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A rare study from the wintering grounds provides insight into the costs of malaria infection for migratory birds

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Malaria parasites can have strong effects on the population dynamics and evolution of migratory bird species. In many species, parasite transmission occurs on the wintering grounds, but studies to determine the consequences of infection have taken place during the breeding season, when malaria parasites circulate at chronic levels. We examined the predictors of malarial infections for great reed warblers during the northern winter in Africa, where active parasite transmission is thought to occur and naïve individuals experience acute infections. Counter to expectations, we found that winter infection intensities were lower than those encountered on the breeding grounds. One potential explanation is that reduced immune function during breeding allows parasites to persist at higher chronic intensities. We found no relationships between the incidence or intensity of infection on condition (as measured by scaled mass index, plasma metabolites, and feather corticosterone), spring migration departure dates, or home range sizes. We also tested a prediction of the Hamilton–Zuk hypothesis and found that male ornament (song) quality was unrelated to parasitic infection status. Overall, our results provide the first evidence that long-distance migrants captured on their wintering grounds are in the chronic stage of infection, and suggest that winter studies may fare no better than breeding studies at determining the costs of acute malarial infection for great reed warblers.

Parasites have powerful effects on the dynamics and evolution of animal populations, comparable to selective forces such as predation and competition (Sheldon and Verhulst 1996, Lively and Dybdahl 2000, Jog and Watve 2005, Anderson and May 2009). Haemosporidians are common blood parasites in birds, and experimental studies have successfully detected costs associated with infection (Siikamäki et al. 1997, Merino et al. 2000, Marzal et al. 2005, Knowles et al. 2010, Puente et al. 2010). Yet there is still much uncertainty as to the type and severity of these costs (Bensch et al. 2007, Knowles et al. 2010, Asghar et al. 2011, Kulma et al. 2014), particularly at different stages throughout the annual cycle and under varying environmental conditions. Some of this uncertainty may be due to different effects on the host throughout the course of an infection. Initially, hosts experience the acute stage of infection when parasites circulate in the blood at high densities and parasite-induced mortality is marked (Atkinson and Van Riper 1991, Valkiūnas 2004). But for those individuals that survive, the acute stage of infection soon transitions into the chronic stage (Asghar et al. 2012), during which host immune systems can control infection and blood parasite densities are maintained at low levels, with some variation among individuals (Asghar et al. 2011). It is during the breeding season, when hosts enter the chronic stage of infection and therefore may experience very slight effects of infection, that most wild-caught birds are sampled (Bensch et al. 2007). Consequently the costs of malarial infections at other stages of the annual cycle remain unknown for most migratory species.

One of the best-studied species with regards to malarial infections in populations of wild birds is the great reed warbler *Acrocephalus arundinaceus*. Previous work has concluded that active parasite transmission of the three most common malaria parasite lineages (*Haemoproteus nucleocondensus*, lineage GRW1; *Plasmodium ashfordi*, GRW2; *P. relictum*, GRW4) takes place in Africa, rather than on the European breeding quarters, since first-year birds still on the breeding grounds (in Sweden, Bulgaria, and Portugal) were never found to carry infection (Hasselquist et al. 2007, Zehtindjiev et al. 2008, Ventim et al. 2012). However, in the MalAvi database there are two recorded instances of infection in resident species (house sparrow *Passer domesticus*; blue tit *Cyanistes caeruleus*) which could

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indicate sporadic but very rare transmission in Europe (Bensch et al. 2009). Studies of great reed warblers during the breeding season have found negligible costs of chronic infection on annual reproductive success and survival over the short term (Bensch et al. 2007, Asghar et al. 2011, 2012). Nonetheless, it has recently been shown that individuals with persistent low-level chronic infections have reduced lifespan and lifetime reproductive success (Asghar et al. 2015). Because species population dynamics via parasite-induced mortality and reduced fitness should be more apparent in areas of active parasite transmission where acute infections occur, there have been calls to study great reed warblers in Africa rather than Europe (Bensch et al. 2007).

We sought to clarify the fitness consequences of blood parasite infections by studying great reed warblers on their wintering grounds in Africa. We hypothesised that fitness costs to the host may be more evident in areas of active parasite transmission where acute infections take place. This predicts, first, that the prevalence and intensity of infection in Africa should be higher than on the breeding grounds, and especially so for juveniles since they are naïve to infection. Irrespective of their potential fitness costs, we also expected that the species diversity comprising *Haemoproteus* and *Plasmodium* infections should be at least as great as the high levels found in previous studies conducted in Europe (Bensch et al. 2007), since transmission rarely (if ever) occurs outside Africa.

Second, because acute infection is known to reduce feeding, movement, and delay spring migration arrival schedules on the breeding grounds (Valkiūnas 2004, López et al. 2013), we predicted that infected birds with the highest infection intensities would be in the poorest condition (as measured by scaled mass index, plasma metabolites, and corticosterone in feathers '$CORT_f$'), leave latest for spring migration, and have the smallest home range sizes.

Third, the Hamilton–Zuk hypothesis predicts that elaborate male sexual ornaments should reflect improved parasite resistance (Hamilton and Zuk 1982). Bensch et al. (2007) found no association between parasite prevalence and song quality (a sexual ornament) for great reed warblers breeding in Sweden; however, in Africa, where active parasite transmission occurs, this association may become evident. Recent evidence has found that winter singing may function for song improvement (Sorensen et al. 2016b) and, therefore, males capable of investing more in winter song should have improved future reproductive success (Hasselquist et al. 1996, Bell et al. 2004). Support for the Hamilton–Zuk hypothesis has been found within species, such that more elaborate ornamentation is associated with parasite-free males (Milinski and Bakker 1990, Buchanan et al. 1999); however, evidence across species has been much more mixed, partially reflecting difficulties in making clear predictions (Read and Harvey 1989, Garamszegi and Møller 2012). Since winter song may reflect subsequent breeding song quality, following Bensch et al. (2007) we asked whether parasitised males have simpler (less elaborate) songs than unparasitised males, as would be expected if song quality reflects a male's superior ability to eradicate infections. Additionally, not all males sing in our study population and singing males show several indicators of higher quality than those that do not sing (Sorensen et al. 2016b). The Hamilton–Zuk hypothesis might therefore predict that infection prevalence should be lower for males that are able to invest in a costly sexual signal on the non-breeding grounds, than for those males that do not.

Methods

Study site and field data

We conducted fieldwork in southern Zambia, near Choma, in ca 280 ha of grass and reed bed habitat around two dams and connected watercourses (site 1: 16°39′S, 27°00′E; site 2: 16°37′S, 26°59′E), from January–April in 2011 and 2012. Individual birds had widely overlapping home ranges rather than restricted individual territories (Sorensen et al. 2016b). Nearby habitat is composed of woodland, thornbush savannah, and tobacco, maize and wheat cultivation. Great reed warblers are common across their European and sub-Saharan wintering ranges and engage in a two-stage autumn migration pattern, moulting their flight feathers at an initial staging site before continuing to Zambia where they arrive between mid-December and mid-January and complete their moult (Dowsett et al. 2008).

Great reed warblers were captured passively using 8–10 mist-nets set daily from 05:30–11:00 h. Mist-nets were moved to different locations every 1 or 2 d to ensure that sampling occurred throughout the entire study site. Playbacks or decoys were not used; this avoided biases towards the most aggressive individuals, which are unlikely to be those at the acute stage of infection. At the time of capture, tarsus length, bill length, and wing length were measured with digital calipers ($\pm$ 0.01 mm) and all individuals were weighed with an electronic scale ($\pm$ 0.01 g). All measurements were performed by the same individual to reduce observer effects. We used the scaled mass index as an estimate of body condition following (Peig and Green 2009). This index scales the mass of all individuals to that expected if they were all of identical body size, using the equation of the linear regression of log-mass on log-length estimated by type-2 (standardized major axis) regression. For this equation we chose wing length as a single measure of structural size since it was correlated most strongly with mass ($r = 0.46$, $p < 0.001$). A single measure of size is preferable to using a principle component analysis (PCA) of several different measures (Peig and Green 2009). Within 10 min of capture, a 100 μl blood sample was also taken for molecular sexing and metabolite analyses (see Plasma metabolites below); and 5–8 contour feathers (grown on the Zambian winter site) were sampled for corticosterone analyses (see below). Because great reed warblers are sexually monomorphic, sex was determined using a molecular technique. Primers for molecular sexing were modified from the primers 2550F and 2718R first described by Fridolfsson and Ellegren (1999); see Round et al. (2007) for details.

Radio telemetry

From 28 January–3 March 2012, 25 great reed warblers were captured and fitted with radio transmitters (Holohil BD-2, ON, Canada; 1.25 g; 4.3% body weight; transmitter life 9 weeks) using a leg harness (Rappole and Tipton 1991). Radio receivers and two element 'H' antennas (Telonics, AZ,

USA) were used to acquire daily locations for each tagged individual. Home ranges were calculated using 95% minimum convex polygons. Spring migration departure dates were taken as the last date each radio-marked bird was detected. Radio signals were checked for two subsequent days after departure to ensure that signals had not simply been undetected. Lemke et al. (2013) tracked the migration of Swedish breeding great reed warblers using geolocators and found that spring migration departure dates were correlated with spring arrival dates (i.e. birds that were the earliest to depart African winter sites were also the first to arrive on breeding grounds in the spring). The date of arrival to breeding grounds has important consequences for reproductive success, as the earliest arriving males have improved pairing success, fledgling success, and number of offspring recruits to the breeding population the following summer (Hasselquist 1998). Therefore, we used spring departure dates as an additional metric of over-winter success (in conjunction with over-winter body condition measured via $CORT_f$, metabolites, and scaled mass index).

Molecular quantification of incidence and intensity of infections

In order to determine the prevalence and strain of infection, genomic DNA was extracted and diluted to 25 ng μl^{-1} for use in a polymerase chain reaction (PCR). We used a two-step nested PCR approach as described in Waldenström et al. (2004), to amplify the cytochrome *b* gene of avian malaria parasites in the genera *Plasmodium* and *Haemoproteus*. For the first PCR we used the primers HAEMNF (5′-CATATAT TAAGAGAATTATGGAG-3′) and HAEMNR2 (5′-AGAG GTGTAGCATATCTATCTAC-3′) of which final product 1 μl was taken as a template in a second PCR with the primers HAEMF (5′-ATGGTGCTTTCGATATATGCATG-3′) and HAEMR2 (5′-GCATTATCTGGATGTGATAATGGT-3′). We used multiple negative controls in both PCRs. Positive amplifications, as evaluated by running a 2.5 μl aliquot of the final PCR on agarose gels, were sequenced directly with the primer HAEMF. Electropherograms were manually inspected and the proofread sequences were compared with sequences held in MalAvi (Bensch et al. 2009).

Quantitative PCR

Parasite quantification was carried out by real-time quantitative PCR (qPCR) in Mx3000P QPCR system (Stratagene). Each sample was diluted to 1 ng μl^{-1} for the quantification of infection intensities. We used primers 343F (5′GCTCACGCATCGCTTCT3′) and 496R (5′GACCG GTCATTTTCTTTG3′) that amplify a portion of the cytochrome b gene of all lineages present (Fallon et al. 2003). It is possible that some individuals were infected with more than one lineage, Asghar et al. (2011) found that 4.4% of infected great reed warblers had double infections; therefore, the lineage-specific intensities presented in Fig. 1 may be overestimates due to mixed infections. Total DNA contents were measured by real-time quantitative PCR (qPCR) using host-specific primers (*sfsr*/3Fb 5′-ACTAGCCCTTTCAGCGTCATGT-3′ and *sfsr*/3Rb 5′-CATGCTCGGGAACCAAAGG-3′) that amplify an ultra-conserved single-copy nuclear sequence (Bejerano 2004, Asghar et al. 2011). Previous studies showed that this assay gives very good agreement with parasitemia estimated by traditional microscopic methods (Zehtindjiev et al. 2008, Asghar et al. 2011). Each reaction of 25 μl included 5 μl DNA template (1 ng μl^{-1}), 12.5 μl Supermix (Platinum SYBR-green q-PCR SuperMix-UDG, Invitrogen), 0.1 μl ROX, 1 μl of each primer, 1 μl $MgCl_2$ only for 343F/496R, and ddH2O. After an initial incubation at 50°C for 2 min and 95°C for 2 min, thermal cycling followed by 42 cycles at 95°C for 15 s, 55°C for 30 s (57°C for *sfsr*3) and at 72°C for 30 s. Standard curves were produced by diluting samples of known parasitemia with uninfected great reed warbler DNA and for quantification of host DNA with ddH_2O in five step – 5 × dilutions (25 ng, 5 ng, 1 ng, 0.2 ng, 0.04 ng). Each qPCR plate contains samples and serially diluted standards in duplicate along with two negative controls. We discarded and re-ran experiments producing standard curves that were outside the range of 100 ± 15% qPCR efficiency and relative parasitemia was recalculated after adjusting for the total DNA content in each reaction (Asghar et al. 2011).

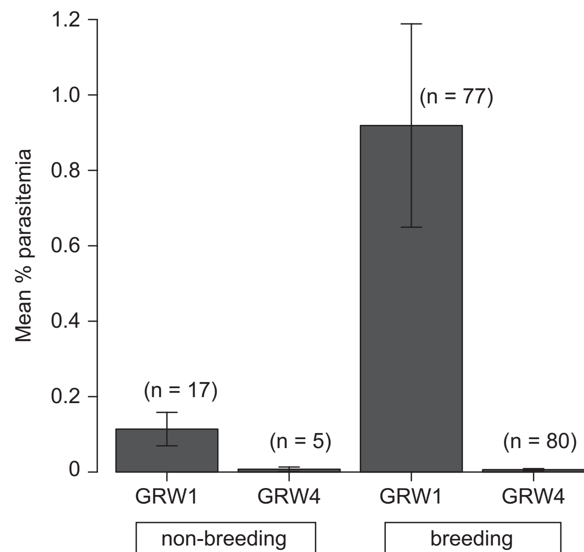

Figure 1. Mean infection intensity (% infected erythrocytes) of GRW1 and GRW4 haemosporidian lineages for great reed warblers that carried infection on their wintering grounds in Zambia (non-breeding), compared to a Swedish breeding ground (Lake Kvismaren; 59°10′N, 15°25′E). Error bars indicate standard error, and sample sizes are indicated in parentheses. GRW2 was not included for comparison since only one case was found in Zambia.

Plasma metabolites

B-hydroxybutyrate (hereafter ‘BUTY’) is an indicator of fasting since BUTY is synthesized from fatty acids and replaces glucose as fuel when glucose is unavailable (Jenni-Eiermann and Jenni 1991, Féry et al. 1996). Circulating triglycerides (hereafter ‘TRIG’) reflect fat deposition rates, at least in the short term (Jenni-Eiermann and Jenni 1994, Guglielmo et al. 2005). To quantify plasma metabolite levels, we took a 50 μl blood sample within 8 min

of capture. Blood was collected into heparinized capillary tubes from the brachial vein. Samples were then centrifuged for 10 min (maximum speed of 8000 rpm) within 4 h of collection, and the separated plasma was frozen at –20°C until analysis. Samples were transported on ice to the Schweizerische Vogelwarte, Sempach, Switzerland, for quantitative determination in May 2012. All metabolites were determined in the plasma using standard test-combinations, the Wako Auto-Kit for B-hydroxybutyrate (cyclic enzymatic method), and the enzymatic colorimetric test for triglycerides including free glycerol (Invicon HIT 917, PAP-method). Dilution curves of great reed warbler plasma were run for both metabolites to test for linearity. The triglyceride assay was adapted to small volumes (5–20 µl per determination). All samples were measured in duplicates and repeatability for both metabolites was high (0.985 ± 0.006 SE, $p = 0.001$ for BUTY; 0.961 ± 0.054 SE, $p = 0.001$ for TRIG).

Feather corticosterone

Corticosterone was extracted from feathers using a methanol-based procedure (Bortolotti et al. 2008) that has been replicated in passerines (Fairhurst et al. 2013, 2014). Because feathers grow slowly (estimated 51 d total moult duration for great reed warblers; Bensch et al. 1991), $CORT_f$ is an ecologically relevant measure of physiological responses to long-term stressors such as infection at the Zambian wintering site (where contour feathers are grown). Multiple contour feathers were collected from all individuals; therefore, two contours per bird were extracted to ensure that $CORT_f$ measurements were well within limits of detectability (Fairhurst et al. 2015). Following removal of the calamus, the length of each feather sample (proximal cut to distal tip of vane) was measured flat against the edge of a ruler. Feather samples were then placed in glass vials and cut into tiny pieces (ca 5 mm^2). Ten ml of methanol (HPLC grade; Fisher Scientific, Fair Lawn, NJ, USA) was added to each vial and samples were sonicated at room temperature for 30 min, followed by overnight incubation at 50°C. Using a glass funnel fitted with polyester fibre, vacuum filtration was employed to separate the methanol extract from the feather pieces. Collected methanol extracts were evaporated and residues were reconstituted in 600 µl of phosphate-buffered saline (0.05 M, pH 7.6) and frozen at –20°C until analysed by radioimmunoassay (RIA). Samples were extracted in two batches and the recovery efficiency of each extraction was assessed by spiking three sample extracts with approximately 5000 cpm of ^{3}H-labelled CORT. The average recoveries were 98.6% and 93.2%. Serial dilutions of feather extracts were parallel to the CORT standard curve. Samples were analysed in duplicate in two RIAs using a commercial antiserum (Sigma-Aldrich, St Louis, MO; product# C8784). The variability of each assay was assessed using six replicates of the same internal standard, and average intra-assay coefficient of variation (CV) was 7.4 (SD = 1.1) % and inter-assay CV was 5.5%. Average detection limit (%B/B_0) was 11.0 (SD = 1.5) pg CORT/100 µl of extract and all samples were above this limit. $CORT_f$ measurements were corrected for total feather sample length, reflecting the time-dependent deposition of CORT into feathers, and were thus expressed as pg CORT mm^{-1} (Bortolotti et al. 2008, Bortolotti 2010, Jenni-Eiermann et al. 2015).

Song complexity

We measured song complexity from 18 individual males that sang at our study site from Jan–Apr 2011 and 2012. Recordings were made with a Sennheiser ME66 directional microphone from within 15 m (or less) of the focal individual. We produced spectrograms using Sonic Visualiser (Cannam et al. 2010) and measured syllable repertoire size, syllable repeats, song duration, and song rates (songs min^{-1}). The number of different syllables (different sounds) sung by each male were counted visually, a method commonly used for great reed warblers and other *Acrocephalus* species (Catchpole 1986, Hasselquist 1998). Syllable repertoire size levels off after 10–12 song strophes on the breeding grounds (Catchpole 1983); to guard against any different patterns on the wintering grounds, we analysed song over at least 30 strophes.

Statistical analysis

We used two-way ANOVAs to test for relationships between malaria prevalence, sex, and age (categorical variables) and over-winter body condition (continuous response variables: scaled mass index, BUTY, TRIG, $CORT_f$). All model residuals were normally distributed. Because the sample size was not large enough to include both sex and age in the same model, we ran two separate models (A = age and malaria prevalence, B = sex and malaria prevalence; Table 1).

We used generalised linear models to test for relationships between malaria intensity (continuous variable), sex, and age (categorical variables) and over-winter body condition (continuous response variables: scaled mass index, BUTY, TRIG, $CORT_f$). As for malaria prevalence models (above), two separate models were run for sex and age (Table 2). All analyses were conducted in R ver. 2.15.0 (R Core Development Team).

To test for differences between groups, we performed unequal variance (Welch's) t-tests on ranked data, following Ruxton (2006). The test statistic for unequal variance t-tests is reported as t'.

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.5sh35> (Sorensen et al. 2016a).

Results

Overall parasitic diversity, prevalence and intensity

The prevalence of *Haemoproteus* and *Plasmodium* combined was relatively high: 45.1% involving seven different parasite lineages, including one new lineage named GRW18 (28 cases total; GRW1 = 17, GRW4 = 5, GRW6 = 2 and one case each of GRW2, GRW5, GRW8, and GRW18; n = 62 birds). This prevalence is similar to that found in studies on the breeding grounds (Asghar et al. 2011: 37.4%, Bensch et al. 2007: 43.2%). Winter infection intensities (% of infected erythrocytes) for GRW1 were lower than those measured on

Table 1. Two-way ANOVA results testing for relationships between malaria prevalence and over-winter body condition. The parameter values for malaria prevalence were similar for the two different models (A = age × prevalence, and B = sex × prevalence), and therefore we present a range of *F* and p values.

Response	Model	Parameter	*F*	p	DF
Scaled mass index	A–B	Prevalence	0.04–0.14	0.84–0.94	
	A	Age	2.09	0.15	53
		Age × Prevalence	0.72	0.39	
	B	Sex	17.1	< 0.001	54
		Sex × Prevalence	0.29	0.59	
B-hydroxybutyrate	A–B	Prevalence	0.50–0.81	0.37–0.48	
(BUTY)	A	Age	0.58	0.45	54
		Age × Prevalence	0.49	0.49	
	B	Sex	0.14	0.70	55
		Sex × Prevalence	0.84	0.36	
Triglyceride	A–B	Prevalence	0.93–1.0	0.31–0.33	
(TRIG)	A	Age	2.33	0.13	50
		Age × Prevalence	0.04	0.83	
	B	Sex	1.06	0.31	51
		Sex × Prevalence	0.78	0.38	
Feather corticosterone	A–B	Prevalence	0.01–0.02	0.87–0.90	
($CORT_f$)	A	Age	0.27	0.61	52
		Age × Prevalence	0.76	0.34	
	B	Sex	1.55	0.22	53
		Sex × Prevalence	0.89	0.35	

the breeding grounds (winter = 0.114% ± SE 0.04; summer = 0.919% ± SE 0.27; $t'_{30.3} = 3.05$, $p = 0.004$), whereas for GRW4 there was no difference in infection intensity between summer and winter (winter = 0.007% ± SE 0.006, summer = 0.006% ± SE 0.002; $t'_{4.2} = 0.09$, $p = 0.93$; Asghar et al. 2011; Fig. 1). Juveniles (first-winter birds, approx. 8 months old at the time of sampling) had marginally higher prevalence of infection than adults, but not significantly so (juvenile = 53.3%; adults = 43.4%; Fisher exact test: $p = 0.56$). Counter to expectations, infection intensity in juvenile birds was not higher than in adults ($t'_{12.8} = -0.27$, $p = 0.79$). Interestingly, intensity and prevalence of infection did not differ between the sexes (intensity: $t'_{14.2} = 0.53$, $p = 0.60$; prevalence: Fisher exact test: $p = 1$). Three birds were caught and sampled in both years: two were infected with similar intensities of GRW1 in both years, and one was free of malaria in 2011 but carried a low intensity GRW8 infection in 2012.

Does parasite infection correlate with indicators of over-winter success?

We measured three indices of body condition: scaled mass index (mean = 30.8 g, range 26.2–35.1 g), $CORT_f$ (mean = 21.0 pg mm^{-1}, range = 11.5–30.7), and plasma metabolites (BUTY mean = 1.3 mmol l^{-1}, range 0.6–1.9;

Table 2. Generalised linear model results testing for relationships between malaria infection intensity (= parasitemia, corresponding to % infected erythrocytes) and four measures of over-winter body condition. The parameter values for malaria prevalence were similar for the two different models (A = age × parasitemia and B = sex × parasitemia), and therefore we present the range of *t* and p values.

Response	Model	Parameter	*t*	p	DF
Scaled mass index	A–B	Parasitemia	(–0.52)–(–0.03)	0.60–0.97	
	A	Age	–0.14	0.89	25
		Age × Parasitemia	1.06	0.30	
	B	Sex	2.51	0.02	25
		Sex × Parasitemia	0.65	0.52	
B-hydroxybutyrate	A–B	Parasitemia	0.19–0.61	0.54–0.84	
(BUTY)	A	Age	–1.12	0.27	23
		Age × Parasitemia	0.37	0.71	
	B	Sex	–0.24	0.81	23
		Sex × Parasitemia	–0.14	0.89	
Triglyceride	A–B	Parasitemia	0.28–0.42	0.68–0.78	
(TRIG)	A	Age	–0.82	0.42	24
		Age × Parasitemia	–0.14	0.89	
	B	Sex	1.24	0.23	24
		Sex × Parasitemia	–0.34	0.74	
Feather corticosterone	A–B	Parasitemia	0.38–1.27	0.22–0.71	
($CORT_f$)	A	Age	1.36	0.19	25
		Age × Parasitemia	–1.53	0.14	
	B	Sex	–0.65	0.53	25
		Sex × Parasitemia	–0.52	0.61	

TRIG mean = 1.2 mmol l^{-1}, range 0.3–1.8). With respect to all three measures, infected birds were not in poorer body condition than non-infected birds, even when modelling effects of age and sex on condition (Table 1). Intensity of infection was also unrelated to body condition (Table 2). Males had higher scaled mass indices than females (Table 1 and 2). Spring migration departure dates did not differ between infected and non-infected birds ($t'_{7.4} = 0.45$, $p = 0.66$), and nor did home range size ($t'_{8.6} = -0.75$, $p = 0.47$).

Testing a prediction of the Hamilton–Zuk hypothesis

Among the singing males (we were able to make high quality recordings for 18 of 21 singing males; 64.7% of these 18 males carried infection), there was no relationship between song complexity (syllable repertoire size) and either malaria prevalence or intensity (prevalence: $t'_{9.3} = 0.07$, $p = 0.94$; intensity: $r^2 = 0.12$, $F = 1.3$, $p = 0.28$). Considering all sampled males, 60% of singing males (21 out of 43 males sang in Zambia) carried infections, compared to 33% of non-singing males; however, this trend was not significant (Fisher exact test: $p = 0.12$).

Discussion

Understanding the consequences of blood parasites for the survival and reproductive success of Palearctic-African migratory birds has been limited to breeding season studies, when long-term chronic infections are present. However, acute infections and parasite transmission often occur on the wintering grounds where fitness costs might be expected to be greater, but where migrants are also inherently difficult to study (Bensch et al. 2007). Contrary to expectations, we found that great reed warblers wintering in Zambia had even lower infection intensity than the already low chronic levels found in breeding birds, and that spring migration departure dates and three indices of individual condition were unrelated to infection prevalence and intensity. The overall low levels of intensity suggest that the birds we sampled had, as in breeding studies, already entered the chronic stage of infection. They may therefore represent a non-random subset of the population: first, because birds in the acute stage of infection are likely to be difficult to catch using mist-nets due to reduced movements (Valkiūnas 2004) and second, because parasite-induced mortality may quickly remove individuals with high parasite intensity from the population before they can be sampled. As a result, the effects of winter infection on wild populations remain exceptionally difficult to quantify, even when research is carried out in Africa. Finally, we did not find support for the Hamilton–Zuk hypothesis in our data, as more ornamented males (males with higher song complexity, or males that sang compared to those that did not) did not carry fewer infections than less ornamented males (Hamilton and Zuk 1982).

Several hypotheses may explain the lower levels of chronic infection intensity that we observed during the winter. First, this may reflect reduced energetic demands outside of breeding, allowing resources to be mobilised towards immune defence. Reproduction is one of the most energetically demanding periods of the avian annual cycle (Williams 1966), and a trade-off between reproductive effort and immune function has been documented in several bird species (Siikamäki et al. 1997, Knowles et al. 2009). Therefore, when birds are relieved of reproduction during the non-breeding season, resources can be re-directed towards immune system function, maintaining parasitic infections at lower, chronic levels (Gustafsson et al. 1994). This interpretation is supported by the lack of sex differences we found in infection intensities, contrasting with the breeding season when great reed warbler females have higher intensity chronic infections than males (Asghar et al. 2011), likely due to their higher investment in reproduction via egg laying, nest construction, and feeding nestlings (Sejberg et al. 2000). Interestingly, lower chronic infection intensity in winter was restricted to *Haemoproteus* (GRW1), whereas *Plasmodium* (GRW4) infection intensity was similar in both seasons. It is possible that great reed warbler defenses may be more efficient against the higher virulence of *Plasmodium* parasites (Atkinson and Van Riper 1991); therefore, infection intensities may be kept at low levels even under a taxed immune system. While this interpretation that chronic infections change in intensity throughout the annual cycle in response to competing energetic demands is intriguing, it remains speculative at present. In order to test it conclusively, individuals would need to be followed throughout the annual cycle, in conjunction with experimental manipulations of energetic state.

Second, the observed difference between breeding and wintering ground infection intensity may be due to natural variation between populations. One possible factor is different wintering locations and associated local variation in the abundance and community of vectors. Individuals from the Swedish breeding population are known to winter across a large area from west to central Africa (Lemke et al. 2013), and therefore are likely to experience a variety of different winter conditions, while birds in our study all wintered in a small area in Zambia. Variation in wintering conditions may be associated with variation in vector abundance (for example, wetter habitats have been linked to higher infection prevalence, Von Rönn et al. 2015) and infection intensity (experimental evidence has shown that exposure to high vector abundance increases blood parasitemia, Cornet et al. 2014). This, in addition to other factors, may influence selection on immune defences and contribute to differences in observed infection intensity between populations. Clearly, understanding the influence of parasites throughout the annual cycle for wild populations is important for determining the effects of specific parasites on population dynamics. However, in order to test this directly, individual parasite intensity should be monitored throughout the annual cycle, which is a considerable practical challenge using currently available tracking technology.

As noted above, it is surprising that all the infections we observed in Zambia were chronic rather than acute in nature. One possible explanation is that parasite transmission occurs on autumn migration staging sites rather than on final winter sites. Great reed warblers complete a two-stage autumn migration, first stopping at northern moulting sites for several weeks to months before continuing on to their final winter sites (Zambia in this case; Lemke et al. 2013). It is possible that parasite transmission in Africa occurs

primarily at long-term staging sites, as has been suggested from an association between feather stable isotope values and malaria infections scored at a breeding site in Sweden (Yohannes et al. 2008). Moreover, great reed warblers are known to spend up to three weeks at scattered stopover sites in central and south-eastern Europe before crossing the Sahara (Lemke et al. 2013); therefore, the possibility of parasite transmission at these sites cannot be excluded. The moulting area of the birds in the present study has been estimated from stable isotope analysis of feathers to be 678–2000 km north of the Zambian study site (Sorensen unpubl.). If great reed warblers were captured at these sites, it is possible that acute infection stages could be detected. However, it is entirely possible that reduced movements of birds with acute infections may also hinder the detection of acute infections (Valkiūnas 2004).

In summary, our results provide the first evidence that long-distance migrants captured on their wintering grounds are in the chronic stage of infection, and that winter studies may fare no better than breeding studies at determining the costs of acute infection for great reed warblers. The intensity of chronic infection was even lower during the northern winter than during the breeding season, and was unrelated to our measures of over-winter success. Despite the logistical challenges, measuring the costs of primary infections when their consequences for fitness are likely most severe remains essential for determining how parasites may affect species population dynamics.

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