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Circannual variation in blood parasitism in a sub-Saharan migrant passerine bird, the garden warbler

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Abstract

Knowing the natural dynamics of pathogens in migratory birds is important, for example, to understand the factors that influence the transport of pathogens to and their transmission in new geographical areas, whereas the transmission of other pathogens might be restricted to a specific area. We studied haemosporidian blood parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in a migratory bird, the garden warbler *Sylvia borin*. Birds were sampled in spring, summer and early autumn at breeding grounds in Sweden, on migration at Capri, Italy and on arrival and departure from wintering staging areas in West Africa: mapping recoveries of garden warblers ringed in Fennoscandia and Capri showed that these sites are most probably on the migratory flyway of garden warblers breeding at Kvismaren. Overall, haemosporidian prevalence was 39%, involving 24 different parasite lineages. Prevalence varied significantly over the migratory cycle, with relatively high prevalence of blood parasites in the population on breeding grounds and at the onset of autumn migration, followed by marked declines in prevalence during migration both on spring and autumn passage. Importantly, we found that when examining circannual variation in the different lineages, significantly different prevalence profiles emerged both between and within genera. Our results suggest that differences in prevalence profiles are the result of either different parasite transmission strategies or coevolution between the host and the various parasite lineages. When separating parasites into common vs. rare lineages, we found that two peaks in the prevalence of rare parasites occur; on arrival at Swedish breeding grounds, and after the wintering period in Africa. Our results stress the importance of appropriate taxonomic resolution when examining host-parasite interactions, as variation in prevalence both between and within parasite genera can show markedly different patterns.

Introduction

For many bird species, migration is a phenomenon that either occurs at an intra-continental scale, or between continents, where species migrate between temperate and tropical areas (Alerstam, 1990). With the migration and movement of hosts follows an increased probability for the transport of parasites to new geographical areas, hence enabling contact with new potential host populations (Smith *et al.*, 1996; Waldenström *et al.*, 2002; Mackenzie *et al.*, 2004; Ishiguro *et al.*, 2005; Ricklefs *et al.*, 2005; Olsen *et al.*, 2006). The transmission of parasites and diseases has traditionally been studied in

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systems in which a novel introduction event has already occurred, for example, during an ongoing outbreak (Mackenzie *et al.*, 2004; Ishiguro *et al.*, 2005; Stenseth *et al.*, 2008), or by analysing patterns over an evolutionary time scale (Hellgren *et al.*, 2007).

However, few studies have investigated the dynamics of pathogens in migrant bird hosts under natural conditions over their full migratory cycles (but see some studies of avian influenza; Latorre-Margalef *et al.*, 2009; Munster *et al.*, 2007). Such considerations are important to understand why some pathogens might be transferred by migratory hosts to new geographical areas where they may achieve transmission, whereas the transmission of others may be confined to a specific area (Waldenström *et al.*, 2002; Hellgren *et al.*, 2007). Here, we provide one of the first studies that examine the dynamics of globally transmitted pathogens (i.e. avian blood parasites belonging to the genera *Haemoproteus*, *Plasmodium* and *Leucozytozoon*) during a full migratory cycle in a long-distance migratory bird species.

Blood parasites of the genera *Haemoproteus*, *Plasmodium* and *Leucozytozoon* constitute a highly diverse group of vector-borne blood parasites (Bensch *et al.*, 2004, 2006a; Beadell *et al.*, 2006; Perez-Tris *et al.*, 2007), which has a global distribution, with the exception of Antarctica (Valkiunas, 2005; Beadell *et al.*, 2006; Hellgren *et al.*, 2007; Bensch *et al.*, 2009). It was presumed that parasite species of the genera *Haemoproteus*, *Leucocytozoon* and, to a lesser degree, *Plasmodium*, were highly host specific; that is, that each parasite species was confined solely to a certain host species (summarized in (Valkiunas, 2005)). With increased sampling and unambiguous identification of parasite lineages due to the introduction of PCR-based identification methods, large variations in host specificity have been observed at the level of mitochondrial cytochrome b lineages for all three genera (Ricklefs & Fallon, 2002; Beadell *et al.*, 2004; Bensch *et al.*, 2004, 2009; Hellgren, 2005; Krizanauskiene *et al.*, 2006; Hellgren *et al.*, 2009). In extreme cases, particular parasite lineages have been found in resident birds from areas as far apart as sub-Saharan Africa and temperate regions of Scandinavia (Hellgren *et al.*, 2007). Although host specificity may vary between haemosporidian genera, all three genera have been found to include parasites occurring in birds from different families: the lineage BT2 (*Leucocytozoon*) has to date been found in eight species belonging to four different families, the lineage WW2 (*Haemoproteus*) in 17 species belonging to five families and SGS1 (*Plasmodium*) in 55 species belonging to 19 different families (data retrieved 21 May 2012 from the MalAvi database (Bensch *et al.*, 2009)).

In this study, we examine circannual variation in the prevalence of 24 blood parasite lineages belonging to the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in the garden warbler *Sylvia borin*, over its full migratory cycle. The garden warbler is a long-distance migratory passerine bird species, breeding in temperate Europe and Western Asia and wintering in Western and Central Africa (Cramp, 1988). We sampled garden warblers for blood parasites at four geographical sites over the annual cycle: in chronological order, (i) early-spring departure from winter staging in sub-Saharan Africa in Amurum, Nigeria, (ii) mid-spring migration stopover on the Mediterranean island of Capri, Italy, (iii) late-spring migration on stopover in Ottenby, Sweden, (iv) late breeding-season and onset of autumn migration at Lake Kvismaren, Sweden, (v) early-autumn migration stopover in Ottenby, Sweden, (vi) mid-autumn migration stopover at Capri, Italy, (vii) late-autumn arrival to Amurum, Nigeria and (viii) early-spring departure from Amurum in sub-Saharan Africa the following calendar year. The four sampling sites were selected to represent breeding grounds, migratory stopover sites and winter staging sites used by the same garden warbler population. We based our choices on the connectivity between these areas as demonstrated by recoveries of garden warblers ringed in Fennoscandia and at Capri, Italy (see supplementary information). This approach to demonstrating migratory connectivity is necessary, and perhaps the only approach available until cost-effective tracking devices are available for such small birds, because so little bird ringing has been carried out in West Africa.

In this study, we examined (i) how overall infection rates vary over the migratory cycle, (ii) whether the different parasite genera and their component lineages show different prevalence patterns over the migratory cycle, indicating different transmission strategies and coevolutionary dynamics and (iii) whether geographical areas, or periods during the migration, are associated with accumulation of, for the species, rare parasite lineages.

Materials and methods

Study species and sampling

The garden warbler is a small passerine bird breeding across most of Europe (except the Mediterranean) and eastwards into Russia east of the Urals (Cramp, 1988). It is primarily a woodland bird, preferring deciduous forest. It is an obligate migrant: all populations winter in sub-Saharan Africa, mainly in forested areas, from the Guinea savannah region of West and East Africa down to South Africa (Cramp, 1980, 1988). West European populations of garden warblers winter in West Africa and eastern birds winter in Eastern and Southern Africa. We sampled birds breeding in Sweden and aimed to follow North European populations during migration through Europe to Nigeria in West Africa. The different populations cannot be distinguished by plumage characters, but we analysed ringing recovery data from birds ringed in Fennoscandia and at Capri, Italy, to examine connectivity between the sites. In total 1 110 ringing

recoveries, of which 19 were from sub-Saharan Africa, show the general migratory flyways of North European breeding populations via Southern and Southwestern Europe, to Western Africa and south of the Congo River basin. We further restricted the selection of recoveries to birds that were ringed or found within 100 km from either of the European sampling sites, or in the West African countries Ghana, Togo, Benin, Nigeria or Cameroon. The 184 remaining records illustrate that birds of a single population often do not migrate along a narrowly defined corridor; but that the sampling sites are clearly interconnected and thus should be regarded as sites visited by a Northern European population of garden warblers.

The sub-Saharan sampling site, Amurum, is situated in Guinea savannah and does not represent the final winter destination for North European garden warblers. It is rather used as staging area between the trans-Saharan desert crossing and the wintering grounds in, or south of the Congo River basin (Ottosson *et al.*, 2005; Iwajomo *et al.*, 2011). This is also indicated by sub-Saharan ringing recoveries: of 16 birds with credible recovery dates, six are from West Africa (as defined above), where the mean recovery date is 19 December (10 October–24 January, SD 40 days), whereas 10 recoveries are reported from the Congo basin and south thereof on average 1 month later, 19 January (12 November–15 February, SD 29 days, $n = 10$; t-test $t = 1.834$, d.f. = 14, $p_{\text{one-tailed}} = 0.044$). In contrast, garden warblers breeding in Western Europe, including Britain, seem to winter further to the west in West Africa, with six winter recoveries in Ghana and one in western Nigeria (Wernham *et al.*, 2002).

In 2003 and 2004, we sampled garden warblers for haemosporidian parasites at Lake Kvismaren in Sweden (during late breeding period and onset of autumn migration), at Ottenby Bird Observatory, Sweden (in early autumn just after leaving, and in late spring just before arriving at, the breeding grounds), on the island of Capri Italy (in autumn just prior to, and in spring just after, the migratory journey over the Mediterranean Sea) and at Amurum, Nigeria (in late autumn when arriving at, and in early spring when leaving, the winter staging area). For sampling dates and number of birds sampled, see Table 1. Birds were caught using mist nets at all sites, and also using funnel traps at Ottenby Bird Observatory. Each bird was individually ringed, ensuring no bird was sampled twice. From each individual, a small blood sample was taken, under licence, from the wing by brachial venepuncture. The blood samples were stored at ambient temperatures in SET buffer (0.015 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) during the field work, before storage at −80°C until DNA extraction. Total DNA was extracted using standard phenol/chloroform protocols (Sambrook *et al.*, 2002) or amino acetate protocols (Richardson *et al.*, 2001). Total extracted DNA was used for amplification of DNA from either of the genera *Plasmodium, Haemoproteus* and *Leucocytozoon* following the protocol and primers in Hellgren *et al.* (2004). The protocol amplifies a 480-base-pair (bp) fragment of the parasite's mitochondrial cytochrome b gene. Amplified PCR products were sequenced to assign each parasite infection to a parasite lineage. Parasite lineages were considered unique even with only a one-nucleotide substitution, as two parasite lineages with such a small difference may still show different ecological properties (Perez-Tris & Bensch, 2005a,b; Reullier *et al.*, 2006). Parasite lineages were assigned as rare if their prevalence was $\leq 2\%$ in the whole dataset. In some cases, PCR methods have been found to underestimate the occurrence

Table 1 Sites, dates and numbers of sampled garden warblers. Site-specific prevalence is shown for all haemosporidian parasites pooled (i.e. *Haemoproteus, Plasmodium* and *Leucocytozoon* spp) as well as genus-specific prevalence for the different sites.

					Prevalence				Number of lineages			
Migratory phase	Study site	Year	Date	No.	Pooled	*Haem.*	*Plas.*	*Leuco.*	Pooled	*Haem.*	*Plas.*	*Leuco.*
Arrival winter staging, autumn migration	Nigeria, Amurum	2003	18 October–7 November	57	0.53	0.26	0.14	0.16	11	1	7	3
Leaving winter staging, spring migration	Nigeria, Amurum	2004	2 February–14 April	48	0.40	0.06	0.13	0.21	9	1	4	4
Mid-spring migration	Italy, Capri	2004	27 April–4 May	60	0.33	0.10	0.05	0.18	8	1	3	4
Late spring migration	Sweden, Ottenby	2004	10–27 May	32	0.53	0.25	0.06	0.31	8	4	2	2
Breeding grounds	Sweden, Kvismaren	2004	13 July–15 August	51	0.59	0.57	0.02	0.08	7	4	1	2
Early autumn migration	Sweden, Ottenby	2004	1–25 August	41	0.41	0.10	0.00	0.34	6	3	0	3
Mid-autumn migration	Italy, Capri	2004	10 September–5 October	26	0.23	0.08	0.08	0.08	5	2	2	1
Arrival winter staging, autumn migration	Nigeria, Amurum	2004	23 September–27 October	31	0.35	0.19	0.13	0.16	10	3	4	3
			Total	346	0.39	0.18	0.06	0.19	24	7	9	8

of mixed infection (Valkiūnas *et al.*, 2006). Unfortunately, we had no access to blood slide for morphological identification or control of mixed infection. In our case, we identified eight cases of double infection by multiple peaks in the chromatogram (Perez-Tris & Bensch, 2005a,b); in five of these, one lineage was clearly resolvable and the 'main lineage' was used in the analysis, in three cases, we were not able to disentangle the parasite lineages and these three individuals [one caught in Nigeria (spring), one in Nigeria (autumn) and one at Ottenby (spring)] were discarded from the analysis. This might have caused us to underestimate the diversity slightly, but it is our belief that it did not affect the overall results of the study.

Circannual variation in prevalence

To decompose circannual variation in blood parasite infection into variation between and within parasite genera over the migratory cycle, we examined parasite prevalence categorized as (i) the pooled prevalence of all observed haemosporidian infections, (ii) genus-specific prevalence (i.e. *Haemoproteus*, *Plasmodium* and *Leucocytozoon*), (iii) lineage-specific prevalence using the most common lineages in each genus and iv) the prevalence of rare lineages in the dataset of infected individuals (i.e. those lineages with a total prevalence less than 2%, over the whole circannual sample). To allow for potential nonlinear patterns of parasite prevalence over time, we analysed infection status (a binary response) using Generalized additive modelling (GAM) (Wood, 2004, 2006). A GAM is, in essence, a generalized linear model in which a smoothed function of a covariate, in this case sample date, can be considered alongside conventional linear predictors and their interactions. More complex nonlinear functions are penalized such that a linear function would be retained if it would be more parsimonious, with smoothing parameters automatically selected by penalized likelihood maximization using generalized cross-validation (Wood, 2004). The smoothed term uses a cyclic spline for continuity between the end and beginning of the year (in this case, leaving wintering grounds in Nigeria). We incorporated a smoothed function of sampling date as a model predictor, using binomial errors and a logit link. Patterns of prevalence were visualized by constructing predicted response GAMs of sample date on parasite infection (Wood, 2006; Crawley, 2007). This approach applies the estimated model effects (Fig. 1b) to a hypothetical range of daily sampling occasions to produce the predicted response and associated confidence estimates (Fig. 1c). For direct comparisons of seasonal patterns of prevalence between genera or lineages, we used generalized additive mixed models (GAMM) (Wood, 2006), in which each host individual was represented by multiple data points reflecting infection with each genus or lineage, individual identity fitted as a random effect, and varying coefficient smoothing with respect to infection with each genus/lineage (Knowles *et al.*, 2011) Appropriate *a posteriori* treatment

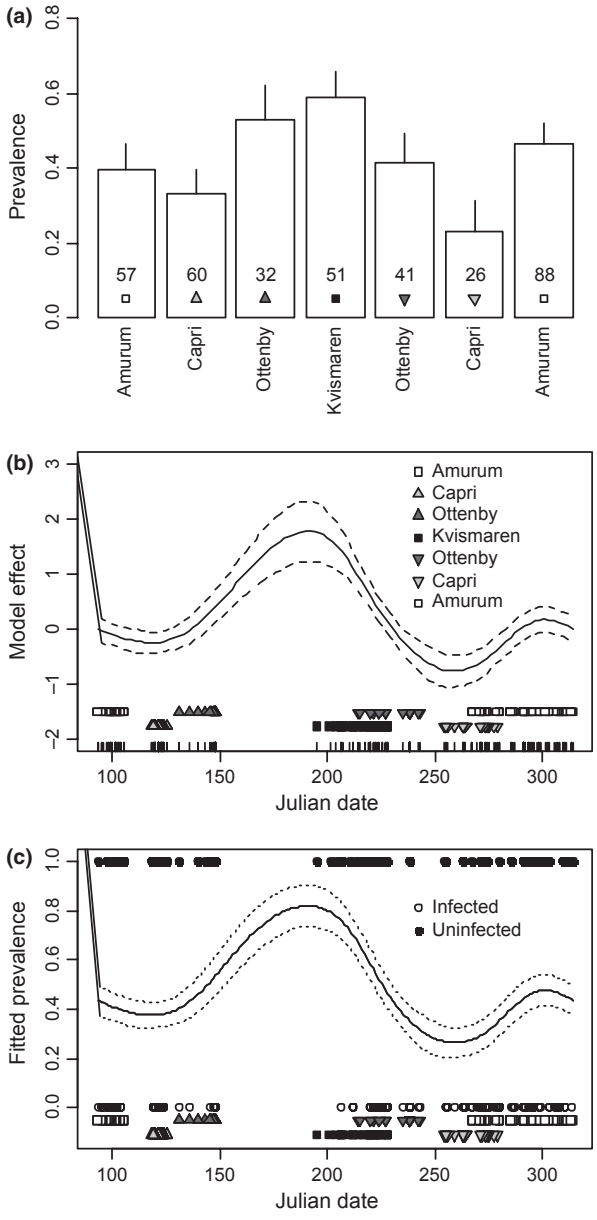

Fig. 1 Analysing nonlinear variation in circannual blood parasite prevalence. In this case, (a) raw prevalence data (shown here categorized by study site, see Table 1 for details of migratory stage) are summarized by (b) a generalized additive model (GAM) of prevalence as predicted by Julian date (using penalized least squares regression, estimated model effect plotted ± 1 SE). (c) The GAM may be visualized by examining the fitted relationship between infection status (infected/uninfected) with the predictor (Julian date, which starts with 0 on the first of January each year.). The predicted response model is presented to visualize circannual variation in prevalence (model fit ± 1 SE). See Methods for further details.

contrasts were made within factor levels of parasite genus or lineage, limited to contrasts of biological interest as indicated by preceding analyses. For comparison with selected parasite prevalence curves, a zero prevalence variable was simulated by assigning the infection status of each individual to be zero.

These analyses were conducted using the packages mgcv 1.7–13 and gamm4 0.1–5 in R 2.15.0 (Wood, 2012). Means are presented ± 1 standard error.

Results

In total, we sampled 346 garden warblers at the different sampling sites, with an average of 43 individuals per site (Table 1). The overall prevalence of haemosporidian parasites was 39%, with the highest prevalence on breeding grounds (59%) and the lowest prevalence during mid-autumn migration (23%; Table 1). We identified a total of 24 different parasite lineages, of which seven were *Haemoproteus*, nine *Plasmodium* and eight *Leucocytozoon* spp. lineages. Five of the 24 lineages were found on all the sampling locations (either during spring or autumn migration; i.e. lineages SYBOR1, WW2, SGS1, SYBOR6, SYBOR7; Fig. 2). A total of 11 lineages were only found in a sample of a single individual. Twelve of the lineages have also been found infecting species other than garden warbler (Fig. 2) and 12 lineages have, to date, been found exclusively in garden warblers. For Genbank accession numbers, see MalAvi database (Bensch *et al.*, 2009).

Circannual variation in overall prevalence

A complex smoothed function of sample date was a highly significant, and the most parsimonious, predictor of overall infections, indicating that haemosporidian infections in garden warblers show significant circannual variation in overall prevalence ($\chi^2 = 18.1$, $P = 0.0032$; Fig. 1b, 3). Overall prevalence over the migratory cycle was at its highest on arrival near the breeding grounds in Sweden, during breeding and at the onset of the southbound migration. Both the spring and autumn migration showed dips in prevalence, and although the prevalence in the winter staging areas

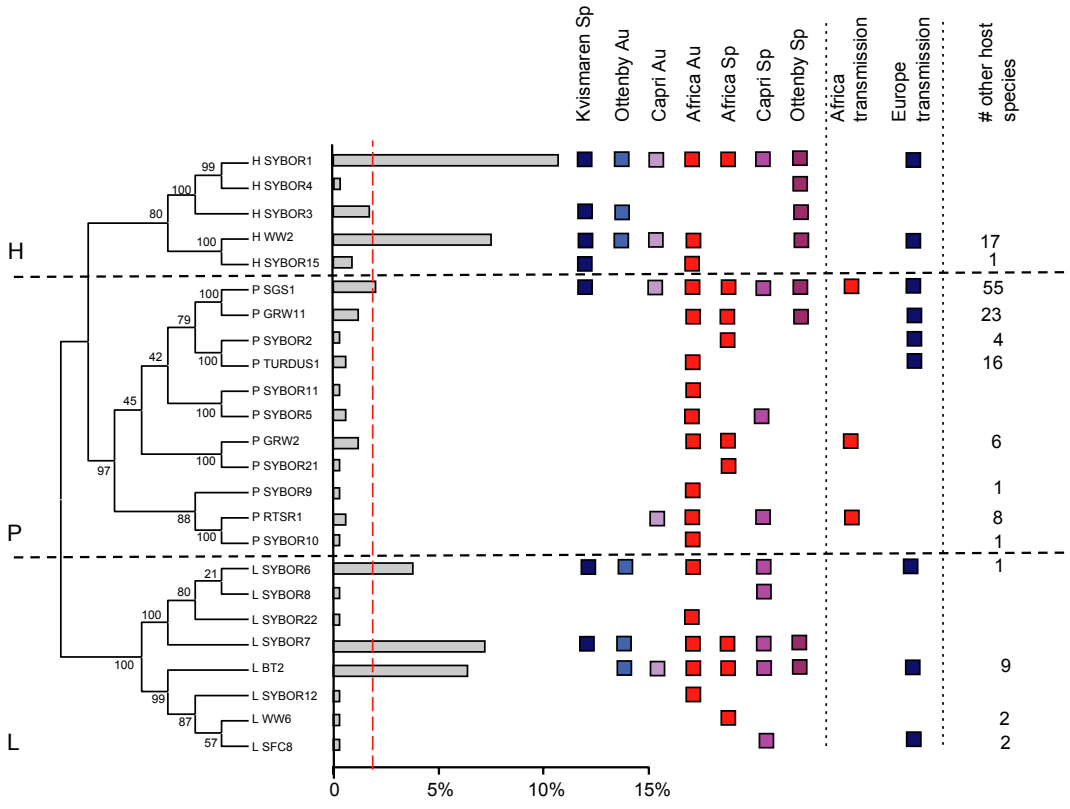

Fig. 2 Neighbour-joining tree of haemosporidian parasite lineages found in the garden warbler. Bars represent total prevalence for each lineage, coloured boxes show the sampling sites at which each lineage was found in this study. Transmission areas are determined by the presence of the lineage in either (i) a juvenile bird before migration, or (ii) in a resident bird species in either Africa or Europe. The number of additional host species in which each lineage has been found is displayed in the right column. For Genbank accession numbers, see the MalAvi database (Bensch *et al.*, 2009). The vertical dashed (red) line represents 2% total prevalence, under which we consider a lineage to be 'rare'.

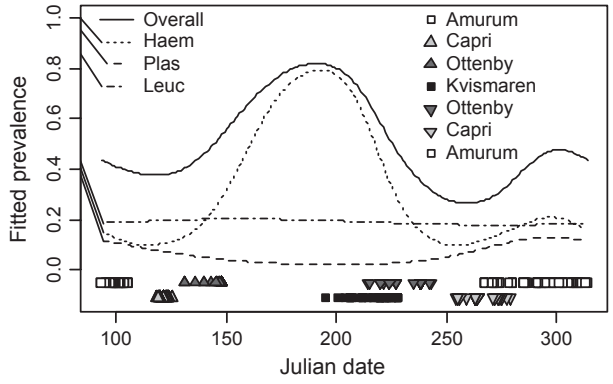

Fig. 3 Circannual variation in haemosporidian prevalence between genera. Fitted prevalence functions for overall infections, *Haemoproteus* (Haem), *Plasmodium* (Plas) and *Leucocytozoon* (Leuc) infections. Julian day starts with 0 on the first of January each year. Prevalence is written presented as a proportion between 0 and,1 where 1 represents a prevalence of 100%.

was somewhat higher than during migration, it was still lower than on the breeding grounds (Figs 1 and 2).

Separate prevalence profiles of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infections

Seasonal patterns showed significant variation between genera (Fig. 3; GAMM: $\chi^2 = 23.2$, d.f. = 2, $P < 0.0001$). The prevalence of two of the three parasite genera was predicted by smoothed sampling date: both *Haemoproteus* (GAM: $\chi^2 = 38.2$, $P < 0.0001$) and *Plasmodium* ($\chi^2 = 7.58$, $P = 0.038$) showed significant circannual variation, and the seasonal patterns of these two genera were significantly different to those of other genera (GAMM date:genus interactions, *Haemoproteus* vs. others $\chi^2 = 41.9$, $P < 0.0001$, *Plasmodium* vs. others $\chi^2 = 7.83$, $P = 0.021$). *Leucocytozoon* showed no such seasonal pattern (GAM: $\chi^2 = 0.095$, $P = 0.76$), being of a steady prevalence (mean 18.8 $\pm$ 0.021%), significantly different from zero (GAMM: $\chi^2 = 9.68$, $P = 0.0019$).

The circannual prevalence profile of *Haemoproteus* infections (Fig. 4a) showed a similar pattern to the overall prevalence although at slightly lower levels. The annual patterns of *Plasmodium* and *Leucocytozoon*, however, show strikingly different patterns. The prevalence of *Plasmodium* is lowest during breeding and onset of migration and then increases slightly when birds arrive at or leave the winter staging areas (Fig. 5). The overall *Leucocytozoon* prevalence was at an almost constant level all over the annual cycle (Fig. 6a).

Disentangling variation within genera

Lineages belonging to the same genus can have widely different prevalence profiles in a population over a certain year (Wood *et al.*, 2007; Cosgrove *et al.*, 2008). We

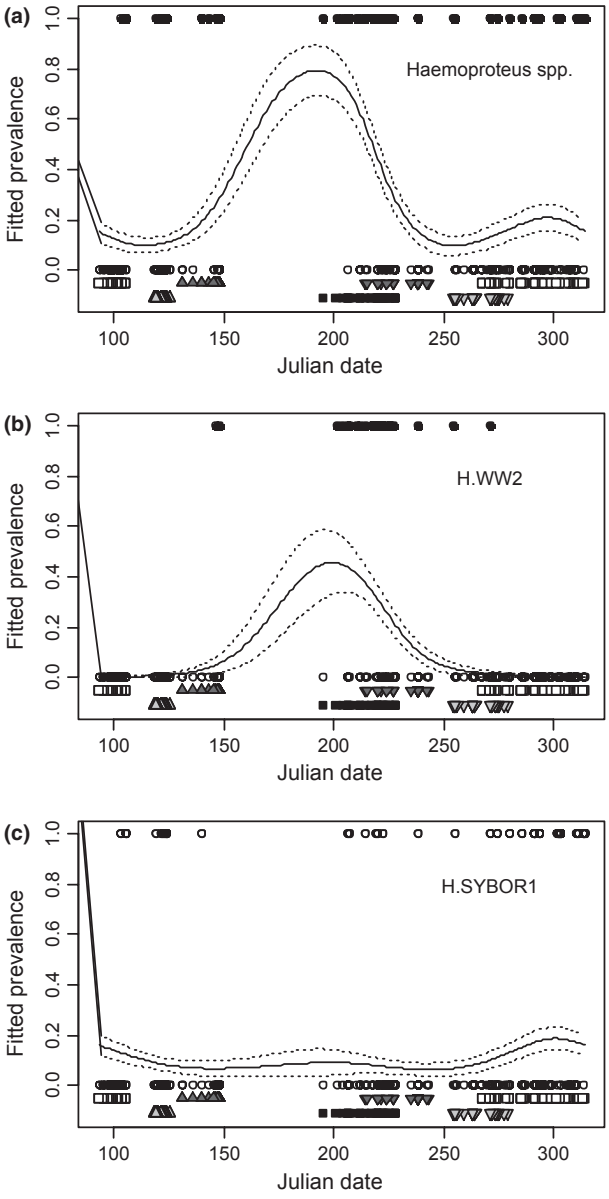

Fig. 4 Circannual variation within genera: *Haemoproteus*. Fitted prevalence functions for (a) pooled *Haemoproteus* infections, (b) *Haemoproteus* lineage WW2, (c) *Haemoproteus* lineage SYBOR1. Infection status is plotted as circles, migratory stage by squares and triangles (see Fig. 2). Smoothed functions are plotted $\pm$ 1 SE.

examined the most prevalent lineages in each genus to disentangle lineage-specific transmission patterns and coevolutionary traits. The two most common parasite lineages of *Haemoproteus* (WW2 and SYBOR1) displayed very different annual patterns. WW2 showed a highly significant circannual variation (GAM: $\chi^2 = 25.6$, $P < 0.0001$), with high prevalence during breeding and the onset of migration, and absence during the wintering period (Fig. 4b). SYBOR1, however, did not vary

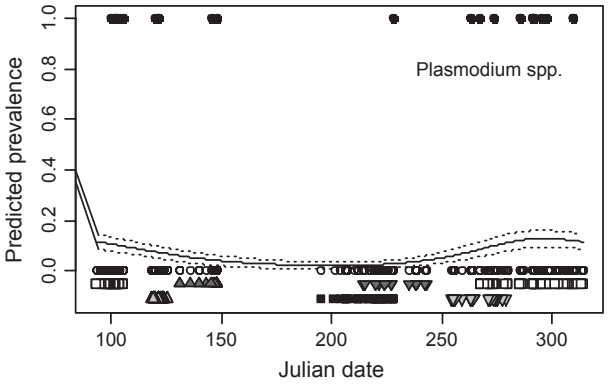

Fig. 5 Circannual variation within genera: *Plasmodium*. Fitted prevalence functions for (a) pooled *Plasmodium* infections. Infection status is plotted as circles, migratory stage by squares and triangles (see Fig. 2). Smoothed functions are plotted ± 1 SE. Prevalence is written presented as a proportion between 0 and 1, where 1 represents a prevalence of 100%.

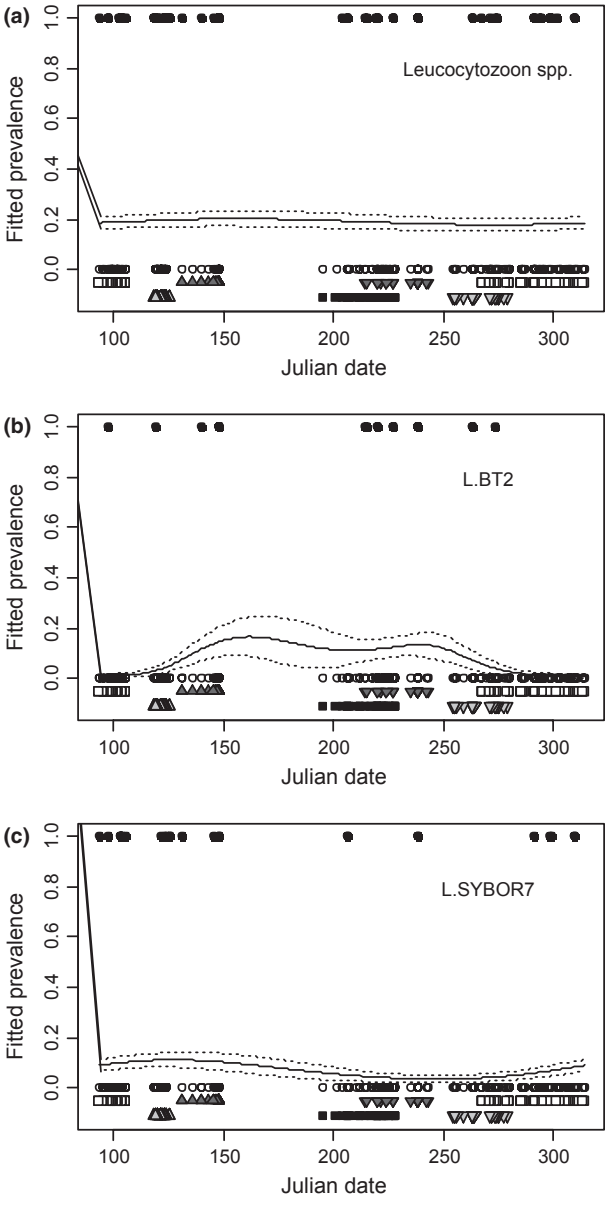

Fig. 6 Circannual variation within genera: *Leucocytozoon*. Fitted prevalence functions for (a) pooled *Leucocytozoon* infections, (b) *Leucocytozoon* lineage BT2, and (c) *Leucocytozoon* lineage SYBOR7. Infection status is plotted as circles, migratory stage by squares and triangles (see Fig. 2). Smoothed functions are plotted ± 1 SE. Prevalence is written presented as a proportion between 0 and 1, where 1 represents a prevalence of 100%.

significantly in prevalence over the year (GAM: $\chi^2 = 7.21$, $P = 0.11$), instead prevalence was level – and significantly different from zero (GAMM: $\chi^2 = 9.68$, $P = 0.0019$) – over the migratory cycle with a small increase in prevalence in winter (Fig. 5c). These two *Haemoproteus* seasonal patterns were significantly different from each other (GAMM *a posteriori* contrast: $\chi^2 = 9.68$, $P = 0.0019$). The higher prevalence of *Plasmodium* spp. in winter (Fig. 5) was not explained by variation in the most common *Plasmodium* lineage, SGS1, which showed no significant circannual variation. Pooled *Leucocytozoon* infections showed no circannual variation in prevalence, however, considering that the two most common *Leucocytozoon* lineages revealed some evidence for a contrasting pattern: BT2 varied circannually in prevalence ($\chi^2 = 11.8$, $P = 0.020$) with a bimodal distribution of one peak in late spring migration and another during early autumn migration (Fig. 6b), whereas SYBOR7 showed a more evenly distributed prevalence over the migratory cycle, although this circannual pattern only approached statistical significance ($\chi^2 = 5.78$, $P = 0.062$; Fig. 6c). This is weak evidence for contrasting seasonal patterns between these two lineages, because the seasonal patterns of these two *Leucocytozoon* lineages were not significantly different from each other (GAMM *a posteriori* contrast: $\chi^2 = 1.08$, $P = 0.30$), and SYBOR7 was of such low and even prevalence that its circannual pattern was not significantly different from zero (GAMM *a posteriori* contrast: $\chi^2 = 0.0013$, $P = 0.97$).

Rare parasite lineages and lineage diversity

In the case of *Plasmodium* and *Leucocytozoon*, 14 of 15 rare lineages occurred only in winter staging areas or the following spring migration stopover in the Mediterranean; whereas two rare lineages of *Haemoproteus* occurred only on or close to breeding grounds and one occurred on breeding grounds and winter staging areas, but not during migration (Fig. 2). When analysing the overall occurrence of rare lineages over the migratory cycle, the highest probability of finding a rare lineage

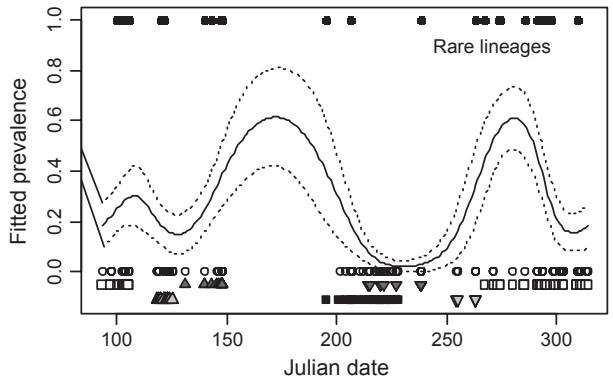

Fig. 7 Circannual variation in the prevalence of rare parasite lineages. Rare parasite lineages were defined as those with less than 2% prevalence. Infection status is plotted as circles, migratory stage by squares and triangles (see Fig. 2). A fitted prevalence function was estimated only among infected individuals. Prevalence is written presented as a proportion between 0 and 1, where 1 represents a prevalence of 100%.

occurred when garden warblers were sampled in Africa or when they arrived on the Swedish breeding grounds ($\chi^2 = 21.42$, $P = 0.006$, Fig. 7). Seven of these rare lineages are known to be transmitted in Europe, because the lineages have either been found in juvenile migrants before autumn migration, or in a resident European bird species (data extracted from MalAvi (Bensch *et al.*, 2009 & Hellgren *et al.*, 2007), based on 161 different studies, conducted in 92 different countries including 659 different host species (MalAvi 2012-11-23). Three other rare lineages have been found in African resident bird species, thus confirming transmission in Africa (Fig. 2). However, with increased sampling of avian haemosporidians, some of these lineages might be confirmed to have transmission both in Europe and in Africa in the future. However, based on strong phylogenetic signals of transmission areas for both *Haemoproteus* and *Leucocytozoon*, transmission area (i.e. Europe or Africa) seems to be an evolutionary robust trait (Hellgren *et al.*, 2007).

Discussion

We have shown that the prevalence of haemosporidian blood parasites in a migratory bird species varies significantly over the annual cycle, with high overall prevalence in the population on the breeding grounds and at the onset of autumn migration, followed by marked decreases in prevalence during mid-migration both in spring and autumn. When disentangling the patterns, differences in prevalence profiles emerged between the genera and well as within genera for the *Heamoproteus* lineages WW1 and SYBOR2. Our results points towards the possibility that different parasites lineages have evolved different transmission strategies or cues that make them leave dormant tissue stages during different parts of its host's migratory cycle and that this is reflected by the significant difference in circannual prevalence profiles both between parasite genera, but also within. Therefore, we would like to raise the awareness that a range of different host-parasite interactions might underlie apparent variation in overall parasite prevalence.

The absence of a parasite in the bloodstream might be because either the host individual is not infected, the parasite is dormant and found in tissues and not the bloodstream (Valkiunas, 2005), or that it occurs in such low intensities in the blood that it is not detectable by PCR screening. If the parasite is found in the blood of the host, it is, in the case of *Haemoproteus* and *Leucocytozoon*, always as gametocytes, that is, at the final (sexual) transmission stage of the parasite (Valkiunas, 2005), whereas in the case of *Plasmodium*, the blood can also include asexual reproduction stages of the parasites. When present in the blood, haemosporidian parasites infect blood cells which are lysed to different degrees, potentially causing different degrees of anaemia (Atkinson & van Riper, 1991). Thus, there might be a trade-off for the parasite, either (i) to be in the bloodstream and potentially harm the host but also being available to be transmitted by a vector or (ii) staying dormant in host tissues; probably causing less severe fitness effects, but thereby loosing the potential of being transmitted. The outcome of this trade-off for the parasite is likely to be influenced mainly by the probability of parasite transmission, which in turn is influenced by the abundance of compatible vectors and the effects which the infection has on the host.

When investigating parasite prevalence in correlative studies of wild populations, it is difficult to identify the processes behind the observable patterns. For example, low prevalence could result from (i) the absence of infected individuals due to high parasite-induced mortality of the hosts, (ii) the parasite's strategy not to be in the blood stream at a given point in the migratory cycle or (iii) individuals either not having been exposed to the parasite or having recovered from the infection. Similarly, high prevalence can be caused by several mutually operating processes, such as (i) an active strategy of the parasite to be present in the bloodstream and therefore enable transmission, (ii) physical stress of the host which suppresses immune function and allows the parasite to proliferate, and (iii) a high rate of exposure of the host to the new infections of parasite in question. We will discuss our observed prevalence pattern in the light of these scenarios.

Overall prevalence

It is a well-studied phenomenon that seasonal relapses occur in haemosporidian parasites in temperate regions. This has been shown for parasites belonging to all three

of the genera (Cosgrove *et al.*, 2008; Valkiunas, 2005; p185-187 and references therein). These relapses might either be seasonal, with relapse often occurring in the spring and might be induced by physiological cues in the host (Applegate, 1971), which in turn might be influenced by abiotic cues such as day length and temperature. Such relapses increase the infectivity of the parasite by being more infective for the vectors (Applegate *et al.*, 1971). Relapses might also be nonseasonal and are then often found in haemosporidian parasites transmitted in warmer climates. However, relapses are not found in all species of haemosporidians (e.g. Hatchwell *et al.*, 2000; Valkiunas, 2005).

Being a long-distance migrant, garden warblers might be exposed to avian blood parasites over the whole calendar year, including both parasites with seasonal relapses as well as parasites with nonseasonal relapses. In the case of the garden warbler, the pooled prevalence patterns reveal that a proportion of the population carry active infections of some kind of blood parasite throughout the whole annual cycle (Fig. 3). This difference between resident and migratory species could stem from one of two differences. On one hand, the lack of parasites during winter in the resident bird species could be a result of clearance of the infection during winter, followed by reinfections in spring. On the other hand, the adaptive strategy of parasites infecting resident species may involve leaving the blood stream for dormancy in the tissues during winter, due to absence of vectors and thus no possibilities of transmission, and subsequently relapsing in spring when transmission is enabled again with the return of vectors (Applegate *et al.*, 1971; Valkiunas, 2005). In the garden warbler, the occurrence of winter infections could thus be due to the fact that some parasites have different transmission periods to match patterns of vector abundance at different sites or due to the presence of parasites exhibiting nonseasonal relapses.

The overall prevalence pattern showed a peak during the breeding period and at arrival in West Africa, with prevalence troughs during spring and autumn migration periods. During migration, parasites might stay dormant or at levels of parasitaemia below detection for several reasons. First, suitable vectors might be absent from stopover sites, and once the parasites finally have matured in the vector, the majority of hosts might already have passed through. Second, the migration itself reduces the survival chances of the host, and if the parasite is occurring out in the bloodstream, and not in dormant internal organ stages, the survival probability of the host might be further reduced, thus also reducing the survival probability of the parasite without the gain of potential transmission. However, a study of redwings *Turdus iliacus* showed a contrasting pattern, with experimentally induced Zugunruhe (migratory restlessness) resulting in relapses of dormant infections of *Borrelia garnii,* a spirochaete bacterium (Gylfe *et al.*, 2000). One possibility for the contrasting patterns between haemosporidia and *Borrelia* could be due to different effects on host survival leading to different evolutionary strategies, or that *Borrelia* also shares hosts across species that do not migrate (i.e. mammals as well as sedentary and migratory birds).

An alternative explanation for the overall lower prevalence during migration is that it might be a consequence of reduced survival caused by the parasite, such that hosts with detectable parasitaemia suffer higher mortality during demanding migratory journeys, for example, the crossing of the Sahara desert or the Mediterranean, compared with individuals with low levels of infection (Westerdahl *et al.*, 2005). The high prevalence when arriving to the breeding grounds at the final stage of their northward spring migration would then stem from relapses in individuals that were able to keep the intensity of the infection at a low level during migration (Fig. 4).

Lineage-specific prevalence patterns

When dividing total haemosporidian prevalence into prevalence of parasites belonging to any of the three genera, we observed that the mid-migration troughs in prevalence are mainly due to circannual variation in *Haemoproteus* lineages (Fig. 3), and that the wintering peak is to some extent augmented by *Plasmodium* infections. When further dividing the *Haemoproteus* lineages into the two most common lineages, we found two totally different patterns which shed light on the observed mid-migration troughs in prevalence.

The increase in prevalence of the WW2 lineage starts already when birds are arriving to the breeding grounds in spring and the high prevalence lasts until they are leaving the breeding ground in northern Europe in autumn. Moreover, we know that this lineage is transmitted in Europe, whereas we have no indication of transmission in Africa. The lineage is then almost absent, except for two cases one infected bird at Capri during autumn and one individual arriving in Africa, in the population during the mid-migration period as well as on the wintering grounds. This could be a consequence of either the parasite's dormancy in internal host organs, or the impossibility of transmission in Africa due to vector availability or climate. In addition, we cannot exclude the possibility of host recovery from WW2 infections in late summer. However, based on our data, it is more likely that the parasite is dormant during autumn and winter, because we find it in the blood of migrants at the arrival on the breeding grounds (found in two birds in late May). For these birds to have a detectable nondormant infection, the biting midge that infected them must have taken its blood meal in late April, when passing stopover sites in southern Europe.

The second lineage SYBOR1 is found throughout the year (Fig. 2) with no significant peaks or troughs in the

prevalence profile (Fig. 4c). This suggests either that transmission does occur in both the breeding and the wintering areas, or, if no circannual transmission is possible, that the parasite is occurring in the blood stream at periods when transmission cannot occur. Tropically transmitted haemosporidian parasites do occur in the bloodstream of long-distance migrant birds during summer in their European breeding areas without transmission taking place (Bensch *et al.*, 2006b; Hellgren *et al.*, 2007).

Pooling the prevalence of parasites with different transmission strategies may result in spurious circannual patterns in prevalence during migration. For example, in the case of our garden warbler study, a trough in total haemosporidian prevalence during autumn migration may constitute a break point where one lineage (WW2) has dropped in prevalence perhaps because of the difficulty of transmission in Africa, and another lineage (SYBOR1) exhibit a more even distribution throughout the year (Fig. 4a–c). Hence, overall patterns of parasite prevalence might stem from many different parasite lineages that exhibit different prevalence patterns over the annual cycle, thus makes it hard to interpret prevalence patterns based on lineages pooled within genera. Our data show that to understand interactions between blood parasites and their bird hosts, one should to take into account that different parasite lineages might have different transmission strategies and circannual adaptations which in turn might have different effects on its host.

The overall *Leucocytozoon* spp. prevalence remained stable at around 20% over the whole annual cycle in the garden warbler. However, a closer inspection of the two most common lineages reveals that, in fact, circannual patterns also exist for *Leucotyzoon*. The BT2 lineage had a bimodal shape, with peaks when the birds arrived and left the breeding grounds (Fig. 6a–b). The second lineage, SYBOR7 had a more even distribution, which only approached statistical significance ($P = 0.062$).

The prevalence of *Plasmodium* spp. was comprised of many rare lineages, most of them detected mainly during the nonbreeding period (Fig. 5). SGS1, the most common *Plasmodium* lineage, had a prevalence curve, which was apparently independent of time and location, with infected birds found at all locations but one (Fig. 2). This corroborates earlier findings, which have found that the SGS1 lineage is one of very few lineages that can be transmitted both in Africa and Europe, as it have been found in several nonmigratory species both in temperate region in Europe as well as in resident African species (Hellgren *et al.*, 2007; Bensch *et al.*, 2009).

Occurrence of rare parasite lineages

When screening a passerine bird species for avian blood parasites, a common finding is that the parasite community within that host species often is comprised of a few common lineages followed by a tail distribution of rare parasite lineages (found in a few or a single host individuals). This pattern has been found also in other well-sampled European passerine bird species, such as blackcaps (Perez-Tris & Bensch, 2005b), great reed warblers *Acrocephalus arundinaceus* (Bensch *et al.*, 2006b) and house sparrows *Passer domesticus* (Bonneaud *et al.*, 2006). This pattern was also apparent in the garden warbler (Fig. 3). When finding a rare lineage we cannot exclude the scenario that the host is a 'dead-end' for these parasite with the implications that the parasite might not reach maturation in this specific species and might thus not be transmitted further. However, being infected with a novel parasite that you are not adopted to might cause high levels of mortality despite the fact that it cannot be transmitted further (Olias *et al.*, 2011). Importantly, the tail of rare lineages comprised 25% of all infections. For the host, however, rare parasite lineages might also have important evolutionary implications. When hosts are exposed to common parasites this should result in coevolution between parasites and the host, as every evolutionary change in the host or the parasite that increases host survival would also be beneficial for host's offspring, because they are likely to be exposed subsequently to the same common parasite lineages. However, with the uncommon lineages the scenario might be different, because even though the risk of being exposed to and infected by an uncommon lineage is fairly high, the probability of the offspring being infected by the same lineage is low. Several studies have reported on specific immune alleles (i.e. major histocompatibility complex (MHC) alleles)/specific *Plasmodium* lineage associations, both in terms of prevalence as well as intensity of infection (Westerdahl *et al.*, 2005, 2012; Bonneaud *et al.*, 2006; Loiseau *et al.*, 2008). To date, we do not know the exactly specificity level of the immune system against different lineages of haemosporidians, that is, do closely related parasite lineages as WW2 and SYBOR15 (Fig. 2) require unique MHC alleles or what is the level of cross-infection immunity provided for closely related parasite lineages? Therefore, having more knowledge about this question would help us to understand the delicate trade-off in either having a broad defence against a wide array of parasites, or an immune system adapted to the more frequently encountered lineages. In our case, the uncommon lineages were found predominantly in samples from the nonbreeding area (Fig. 2), probably reflecting increased parasite diversity in the African bird community (Møller & Erritzøe, 1998; Hasselquist, 2007). Furthermore, there is a decrease in parasite diversity from Africa (20 lineages) to Europe in spring (14 lineages) and to Europe in autumn (eight lineages) (Fig. 2). One alternative explanation for this decrease in diversity or lineage-specific prevalence could be parasite-induced mortality of certain lineage, a scenario that previously has been put forward in Africa-transmitted *Plasmodium* parasites (Westerdahl *et al.*, 2005). If so, this would

mean that by being a migrant, birds not only increase the time over which they are exposed to parasites (as compared with resident birds in temperate regions that lack parasite transmission during autumn and winter (Cosgrove *et al*., 2008)), but they are also exposed to a higher diversity of parasites by visiting areas with totally different bird communities and their accompanying parasites. Hence, this then constitutes a 'cost of migration' (Waldenström *et al*., 2002) with important implications. For example, being a migrant bird would mean quite different demands on the immune system being exposed to a more diverse parasite fauna, as compared with resident bird species that might be able to adapt to a more stable and homogenous parasite fauna (Hasselquist, 2007). In the case of birds wintering in Africa, one should be aware of the possibility that the occurrence of rare lineages might be a result of sampling different populations of garden warblers, which breeds and migrates outside our sampled areas. If different populations are aggregating at the wintering areas and have population-specific parasite lineages, this might inflate the occurrence of 'rare lineages' at the wintering areas. To pinpoint the exact accumulation of parasites throughout a migratory cycle would call for repeated sampling of the same individuals throughout the migratory cycle, a monumental task at the moment. However, as the tracking devices for passerine birds species are getting smaller and more efficient, this might not be an impossible task for future researchers.

Concluding remarks

This is one of the first studies that follow the parasitism in a migratory passerine bird species over the whole annual cycle. By doing so, we have highlighted that the transmission strategies of a parasite might have strong effects on its potential to be transported to new areas. For example, a parasite adapted to transmission in Europe during summer and which is not present in the blood during migration would have very low chances of infecting African bird species. We have further shown that related parasites can have different circannual prevalence patterns in the same host species.

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Supporting information

Additional Supporting Information may be found in the online version of this article:
Figure S1 Recoveries of 1 110 garden warblers ringed in Fennoscandia or at Capri, Italy.

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