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ACTIVE BLOOD PARASITE INFECTION IS NOT LIMITED TO THE BREEDING SEASON IN A DECLINING FARMLAND BIRD

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ABSTRACT: Avian blood parasites can have significant impacts on adult breeding birds, but studies of parasitism outside the breeding season are rare, despite their potentially important implications for host-parasite dynamics. Here we investigate temporal dynamics of blood parasite infection in adult yellowhammers Emberiza citrinella. We screened blood samples collected between December and April of two consecutive winters using PCR. We found a high prevalence of both Haemoproteus and Leucocytozoon parasites, with a mean prevalence of 50% across 2 winters. Prevalence of both parasites was higher during the second, colder, winter of the study. Temporal trends differed between the 2 genera, suggesting that chronic Haemoproteus infections gradually disappear throughout the winter, but that Leucocytozoon infections exhibit a relapse during late winter, possibly coincident with reduced food availability. Our results highlight the difference in temporal dynamics between 2 blood parasite genera infecting the same host population and emphasise the need for accurate assessment of infection status at appropriate time periods when examining impacts of, and associations with, blood parasite infection. We suggest that further research should investigate the implications of over winter infection for birds' physiology, behaviour and survival.

Blood parasites can have a pronounced effect on reproduction in many bird species (Merino et al., 2000; Tomás et al., 2007; Knowles et al., 2009, 2010) and are associated with reduced survival in both naïve (Warner, 1968) and co-evolved hosts (Martínez-de la Puente et al., 2010; van Oers et al., 2010). Avian blood parasite infection can be associated with behavioural traits such as increased exploratory behaviour (Dunn et al., 2011) and morphology in terms of feather length (Rätti et al., 1993), and can also have ecological associations with later arrival date for migratory species (Rätti et al., 1993) and reduced survival (Martínez-de la Puente et al., 2010; van Oers et al., 2010). Despite the wide ranging effects of haemoparasites during the breeding season, such as reduced hatching, nestling provisioning and fledging success (Merino et al., 2000; Tomás et al., 2007; Knowles et al., 2010), the potential for them to affect hosts during the inter-breeding period has seldom been investigated (Allander and Sundberg, 1997). Environmental stressors may amplify impacts of parasite infection (Clinchy et al., 2004; Sih et al., 2004). Thus, during winter, when environmental stress in temperate climates can to be high due to low temperatures, increased flocking behaviour and a high requirement for scarce food resources, parasites may exert an additional pressure on populations (Barrow, 1963; Valkiūnas, 2005).

Three separate genera of blood parasites commonly infect avian populations: *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. The 3 genera differ in their vectors and, to some extent, in their life cycles (Valkiūnas, 2005), but all 3 have been associated with detrimental impacts on their hosts (e.g. *Plasmodium*: Van Riper et al., 1986; *Leucocytozoon*: Bunbury et al., 2007; *Haemoproteus*: van Oers et al., 2010).

The prevalence of patent blood parasite infection (when parasites can be detected circulating in the blood, rather than dormant in tissues) varies temporally (Bensch and Åkesson, 2003; Cosgrove et al., 2008), and the prevalence of all 3 genera tends to peak at the beginning of the breeding season in temperate climates (Sundberg, 1995; Allander and Sundberg, 1997; Valkiūnas, 2005; Cosgrove et al., 2008). This peak is due to relapse of existing infections as the onset of breeding leads to rising levels of hormones such as corticosterone (Applegate, 1970; Valkiūnas et al., 2004). In tropical and subtropical climates, patent parasite infection can be found throughout the year because transmission can occur continuously; however, in temperate climates transmission tends to cease outside the breeding season as temperatures fall and vector activity ceases (Cosgrove et al., 2008). *Plasmodium* infections tend to clear from the blood completely outside the non-breeding season (Applegate, 1970; Cosgrove et al., 2008). However, gametocytes from chronic *Haemoproteus* and *Leucocytozoon* infections can remain in the blood for many months after initial infection (Valkiūnas, 2005) but relatively little is known about the period for which parasites can be detected in the blood, or the impact that chronic infections may have on host ecology outside the season of active parasite transmission.

Factors outside the breeding season can also cause increased corticosterone levels and may potentially induce relapses of existing parasite infections (Barrow, 1963; Applegate, 1970; Valkiūnas, 2005). Extreme weather conditions (Romera et al., 2000), food restriction (Kitaysky et al., 2001) and poor habitat quality (Marra and Holberton, 1998) can all increase corticosterone levels and these effects may occur at any time of year, suggesting potential interactions with the dynamics of haemoparasite infections (Valkiūnas et al., 2004). Thus, in species where other

stress-inducing factors, such as food shortages or habitat degradation, act outside the breeding season, a stress-induced decrease in immunity might either trigger parasite relapse or cause a delay in clearing parasites from the bloodstream. Multiple stress-inducing factors can have synergistic effects (Clinchy et al., 2004; Sih et al., 2004), both physiologically (Clinchy et al., 2004) and with ecological consequences (Zanette et al., 2003); thus, patent parasite infection may exacerbate the effects of food or weather related stress. Levels of parasite infection might thus be higher during periods of increased stress, such as during colder winters, than during milder winters.

Here, we investigate the temporal dynamics of blood parasite prevalence during the non-breeding season in a population of Yellowhammers Emberiza citrinella, a farmland bird whose downward population trend (Eaton et al., 2011) has been associated with decreased over winter survival (Bradbury et al., 2000). We sampled our population over 2 winters varying markedly in temperature, and we describe the temporal variability of patent infection across our population.

MATERIALS AND METHODS

Study population and blood sampling

Work was carried out within an individually marked population of Yellowhammers near Tadcaster, North Yorkshire (53°53’N, 1°15’W). Birds were caught in static mist nets and whoosh nets (Redfern and Clark, 2001) at an established supplementary feeding site baited sporadically with wheat and weed seeds, within an experimental agroforestry block surrounded by arable farmland. Two hundred and three birds were caught on 30 sampling occasions between November 2007 and April 2009. Nineteen birds were caught and sampled on 2 occasions within this period and 3 birds were caught and sampled on 3 separate occasions more than 2 mo apart.

100 Birds were aged and sexed by plumage (Svensson, 1992; Dunn and Wright,
101 2009). Blood was taken through venipuncture of the brachial vein and stored with
102 EDTA as an anticoagulant prior to freezing.

103 **DNA extraction and detection of blood parasites**

104 DNA was extracted from 30 μl of whole blood using a standard phenol-
105 chloroform extraction followed by ethanol precipitation (Sambrook et al., 1989).
106 Successful DNA extraction was confirmed by using a Nanodrop ND-1000
107 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, Delaware) and
108 extracted DNA was diluted to a working concentration of 25 – 100 ng/μl.

109 Blood parasite presence or absence was determined through PCR using 2
110 established protocols. The presence of Plasmodium and Haemoproteus was
111 established using primers HaemF and HaemR2 nested within HaemNF and
112 HaemNR2 (Waldenström et al., 2004), and Leucocytozoon spp. were detected using
113 primers HaemFL and HaemR2L nested within primers HaemNFI and HaemNR3
114 (Hellgren et al., 2004). All protocols were carried out in a working volume of 25 μl
115 containing 50 – 200 ng template DNA, 1.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.4 μM of
116 each primer, 1 x GoTaq Flexi Buffer (Promega, Madison, Wisconsin) and 1 U GoTaq
117 Flexi (Promega); a positive control of DNA from a bird with known infection and a
118 negative control containing deionised water in place of DNA were included with each
119 PCR reaction to ensure successful amplification, and lack of contamination,
120 respectively.

121 PCR protocols were identical for detection of both parasite genera. First round
122 reactions consisted of a denaturation step of 94 C for 3 min followed by 20 cycles of
123 94 C for 30 sec, 50 C for 30 sec and 72 C for 45 sec, with a terminal extension step of
124 72 C for 10 min; the protocol for second round reactions contained 35 cycles but

otherwise consisted of an identical thermal profile. PCR protocols were carried out on a GeneAmp PCR System 9700 (Applied Biosystems). Non-target DNA can be amplified with nested PCR methods (Szöllösi et al., 2008), so a subsample of 38 positive samples from 34 birds were sequenced using an ABI sequencer at the Core Genomic Facility, Sheffield University (Sheffield, South Yorkshire, UK). Identity of parasites was confirmed by comparison with sequences in GenBank using the NCBI-BLAST database (Altschul et al., 1997).

Blood smears were created from a subset of positive samples (n=44) and examined under an oil immersion x 100 magnification lens. Haemoproteus infection intensity was assessed at the same time as white blood cell (WBC) differentials (results reported in Dunn et al., 2013) and thus was assessed from the number of microscope fields required to find 100 WBCs (mean ± 1 SE: 1242 ± 150 microscope fields; 236927 ± 29595 erythrocytes). We then assessed the number of intracellular parasites in non-distorted erythrocytes to establish Haemoproteus infection intensity and standardised this measure to reflect the number of parasites per 10,000 erythrocytes. We did not assess Leucocytozoon infection intensity.

Statistical analyses

All analyses were carried out in R version 3.0.2 for Mac (R Core Development Team, 2009). Where 2 or more data points existed from the same individual, 1 was selected at random and retained, and the rest deleted to avoid pseudoreplication. To ensure this retained sample was representative, we repeated this three times to ensure results were consistent between datasets. To examine factors influencing variation in parasitism, we used 2 general linear models (for each parasite genus separately) with binomial error structures and infection status as the response variable. The fixed factors we examined were year, day (as a continuous

variable where Nov 1 = 1, allowing for linear and quadratic relationships), age and sex, as well as two-way interactions between year and day to allow for the possibility of year-dependent relationships with day: we examined all possible candidate models using the 'dredge' function in the 'MuMIn' library (Barton, 2012) and ranked models using second-order Akaike's Information Criteria (AICc). AICc measures the relative goodness of fit of a model and takes into account the number of variables within each model, penalising models for the addition of variables, thus selecting for a model with the maximum goodness of fit and retaining the minimum number of explanatory variables (Burnham and Anderson, 2002). Where more than 1 candidate model had delta AIC<2, we averaged these models to provide parameter estimates adjusted for shrinkage according to the number of top models within which each term was found (Burnham and Anderson, 2002).

RESULTS

Parasite prevalence and identity

Two hundred and twenty-five blood samples from 203 birds were screened for the presence of Plasmodium spp. and Haemoproteus spp. Using the protocol of Waldenström et al. (2004), 105 of 225 samples (47 %) tested positive for Plasmodium spp. and Haemoproteus spp. A subset of 195 samples was selected at random and tested using the protocol of Hellgren et al. (2004) for detection of Leucocytozoon spp., and 52 of 195 samples (27 %) contained parasites. Of the 52 birds testing positive for Leucocytozoon spp., only 7 were not infected by Haemoproteus spp. giving an overall parasite prevalence of 50 %. Co-infection by both Haemoproteus spp. and Leucocytozoon spp. occurred more frequently than expected by chance (χ^2_1=44.0, p<0.001): 51 % of birds infected by Haemoproteus

spp. were also infected by Leucocytozoon spp., whereas 89% birds infected by Leucocytozoon spp. were also infected by Haemoproteus spp.

Thirty-eight parasite sequences were obtained from 34 infected birds. Of these, 1 sequence was identified as a novel Leucocytozoon lineage, designated EMCIT01 (Genbank accession number JQ346795), and the remainder were identified as Haemoproteus lineages DUNNO01 and EMRUT01 (Genbank accession numbers DQ991080 and EF380192). The Leucocytozoon lineage was amplified using Hellgren et al. (2004) and all the Haemoproteus lineages were amplified using Waldenström et al. (2004).

Median infection intensity of Haemoproteus spp. in a random subsample of birds (subsample selected based on the quality of the blood smear and blind to the brightness of the PCR band) confirmed as infected through PCR (n=44) was 0.38 parasites per 10,000 erythrocytes (range 0 – 7.03 parasites per 10,000 erythrocytes). Parasites were detected in 24 of the 44 smears.

Associations with host and environmental variables

Day and year strongly influenced the prevalence of both Haemoproteus and Leucocytozoon, being retained in all top models for each parasite genus (Table I). Confidence intervals for day (both linear and quadratic terms) and year did not overlap zero for either parasite genus (Table II), suggesting a strong, non-linear, influence of day on parasite prevalence, as well as a higher prevalence of both parasite genera during the second year of the study (Figures 1 & 2). Haemoproteus prevalence increased from 39% in 2007/08 to 66% in 2008/09, whereas Leucocytozoon prevalence increased from 20% in the winter of 2007/08 to 50% in 2008/09. While both interaction terms were retained in each averaged final model, confidence intervals for all interaction terms spanned zero, suggesting no differences

in temporal variation in prevalence between years. During both years, Haemoproteus prevalence declined gradually throughout the winter, although predicted values suggest a slight increase towards the end of February (day 120; Figure 1). Leucocytozoon prevalence declined gradually until early-February (day 100) and then increased markedly (Figure 2). Predicted prevalence of either parasite did not approached zero in either year.

Host age was retained in only 2 of the 5 models examining Haemoproteus prevalence and 1 of the 7 models examining Leucocytozoon prevalence (Table I), and confidence intervals overlapped zero for both models (Table II). Similarly, host sex was retained in only 1 of the 5 models examining Haemoproteus prevalence, and 3 of the 7 models examining Leucocytozoon prevalence (Table I), and confidence intervals overlapped zero for both models (Table II), so neither variable is considered further.

DISCUSSION

We found an overall parasite prevalence of 50 % in our population during the non-breeding season over 2 yr, which was, on average, low compared to a previous prevalence of 70 % in breeding yellowhammers (Sundberg, 1995). However, Haemoproteus prevalence approached 70 % overall during 2008. Typically, haemoparasite infections relapse at the beginning of the breeding season, when circulating corticosterone levels increase (Sundberg, 1995; Cosgrove et al., 2008). Outside the breeding season in temperate environments, Plasmodium infections tend to disappear from circulating blood rapidly, with over winter prevalence at 0 % (Cosgrove et al., 2008). Less is known about the seasonal dynamics of Haemoproteus and Leucocytozoon in passerine hosts, although a previous study of Haemoproteus in breeding yellowhammers found a pre-breeding relapse during late

April and throughout May, and suggested that infections may last beyond the breeding season albeit at low intensities, although to our knowledge this was not subsequently investigated (Sundberg, 1995; Allander and Sundberg, 1997). Previous studies of haemoparasites in other systems have found various prevalences of infection outside the breeding season: for example, Barnard and Bair (1986) found Leucocytozoon in 16.5 % of 50 bird species, mostly passerines, examined between December and March in Vermont, although no Haemoproteus infections were found between November and April despite the presence of suitable hosts; Haemoproteus prevalence peaked at ~33 % during September. Barnard et al. (2010) found a 42 % Leucocytozoon prevalence during both summer and winter in rusty blackbirds Euphagus carolinus, and found no Haemoproteus infections in breeding rusty blackbirds compared to infections in 3 % of wintering birds. Deviche et al. (2010) found an over winter dip in prevalence of Leucocytozoon fringillinarum infection in white-winged crossbills Loxia leucoptera, but found this parasite genus was absent from sampled birds for only a short period during January, suggesting that the early breeding season of the crossbill initiated a pre-breeding relapse in March and April. Conversely, for Haemoproteus fringillae, this study found parasites were absent from circulating blood between January and April, suggesting that a seasonal relapse in May was indicative of novel infections following an increase in vector activity (Deviche et al., 2010), a pattern also found by Schrader et al. (2003) in red-bellied woodpeckers Melanerpes carolinus, where Haemoproteus prevalence was 0 % in January and February but peaked at 80 % in July. All 4 of these studies used blood smears alone for detection of haemoparasite infections (Barnard and Bair, 1986; Schrader et al., 2003; Barnard et al., 2010; Deviche et al., 2010), suggesting that actual prevalence may be higher because blood smears can be relatively insensitive

compared to PCR for detecting low intensity infections: for example, Fallon and Ricklefs (2008) found 35 % of PCR-detected Haemoproteus infections to not be detected on blood smears. Although PCR cannot distinguish between infective and non-infective stages (Valkiūnas et al., 2011), Haemoproteus species only cast infective gametocytes in the bloodstream and are not found in the blood as non-infective asexual stages (Pérez-Tris and Bensch, 2005; Valkiūnas, 2005), and thus any detection of Haemoproteus in the bloodstream indicates an active infection.

Interestingly, co-infection by multiple parasites was not random, with birds infected by Leucocytozoon 23.1 times more likely to be infected by Haemoproteus than those not infected by Leucocytozoon. Initial infection may be correlated: whilst Haemoproteus and Leucocytozoon are transmitted by different vectors (from the families Ceratopogonidae and Hippoboscidae, and the family Simuliidae respectively; Valkiūnas, 2005), both vectors may be more abundant in wet areas (e.g. Wood et al., 2007). Individual behaviour may make individuals more likely to encounter vectors: for example, female great tits infected by haemoparasites were more exploratory than uninfected individuals, suggesting a behavioural pre-disposition to infection (Dunn et al., 2011).

Examination of temporal trends in infection prevalence in our data suggests different patterns for Haemoproteus and Leucocytozoon infections. Leucocytozoon infections show a gradual decline in prevalence until early-February, after which prevalence starts to increase markedly, unlike Haemoproteus infections which appear to increase slightly towards the end of February. This suggests that, for both genera, we were seeing a gradual decline in patent infections as parasites were cleared from the bloodstream, albeit over a lengthy period. This is supported by our infection intensity data for Haemoproteus that showed levels in our population to be relatively

low compared to other passerine-Haemoproteus spp. systems (e.g. Bensch et al., 2000: 40-790 parasites per 10,000 RBCs; Fallon and Ricklefs, 2008: means of 6.4 and 12.1 parasites per 10,000 RBCs in the West Indies and Missouri Ozarks respectively; Asghar et al., 2011: 90 parasites per 10,000 RBCs) during the summer months. Unfortunately we cannot make direct comparisons with breeding season infection intensity in our study species because, to our knowledge, these data are only presented in Allander and Sundberg (1997) who measured parasites per 100 microscope fields, and did not standardise measurements by erythrocyte abundance, and we did not sample individuals during the breeding season. Future work could test this by collecting more extensive infection intensity data and establishing whether a decline in infection intensity occurs concurrent with reduced prevalence, expected if the pattern we observe is due to declining chronic infections.

After early-February, Leucocytozoon infection prevalence increases markedly, and Haemoproteus infection prevalence increases slightly from the end of February. This suggests a relapse of existing infection rather than a decline in chronic infection. Transmission of all 3 blood parasite genera in temperate regions is thought to be negligible throughout the winter due to a cessation of vector activity (Cosgrove et al., 2008; but see also Klei and DeGiusti, 1975 for high over winter vector activity). However, parasites remain dormant in host tissues (Valkiūnas, 2005) and are activated by stress hormones, usually at the onset of breeding, when relapses occur and parasites can be found circulating in the blood (Applegate, 1971; Allander and Sundberg, 1997). Yellowhammers start breeding relatively late, with mean first egg date on 29 May and the earliest broods being initiated in early May (Bradbury et al., 2000). Our latest sampling point during both years was 23 April, and thus there is minimal overlap between our data collection and the onset of the breeding season,

299 suggesting that this increase in parasite prevalence in our population is independent
300 of breeding-induced relapse. We suggest 3 possibilities for this apparent relapse of
301 infection.

302 Firstly, host immunity can be lowered during the winter (Hasselquist et al.,
303 1999; Møller et al., 2003; Hasselquist, 2007), which may allow a relapse of existing
304 infections because reduced immune function is often associated with increased
305 parasite prevalence (Ots and Hörak, 1998; Barnard et al., 2010). However, a multi-
306 species study including yellowhammers provided little evidence for a reduction in
307 winter immunity in this species, although the sample size was small (Møller et al.,
308 2003).

309 The second possibility is that a reduction in over winter food availability may
310 trigger a relapse of infection through increased circulating corticosterone levels:
311 whilst our population was sampled at a supplementary feeding site, this was baited
312 only sporadically and thus cannot be considered a reliable source of food. A
313 reduction in over winter food availability has been linked to population declines in
314 many farmland bird species, including yellowhammers (Peach et al., 1999; Robinson
315 and Sutherland, 1999; Bradbury et al., 2000) and food availability in the wider
316 countryside is thought to be insufficient from February onwards (Siriwardena et al.,
317 2008). Corticosterone levels can increase at times of low food availability (Kitaysky
318 et al., 2001; Clinchy et al., 2004) and poor weather (Romera et al., 2000), and
319 increased corticosterone levels have been experimentally linked to both an increased
320 parasite prevalence and an increased intensity of infection (Applegate, 1970). It
321 seems plausible, and temporally relevant, that a reduced food supply may either
322 induce relapses of haemoparasite infection or delay clearance of gametocytes from
323 the host blood stream. This possibility is supported by the higher prevalence of both

parasites during the colder winter of 2008/09 than the relatively mild winter of 2007/08. The potential implications of food-stress influencing the temporal dynamics of parasites, and subsequent implications for survival require further exploration.

A third explanation, although one that appears unlikely, is that the infections we observe result from active transmission of parasites resulting in novel infections. Little is known of the ecology of vectors in our population, so we cannot discount continuing vector transmission during the winter months; indeed, where vectors are present, over winter transmission has been recorded (Klei and DeGiusti, 1975). However, if this were the case then we would expect parasite prevalence to be higher during a warmer winter when more vectors would be likely to survive, whereas in fact we found prevalence to be higher during the colder winter of 2008/09, also coincidental with lower bird numbers despite similar sampling effort. This instead supports the idea of a higher incidence of stress-induced relapse in birds that survived the early cold spell during the autumn of 2008 (National Climate Information Centre, 2008), which is likely to have caused high mortality resulting also in the lower number of birds caught during this winter.

The high prevalence of over winter infection found in our study population suggests a previously overlooked and potentially important role of haemoparasites during the non-breeding season, possibly initiated, for Leucocytozoon, by food stress in our population. The implications of this previously over-looked period of active blood parasite infection for both host and parasite life-histories and population dynamics need further investigation. Further understanding the dynamics of Haemoproteus and Leucocytozoon infection emphasises the importance of long-term studies of host-parasite systems that allow repeated sampling of individuals through time.

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FIGURE 1. The prevalence of Haemoproteus infection varied over time and between years. Points show raw data; lines are those predicted from the final models (Table I).

FIGURE 2. The prevalence of Leucocytozoon infection varied over time and between years. Points show raw data; lines are those predicted from the final models (Table I).