32% ($n = 50$)	50% ($n = 22$)	67% ($n = 21$)
Plasmodium	58% ($n = 50$)	45% ($n = 11$)	25% ($n = 20$)
Leucocytozoon	12% ($n = 50$)	14% ($n = 29$)	[a]

[a] Insufficient sample size; 2 of 6 infected males in year 1 returned in year 2; one of the two returning males recovered

($F_{1,100} < 3.59$; $P > 0.06$), infection by two or more parasites was strongly related to EP success. The odds that a male sired EPY was more than four times greater for males with multiple, concurrent infections than males with no or single infections ($e^{\beta} = 4.5$; effect of multiple infection: $F_{1,100} = 6.88$; $P = 0.01$).

Because the occurrence of multiple infections is tightly correlated with age, disentangling their relative effects on extra-pair mating success is difficult. For example, five of seven males in their fourth year sired extra-pair young, but all seven males tested positive for two or more parasites. Among males in their third year, three of eight males with multiple infections sired EPY (27.3%) compared to two of six males (33.3%) with no or single infections (Pearson's $\chi^2 = 0.07$, $df = 1$, $P = 0.79$). Only among males in the second year does EP success appear to be correlated with disease state. Here, and counter-intuitively, 3 of 10 males with multiple infections sired EPY (30%) compared to only 1 of 20 males (5%) with no or single infections (Pearson's $\chi^2 = 3.6$, $df = 1$, $P = 0.058$), but the overall proportion of males achieving extra-pair success (13%) is low.

Discussion

Over the 5 years of our study, we found that the prevalence of infection with three blood parasites—*Trypanosoma*, *Plasmodium*, and *Leucocytozoon*—increased dramatically with age among male common yellowthroats. In cross-sectional comparisons of males, young males in their first or second breeding seasons were likely to be uninfected or infected with only a single parasite. In contrast, more than 90% of males in their fourth or fifth breeding seasons harbored concurrent trypanosome and *Plasmodium* infections and 45% were infected by all three parasites. Age effects were also apparent longitudinally, with the odds of an individual male becoming infected increasing by a factor of 1.7–3.1 each year, depending on the parasite. Although an increased prevalence of hematozoa has been noted among older males in some birds (e.g., Davidar and Morton 1993; Allander and Bennett 1994; Garvin et al. 2006), to our knowledge, this is one of only a handful of studies to characterize the occurrence of infection over time *within* males (but see van Oers et al. 2010; van Rooyen et al. 2013; Marzal et al. 2016). Such studies are important because age-related patterns of infection at the population level can result from the differential loss of individuals (selection), increased exposure or susceptibility to parasites with time, or a combination of mechanisms that can mask or exaggerate the importance of age depending on their strength and direction.

Infections by malarial parasites generally result in an initial, acute phase with high parasitemia followed by chronic infection that may persist for months or years (Garvin et al. 2006; Sarquis-Adamson and MacDougall-Shackleton 2016). Thus, the simplest explanation for the increased prevalence of blood parasites with age is repeated exposure and the accumulation of chronic infections over time (e.g., Allander and Bennett 1994). However, individuals can clear infections (Bensch et al. 2007; van Rooyen et al. 2013), pointing to the possibility of more complex dynamics between infection, recovery, and susceptibility to (re) infection that may be compounded by age-related changes to each of these state-transition variables (Hudson and Dobson 1997). Indeed, we found that the incidence of trypanosome infection among yearling males (32%) was lower than the incidence of new infections among the parasite-free males that survived to the following year (50%), suggesting that the risk of acquiring trypanosomes increases

with age. Further, in the males' second breeding year, we could not detect trypanosomes in most individuals that had been previously infected (Table 3), suggesting that it is the increased risk of infection with age, and not the accumulation of chronic infections per se, that explains the higher prevalence of this parasite in older birds. By contrast, we found the opposite pattern with *Plasmodium*, where the incidence of new infections appears to decline across a male's first 2 years and few males lose the parasite once it is gained (Table 3; cf. van Rooyen et al. 2013).

Interactions among parasites also contribute to age-related patterns of infection. For example, we found that infection with trypanosomes was associated with an increased probability of infection with *Plasmodium* in a male's first year, but a decrease in the probability of infection with *Plasmodium* in a male's second year. Concurrent infection with *Trypanosoma* and *Leucocytozoon* also occurred more frequently than expected given the prevalence of the two parasites. In fact, *Leucocytozoon* was found exclusively in the presence of other blood parasites in three of 5 years (Table 1; also see van Rooyen et al. 2013).

Non-random associations among malarial genera and between malarial parasites and filarial nematodes have been previously described in birds (Clark et al. 2016), but only rarely between trypanosomes and other blood parasites (but see Deviche et al. 2010; Oakgrove et al. 2014). Identifying causal mechanisms for patterns of co-occurrence is notoriously difficult (Johnson and Buller 2011; Fenton et al. 2014; Hellard et al. 2015). One possibility is that the parasites either share vectors or that their different vectors are correlated across space or time (Deviche et al. 2010; Oakgrove et al. 2014). Interestingly, trypanosomes do share vectors with *Leucocytozoon* (black flies) and *Plasmodium* (mosquitoes), but co-transmission does not easily account for the age-reversed pattern we observed. An alternative explanation is that infection with trypanosomes facilitates secondary infection by malarial parasites or vice versa. In such a scenario, age-related trends in co-infection could be attributed to changes in disease state, recovery, and reinfection given the presence/absence of a co-occurring parasite(s), the potential for cross-immunity, and the host's infection history. Unfortunately, we lack sufficient sample size to examine state-transition matrices with any rigor. Identifying causality would require experimental co-infection of birds of different ages and/or infection histories (Dimitrov et al. 2015).

Importantly, age at last reproduction (ALR) was a significant predictor of infection with trypanosomes and *Leucocytozoon*. In fact, every 1-year increase in longevity was associated with a 28% decrease in prevalence of *Leucocytozoon* among same-aged males and a 44% decrease in trypanosome infection. Since nearly all *Leucocytozoon* infections occurred in the presence of one or both of the other parasites, these results suggest that co-infection is also associated with a shorter life span (see Table 2 for marginally NS effect). Similar effect sizes for ALR have previously been described for infection with *Haemoproteus* in house martins (*Delichon urbica*), and our results support growing evidence demonstrating a fitness cost to infection with trypanosomes (Ratti et al. 1993; Dufva 1996; Dyrcz et al. 2005) and *Leucocytozoon* (Merino et al. 2000). Asghar et al. (2015) demonstrated that infection with *Haemoproteus* leads to increased rates of telomere erosion and shorter lifespans in great reed warblers (also see Asghar et al. 2016), perhaps explaining the proximate link between infection and longevity in common yellowthroats.

Female preference for older (infected) males

The age-related patterns of infection and co-infection we describe here have important implications for good genes sexual selection in common yellowthroats. We have previously shown that older males are more likely to produce young outside the pairbond than males in their first breeding season (Freeman-Gallant et al. 2010). Here, we extend that result to show that the odds that a male sires EPY increases >60% per year, even among older, experienced males. Although this pattern could be attributed to older males' ability to create the opportunities conducive to extra-pair paternity, radio tracking of females in Wisconsin demonstrates that females actively pursue extra-pair copulations with neighboring males (Pedersen et al. 2006), suggesting that the increased mating success of older males is the result of female choice. Given the age-related patterns of infection described above, perforce, females must also be allocating fertilizations to infected males. Indeed, infection with multiple parasites is associated with a fourfold increase in the odds of siring extra-pair young, in apparent contradiction to Hamilton–Zuk's hypothesis of parasite-mediated sexual selection (but see Getty 2002).

That covariation between host age and parasite status might confound relationships between male ornamentation, mating success, and health is well appreciated in the literature (Potti and Merino 1996). Thomas et al. (1995) explicitly consider the case where increased mortality of heavily infested males can lead to a spurious, negative relationship between male parasite status and mating success in species where females prefer older males. They call for studies where age effects can be controlled, implying that female preference for older (more or less infected males, depending on the nature of host–parasite interaction) falls outside the Hamilton–Zuk framework for understanding good-genes sexual selection.

To the contrary, parasite-mediated sexual selection can itself lead to female preference for older males. Kokko and Lindstrom (1996), for example, use simulation to show that

females can benefit by preferring older males who have endured episodes of selection across their lifetime, providing support for earlier models that equated age-related sexual displays to viability/longevity indicators (Manning 1985). In the case of parasite-mediated sexual selection, the selective loss of males that cannot withstand infection should increase the frequency of good genes for immune function among surviving males, making it advantageous for females to choose older mates (Davidar and Morton 1993). At the population level, such a preference could generate a negative association between male infection and reproductive success if older, surviving males clear parasites from their system (sensu Thomas et al. 1995) or a positive association if many surviving males continue to harbor chronic infections (this study).

Among common yellowthroats, older age classes are indeed enriched with males who have survived chronic infection(s) or recovered. Although sample sizes are small, the overall pattern is clear. In our population, only 29% of males in their second breeding season ($n = 31$) had, by the time of sampling, survived persistent (chronic) infection by blood parasites or recovered, compared to 64% of males in their third breeding season ($n = 16$), and 80% of males ($n = 10$) in their fourth or fifth breeding seasons. Interestingly, MHC alleles have been linked to the intensity of malarial infection in common yellowthroats, and MHC diversity is also enriched among older, experienced males in some populations (Dunn et al. 2013). Thus, female preference for any trait that is correlated with male age should increase the likelihood that females allocate fertilizations to males who have survived infection and, perhaps relatedly, have greater diversity at the MHC. In common yellowthroats, such traits include the size of the black facial mask, the size and coloration of the UV-yellow bib, and aspects of male singing performance (Thusius et al. 2001; Freeman-Gallant et al. 2010; Taff et al. 2012). Evidence that females allocate fertilizations non-randomly with respect to the MHC is currently lacking, however (Bollmer et al. 2012).

Importantly, any indirect genetic benefits of female preference for older males should pertain even if there is no causal link between infection status and male sexual displays, contrary to most formulations of the Hamilton–Zuk hypothesis. Indeed, whether females should evolve to prefer age-related traits that also reflect parasite status may depend on the relative magnitude of parasite and age effects. For example, if infection reduces the quality of the male's display but the phenotypic distribution of trait values for young versus old males remains distinct, females might benefit from allocating fertilizations to uninfected (fully recovered) individuals among same-aged, older males. However, if the trait is too labile, then the ornaments or display behaviors of chronically infected (but nonetheless surviving) older males may be indistinguishable from the displays of younger, uninfected males whose current health may or may not be related to the presence of good genes, thus eroding the quality of the signal. The evolution of multiple sexual signals, each conveying different information (age, infection status), may be advantageous in this circumstance (Møller and Pomiankowski 1993; van Doorn and Weissing 2004).

We emphasize that our study focuses on only three genera of hematozoa. Although the increased prevalence of multiple infection with age is dramatic, it surely underestimates the true rate given the abundance and diversity of pathogens that infect all free-living organisms. Given the changing patterns of infection and co-infection with age we have observed and the potential for non-additive effects of multiple infection on host fitness (Davidar and Morton 2006; Marzal et al. 2008, 2013), studies of the interrelationship between disease, ornamentation, and female choice might better focus on broader array of pathogens across individuals in known-age populations than on single parasites or even classes of parasites (Balenger and Zuk 2014).

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

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