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Isotope signatures in winter moulted feathers predict malaria prevalence in a breeding avian host

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Abstract It is widely accepted that animal distribution and migration strategy might have co-evolved in relation to selection pressures exerted by parasites. Here, we first determined the prevalence and types of malaria blood parasites in a breeding population of great reed warblers *Acrocephalus arundinaceus* using PCR. Secondly, we tested for differences in individual feather stable isotope signatures (δ^{13}C, δ^{15}N, δD and δ^{34}S) to investigate whether malaria infected and non-infected birds had occupied different areas in winter. We show that birds moulting in Afro-tropical habitats with significantly higher δ^{13}C and δ^{15}N but lower δD and δ^{34}S values were more frequently infected with malaria parasites. Based on established patterns of isotopic distributions, our results indicate that moulting sites with higher incidence of malaria are generally drier and situated further to the north in West Africa than sites with lower incidence of malaria. Our findings are pertinent to the general hypothesis that animal distribution and particularly avian migration strategy might evolve in response to selection pressures exerted by parasites at different geographic scales. Tradeoffs between investment in energy demanding life history traits (e.g. migration and winter moult) and immune function are suggested to contribute to the particular choice of habitat during migration and at wintering sites.

Keywords Stable isotopes · Bird migration · Great reed warbler · Moult · Avian malaria

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Introduction

Since many long-distance migratory bird species cross ecological barriers using long and non-stop flights, such barriers might have minor effects on their patterns of winter distribution. In such species, other ecological or biological factors, such as the selection pressure exerted by pathogenic agents, may play a profound role in determining habitat selection and species ranges (e.g. Brown et al. 1996; Piersma 1997). Due to their higher mobility, migrating birds that breed at northern latitudes and winter in tropical regions have a higher likelihood of exposure to malaria parasites than non-migrating resident species (Waldenström et al. 2002; Fallon et al. 2005). The prevalence of avian malaria parasites (genera *Haemoproteus* and *Plasmodium*) has been shown to differ between habitats and closely located areas (Freeman-Gallant et al. 2001; Bensch and Åkesson 2003; Wood et al. 2007) and even at a micro-habitat scale, e.g. in relation to cavity or colony nest height (Garvin and Remsen 1997).

Blood parasites that succeed in escaping the host's immune system could impose strong fitness costs on their

host (Valkiūans 2005; Zehtindjiev et al. 2008). Complementary to evolving a protective immune system, migrating hosts can defeat parasites by evolving 'avoidance behaviour' such as selecting areas or niches that have lower densities of possible pathogenic reservoirs (e.g. closely related resident species) or avoiding biomes that support higher vector densities (Piersma 1997; Hasselquist 2007).

Stable isotope composition of animal tissues has been widely applied to track migration patterns and distribution (reviewed in: Hobson 1999; Rubenstein and Hobson 2004). Briefly, isotopic ratios of the local environment are incorporated into the organism during nutrient uptake and pass to higher consumers through ecological food chains (De Niro and Epstein 1978). Tissues synthesised in such locations can be used to infer localised and small- to large-scale habitat-specific signals. The relative amount of stable carbon (δ^{13}C), nitrogen (δ^{15}N) and sulphur (δ^{34}S) isotopes in plant and animal tissues can be used to assess xeric versus mesic or marine versus freshwater ecosystems or trophic position in food webs (e.g. Minagawa and Wada 1984; Marra et al. 1998). Global patterns of deuterium (δD or δ^{2}H) in rainfall that transmit through the local food web provides a mechanism to track animal movements within and between continents (e.g. Hobson 2003).

The great reed warbler (*Acrocephalus arundinaceus*) is a long-distance migrant that breeds in reed marshes in Europe and Asia and winters in tropical Africa, in habitats ranging from swamp vegetation to dry low and open scrublands far from water (Cramp 1992). The southward migration of this species is known to occur in two stages. Birds from northern Europe leave their breeding grounds in August but interrupt migration and undergo a complete moult (flight and body feathers) during the first half of the winter (September–December) in sub-Saharan West Africa (Bensch et al. 1991; Hedenström et al. 1993). When the moult is completed, they recommence migration (second stage) to more southerly wintering areas. During migration, winter and moult, the species is exposed to different habitats and a high diversity of Afro-tropical malaria parasites (Waldenström et al. 2002; Hellgren et al. 2007a). Studies of great reed warblers breeding in south Central Sweden recorded 17 lineages of malaria parasites, most of which have transmission in Africa (Bensch et al. 2007; Hasselquist 2007; Hellgren et al. 2007a). Ringing recoveries from this Swedish study site indicate that the moulting range extends from the Ivory Coast in the west to Chad in the east (Fig. 1).

The objective of this study was to investigate the link between the choice of wintering area and malaria prevalence. We used feather stable isotope signatures to track whether great reed warblers wintering in different areas show different patterns of malaria parasite prevalence (though they are sympatric at their breeding grounds). Although little is known on the temporal overlap of migration, moult and malaria infection during the winter, we propose two alternative (but not mutually exclusive) spatio-temporal patterns of wintering/moulting area choice:

1. Habitat separation—a parapatric situation where individual birds rely on different moulting habitats that may abut by an ecotone but do not entirely overlap, i.e. biomes characterised by varying conditions for transmission of malaria.
2. Geographic separation—an allopatric situation where individual birds moult and acquire malaria parasites in different physically separated areas, i.e. exhibit non-overlapping, distinct winter distribution with different levels of malaria transmission.

To distinguish between the two alternatives, we compared the known patterns of regional isotopic distributions to the results of the four elements considered in the study. Most tropical plants that employ C_4 photosynthesis have a mean δ^{13}C value around −12.5‰. In contrast, C_3 plants associated with cooler, moister habitats have more depleted δ^{13}C values (on average: −27‰). C_3/C_4 plant abundance in sub-Saharan Africa is assumed to follow a rapid gradient between ca. 5–15°N (Still et al. 2003; Still, personal communication) with the highest proportion of C_4 plants in a band around 10°N of the equator. Most terrestrial plants have mean δ^{15}N range of 3–15‰, but there is a strong negative correlation between aridity and δ^{15}N, particularly in Africa (e.g. Lajtha and Michener 1994; Ehleringer and Cooper 1988; Cerling et al. 1998). However, δ^{15}N values often vary within regions between nearby habitats (Lajtha and Michener 1994). Environment δD fractions are temperature-dependent, and a strong spatial correlation exists between the annual mean temperature and the mean isotopic ratio δD in precipitation (e.g. Petit et al. 1999). Terrestrial birds often have δ^{34}S values less than +10‰, whereas animals from more marine/coastal areas have values falling between +16 and +18‰. δ^{34}S values register the proximity of the diet to a marine source (also known as the 'sea spray' effect; for review and control experiments, see Richards et al. 2003).

Based on the different expectations for the four elements on the relationships between isotopic values, habitat and geography, we derived the following predictions that would discriminate between the above hypotheses. If the infected and non-infected birds were moulting in the same geographical area but in different habitats (parapatric situation), we would expect differences in δ^{13}C and δ^{15}N but not in δD and δ^{34}S (supporting hypothesis 1). This is because the same geographical region is expected to have the same rainfall pattern (δD) and be exposed to a similar influence from a marine environment (δ^{34}S). Hence, if infected and non-infected birds differ in δD and/or in δ^{34}S, it would

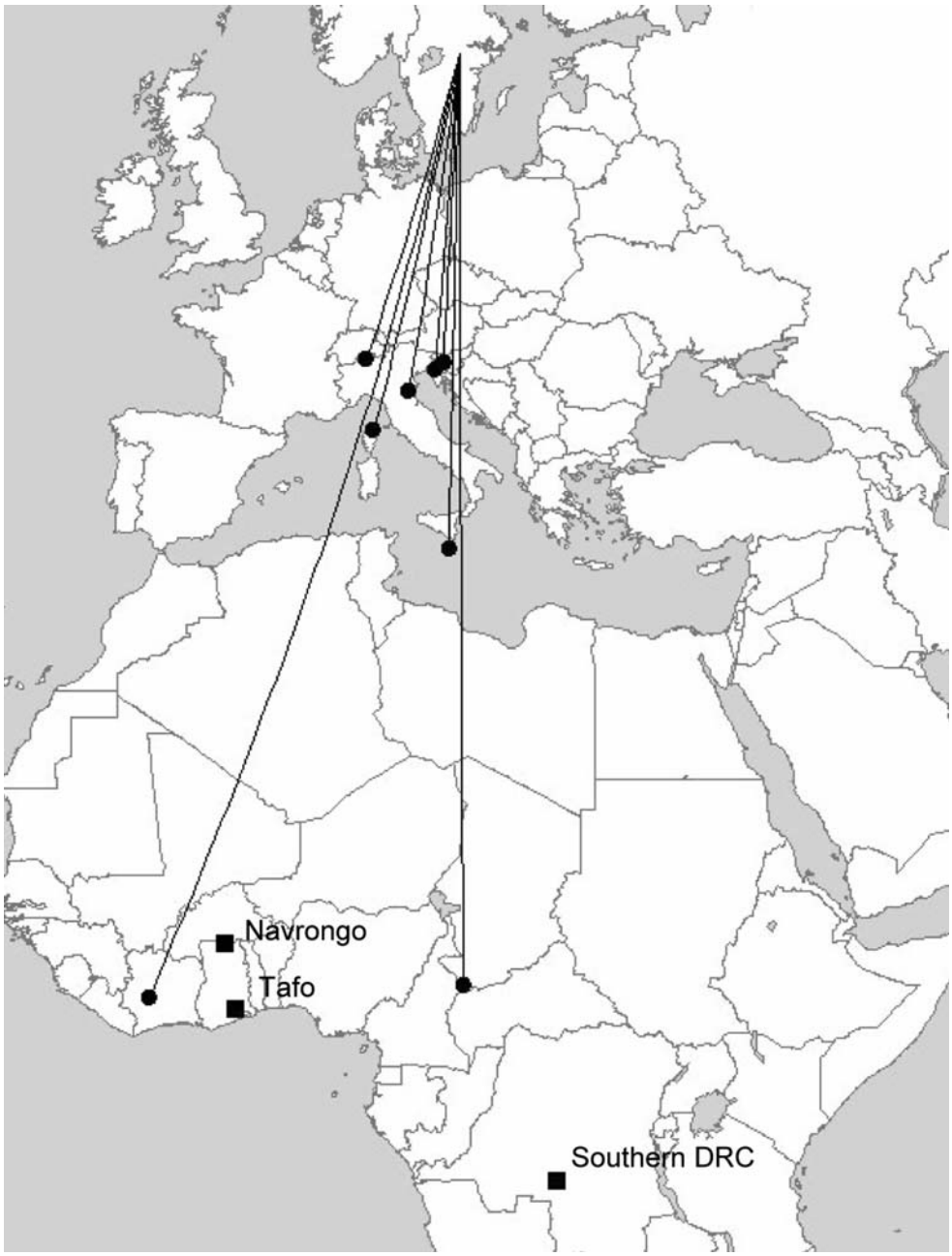

Fig. 1 Ringing recoveries of migratory great reed warblers (*Acrocephalus arundinaceus*) reported outside the breeding site at lake Kvismaren, south Central Sweden. *Dots* represent ringing and recovery sites. *Connecting lines* do not strictly reflect the migratory route and direction. *Square dots* represent winter study sites of great reed warblers in sub-Saharan Africa. At Navrongo, birds moult between September and December whereafter they leave the area (Hedenström et al. 1993). At Tafo, only a few birds occur during moult, but numbers increase from December when freshly moulted birds arrive which stay until March (Hedenström et al. 1993). At Luluabourg in southern Democratic Republic of Congo, birds arrive in fresh plumage during December and stay until March (De Roo and Deheegher 1969)

support the geographic separation hypothesis (2), i.e. that they have moulted in different areas (allopatric situation).

Materials and methods

We detected the prevalence and types of malaria blood parasites (mainly three species that have their transmission strictly in Africa: GRW1, *Haemoproteus payevskyi*; GRW2, *Plasmodium ashfordi* and GRW4, *Plasmodium relictum*) using PCR technique, and determined whether individuals were infected or not infected (Waldenström et al. 2004; Bensch et al. 2007). From each bird screened for malaria, we analysed tail feathers (moulted in Africa) for stable isotopes (δ^{13}C, δ^{15}N and δD) to investigate the relationship between winter moulting habitats (inferred from feather isotope values) and malaria prevalence. A previous study on the same great reed warbler population showed significant repeatability in all three elements, demonstrating that individual birds tend to return to the same moulting area in successive years (Yohannes et al. 2008). To extract further information on habitat use and geographical origin, we analysed stable sulphur (δ^{34}S) isotopes on a subset of the feather samples ($n = 40$), an element not previously investigated in Afro-tropical migratory birds.

Sample collection

Between spring 1999 and 2004, we trapped birds using mist-nets and collected blood (≈20 µl blood from the brachial vein), and feathers (third outermost tail) from 201 great reed warblers breeding at lake Kvismaren (59°10′N, 15°25′E) in south Central Sweden. For details of the study population and field methods see Bensch et al. (1998) and Hasselquist (1998). Each individual was marked with a unique combination of three coloured plastic and one aluminium ring.

Genetic analysis

PCR amplification and malaria screening

Blood samples were stored in 500 µl SET-buffer, and DNA was extracted using a standard phenol–chloroform procedure as previously described (Waldenström et al. 2002). Individual samples were screened for parasites using a nested PCR protocol (primer pair 1: HAEMNF and HAEMNR2; primer pair 2: HAEMF and HAEMR2) targeting a 479-bp fragment (excluding primers) of the parasite cytochrome *b* gene (Waldenström et al. 2004; Bensch et al. 2007). We did direct sequencing with the HAEMF primer of positive reactions and loaded these on an ABI PRISM 310 sequencing robot (Applied Biosystems). This approach allows precise identification of parasitic lineages even when identical parasites occur in different avian host species (Hellgren et al. 2007b). Samples with mixed infections were resolved by TA-cloning of PCR products (Pérez-Tris and Bensch 2005).

Stable isotope measurements

Carbon and nitrogen

Cleaned subsamples of approximately 0.5 mg of feather were weighed into small tin cups and combusted in a Eurovector (Milan, Italy) elemental analyser. The resulting N_2 and CO_2 gases were separated by gas chromatography and admitted into the inlet of a Micromass (Manchester, UK) Isoprime isotope ratio mass spectrometer (IRMS) for determination of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios. Measurements are reported in δ-notation relative to the PDB for carbon and atmospheric N_2 standard in parts per thousand deviations (‰). The standard for $\delta^{15}N$ is atmospheric nitrogen, and for $\delta^{13}C$ is Peedee belemnite (PCB). One egg albumin was used as a laboratory standard for every 11 unknowns in sequence. Hundreds of replicate assays of internal laboratory standards (albumen) indicate measurement errors (SD) of ±0.2‰ and ±0.5‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Deuterium

Deuterium measurements on feathers and standards were performed on H_2-derived Elemental Analyser-Isotope Ratio Mass Spectrometry (EA-IRM) calibrated and controlled against standard reference material. Cleaned and dried feather samples filled on silver capsules were left open for a period of more than 4 days to allow sample exchangeable hydrogen to equilibrate with the moisture in the laboratory air. Moreover, multiple samples of BWB-II (whale baleen) with a known non-exchangeable δ2HV-SMOW value and our eggshell membrane standard, independently measured by Len Wassenaar, NWRI, Saskatoon, Canada, were analysed. Multiple keratin replicate standards, with known non-exchangeable δD values, were used for correcting uncontrolled isotopic exchange between samples and ambient water vapour (Wassenaar and Hobson 2000). Thus, values reported here are equivalent to non-exchangeable feather hydrogen (Wassenaar and Hobson 2003).

Sulphur

Analysis was undertaken by EA-IRMS using tin capsules containing reference (barium sulphate and silver sulphide) or sample material (feathers cut into a powder, in situ) plus vanadium pentoxide catalyst. Barium sulphate and whale baleen were measured as quality control check samples during sample analysis.

Data analysis

A one-way ANOVA followed by Scheffe's post hoc test was performed to detect differences between years (1999–2004). Due to limited data from different years for $\delta^{34}S$, this test was done for feather $\delta^{13}C$, $\delta^{15}N$ and δD only. Based on results of parasite screening, individual birds were scored as either positive (1) or negative (0) for malaria. We used infection (i.e. malaria infected vs non-infected individuals) as a grouping variable, and applied Student's *t* test (with a Bonferroni correction to the probability threshold for significance due to multiple comparisons) on the null hypothesis that the mean feather $\delta^{13}C$, $\delta^{15}N$, δD and $\delta^{34}S$ values of the two groups are equal. The joint relationship of the three isotopes and malaria parasites (presence or absence) was tested with logistic regression. We applied Multivariate analysis of variance (MANOVA) to reveal possible associations between feather isotope values and the three most common avian malaria parasites (GRW1, GRW2, and GRW4). Feather isotope variables were normally distributed (Kolmogorov–Smirnov test: $P > 0.05$).

Table 1 Six years summary of winter grown feather stable isotope (δ^{13}C, δ^{15} N and δD) values of great reed warblers (*Acrocephalus arundinaceus*) controlled at a Swedish breeding ground

Year	Feather stable isotopes (mean ± SE, *n*)		
	δ^{13}C	δ^{15}N	δD
1999	−16.10 ± 0.54 (40)	9.32 ± 0.19 (40)	−66.13 ± 1.13 (41)
2000	−15.57 ± 0.36 (31)	9.31 ± 0.28 (31)	−68.53 ± 1.22 (30)
2001	−16.82 ± 0.55 (27)	8.43 ± 0.33 (26)	−63.35 ± 1.13 (26)
2002	−16.41 ± 0.65 (30)	9.09 ± 0.36 (31)	−65.59 ± 2.26 (29)
2003	−16.32 ± 0.59 (31)	8.58 ± 0.30 (31)	−66.42 ± 2.30 (31)
2004	−15.12 ± 0.66 (24)	8.76 ± 0.40 (24)	−65.04 ± 1.79 (24)

Results

The δ^{13}C, δ^{15}N and δD values in the feathers ($n = 176$) were only weakly correlated (δ^{13}C and δ^{15}N, $r = 0.111$, $P = 0.14$; δ^{13}C and δD, $r = 0.114$, $P = 0.13$; δ^{15}N and δD, $r = -0.146$, $P = 0.05$). The ANOVAs revealed no significant year effect on isotopic values (δ^{13}C: $F_{5,181} = 0.98$, δ^{15}N: $F_{5,180} = 1.57$ and δD: $F_{5,179} = 0.97$, $P > 0.1$ for each), hence we pooled samples from the six study years (Table 1).

Mean δ^{13}C, δ^{15}N and δD values in the feathers of birds with malaria were significantly different as compared to the birds without malaria (δ^{13}C: $t = -2.77$, $P = 0.01$, $n = 160$; δ^{15}N: $t = -2.87$, $P < 0.01$, $n = 160$; δD: $t = -3.48$, $P < 0.001$, $n = 158$; Fig. 2. A logistic regression model with malaria infection (positive or negative) as the response variable and the three element ratios as independent variables was overall highly significant ($\chi^2_3 = 28.8$, $P < 0.001$) and each of the elements contributed significantly to the model (δ^{13}C, $\beta = 0.19$, $P = 0.002$; δ^{15}N, $\beta = 0.24$, $P = 0.029$; δD, $\beta = -0.07$, $P = 0.001$), Table 2. Likewise, mean δ^{34}S values in the feathers of malaria-infected birds were significantly different than malaria-free birds (δ^{34}S: $t = 5.44$, $P < 0.001$, $n = 40$).

Among the 87 individuals scored positive for malaria infection, we detected GRW1, GRW2 and GRW4 in 39, 12 and 32% of the infected population, respectively. A MANOVA revealed no significant relationship between feather isotope values and malaria type ($F_{6,118} = 1.19$, $P = 0.31$).

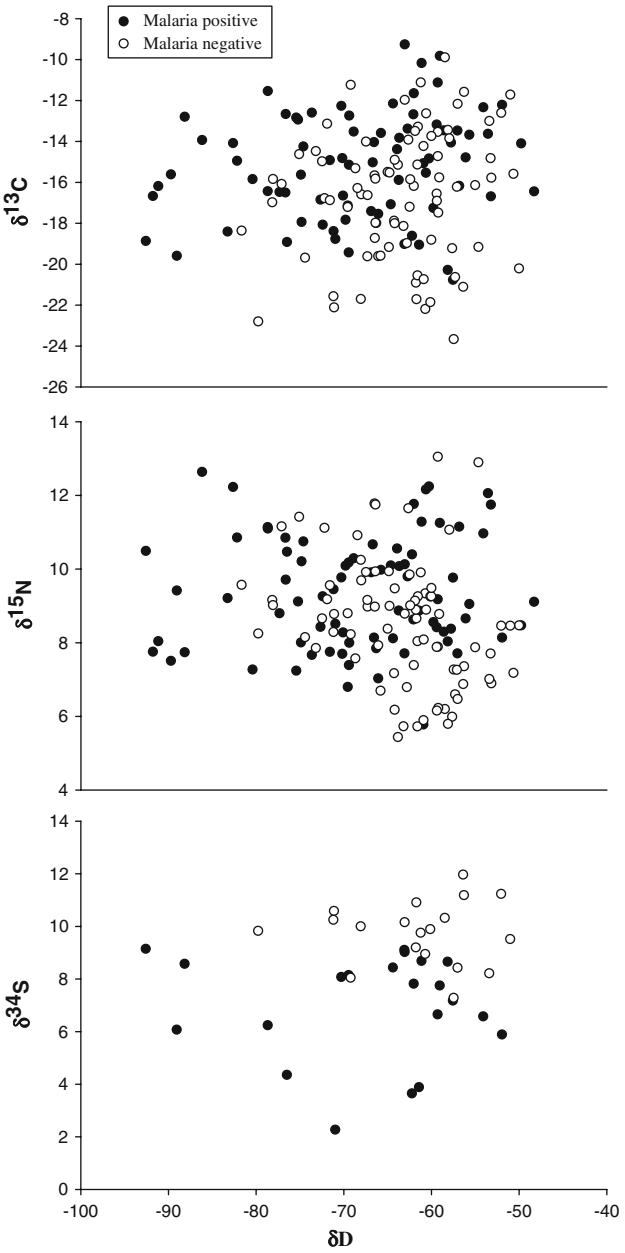

Fig. 2 Feather δ^{13}C, δ^{15} N, δD and δ^{34}S values of malaria infected/positive (1) and malaria free/negative (0) great reed warblers. Birds moulting in areas with relatively higher δ^{13}C and δ^{15} N but lower δD and δ^{34}S values harboured a higher rate of malaria parasites. Analysis of δ^{34}S values was carried out on 40 feather samples that were known to have the highest δ^{13}C values ($n = 21$; 11 malaria infected and 10 malaria free) and lowest δ^{13}C values ($n = 19$; 10 malaria infected and 9 malaria free)

Discussion

We found marked differences in isotopic signatures for each of the four elements when comparing infected and non-infected birds. Great reed warblers moulting in tropical habitats with relatively higher δ^{13}C and δ^{15}N, and lower δD and δ^{34}S values have a higher incidence of malaria infections (Fig. 2). This result shows that birds infected by malaria parasites have moulted in different geographical areas than the non-infected birds. Moreover, the strong differences in δ^{13}C and δ^{15}N suggest that these regions offer different habitats. Our results also show that parasite com-

Table 2 Results from multiple logistic regression analyses between infection status of malaria parasites (0 or 1) and feather stable isotope signatures in great reed warblers. The stable isotope values were *Z*-transformed prior to analyses in order for effect sizes to be compared

Elements	B	SE	*P*
Three elements ($n = 159$)			
$\delta^{13}C$	0.606	0.197	0.002
$\delta^{15}N$	0.401	0.184	0.029
δD	−0.637	0.196	0.001
Four elements ($n = 40$)			
$\delta^{13}C$	0.284	0.350	0.417
$\delta^{15}N$	1.483	0.867	0.087
δD	−0.880	0.665	0.186
$\delta^{34}S$	−4.029	1.542	0.009

position is not related to moulting area. Hence, regardless of the type of parasite involved, the mechanisms employed to contract, avoid or reduce malaria parasites appears similar.

The significantly higher $\delta^{13}C$ and $\delta^{15}N$ values in birds infected with avian malaria parasites suggests that these feathers have been grown in a C_4-dominated and drier habitat than the feathers from uninfected birds. Malaria infected birds had on average ca. 5‰ lower δD values in their feathers compared to non-infected birds. The fact that there is a difference in both δD and $\delta^{34}S$ implies that birds do not segregate into different habitats within the same geographical region, supporting the allopatric situation (hypothesis 2).

In terrestrial systems, feather δD correlate with the local precipitation of δD during the growing season at the birds' moult location (Hobson and Wassenaar 1997). Hence, our data suggest that infected birds have moulted in different geographical regions. The observation of lower δD values in malaria-infected birds is not consistent with the interpretation that these are from drier habitats (since dry habitats are expected to show higher δD values). The result could be explained if the infected birds had been moulting at higher altitudes than non-infected birds, but this is rather unlikely given the small altitude variation in the species' West African wintering range (Fig. 1). An alternative explanation is that the depleted δD values in the malaria-infected birds reflect moulting sites more inland, i.e. further north. Results of feather $\delta^{34}S$ values agree with this explanation. Malaria infected birds showed a lower $\delta^{34}S$ value (mean ± SE: 6.96‰ ± 0.44, $n = 21$) compared to malaria negative birds (mean ± SE: 9.77‰ ± 0.28, $n = 19$) (Fig. 2). These results support the hypothesis that birds moult in geographically separate areas and possibly that the malaria-infected birds are moulting further inland and to the north.

Generally, the results imply that our study population moult in tropical environments dominated by C_4 plants. However, the wide range of $\delta^{13}C$ signatures found in the great reed warbler feathers (range between −23.67 and −9.26‰) suggests that the birds moult across a variable gradient of $\delta^{13}C$ signatures. Based on earlier ringing recovery data (Fig. 1; Bensch 1993) and studies of moulting time and sites in Ghana (Hedenström et al. 1993), our study population is likely to have replaced their feathers in West Africa between 8°N and 17°N latitude and 10°W and 20°E longitude. Feather stable isotope values in this study corresponds very well with values reported for a great reed warbler feather moulted in West Africa, reported by Neto et al. (2006). Aquatic warblers (*Acrocephalus paludicola*) also moult in sub-Saharan West Africa in similar habitats as the great reed warbler. A study based on feathers collected at European breeding grounds (Pain et al. 2004) showed that aquatic warblers have $\delta^{13}C$ values more similar to the malaria-free birds and $\delta^{15}N$ and δD values more similar to the infected ones. Aquatic warblers are believed to moult in the West African floodplains of Mauritania, Mali, and Senegal (Pain et al. 2004), i.e. in the northern part of Sahel, overlapping the supposed moulting range for great reed warblers.

Results of this study indicate that great reed warblers moulting in habitats dominated with C_4 plants and with a dry and warm climate have the highest infection rate. Avian malaria prevalence is associated with the availability of vectors and suitable reservoirs (Valkiūans 2005). An optimal condition created by a combination of such factors may account for inter- and intra-specific habitat, geographic and latitudinal variation in parasite prevalence (e.g. Tella et al. 1999). The winter habitat of the great reed warbler ranges from swampy areas to dry scrublands (Cramp 1992). This probably allows individuals of this species to occupy habitats associated with varying parasite prevalence. Variation in habitat used could either be the result of within-species variation in climatic preferences, or the result of dominance relationships, with subdominant birds forced to use drier, more malaria-exposed areas to the north (see Marra et al. 1998).

Conclusion

We found that the prevalence of avian malaria parasites in a breeding population of great reed warblers differs depending on the winter moulting area. From present knowledge of the distribution of isotopes, our results suggest that birds moulting at drier sites situated further north in West Africa have a higher incidence of malaria than birds moulting at wetter sites closer to the coast. However, the present study cannot exclude that birds obtain the infections after the moulting season when they might be in more southern areas of Africa (De Roo and Deheegher 1969; Hedenström et al. 1993). However, if that is the case, areas used during late winter must be at least partly determined by the moulting

areas. Similar choices of wintering area and malaria prevalence may be an important factor driving assortative mating among individuals with identical (inherited) migration directions and distances. Future studies that apply habitat-specific stable isotope signatures might help to determine whether separated wintering populations of great reed warbler pair assortatively on their breeding grounds.

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