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ORIGINAL PAPER

Hidden haemosporidian infections in Ruffs (*Philomachus pugnax*) staging in Northwest Europe *en route* from Africa to Arctic Europe

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Abstract In their African freshwater wintering habitats, shorebirds show a high prevalence of blood parasites, whereas no parasites are detected elsewhere along the migration route. We looked at two genera of haemosporidian parasites, *Haemoproteus* and *Plasmodium*, in the long-distance migrating Ruff (*Philomachus pugnax*) along a geographical/seasonal gradient to verify the infection pattern and examine possible hidden organ infections at European staging areas. We amplified parasite DNA from blood of 53 healthy birds wintering in Mali, 53 samples of seven organ tissues (spleen, liver, kidneys, heart, lungs, brain, and pectoral muscle) from healthy individuals caught during spring migration, and 18 weak birds found sick in summer in The Netherlands. We confirm that Ruffs wintering in Africa carried blood infections and that some infections developed into hidden organ infections during spring migration. Moreover, sick birds either had new infections (in one juvenile) or relapses (in an adult harboring an African lineage). Our results suggest that some parasites develop latency. This strategy may be beneficial for the parasite as it may take control over reappearance in the blood to help further transmission.

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Introduction

Recent studies on the ecology of bird–haemosporidians interactions have been largely based on single blood samples (Waldenström et al. 2002; Mendes et al. 2005; Pagenkopp et al. 2008; Beadell et al. 2009; Yohannes et al. 2009). However, haemosporidians have complex life cycles involving both dipteran vectors and vertebrate hosts and several stages in the blood and other organs of the vertebrate host (Atkinson and Van Riper 1991; Valkiūnas 2005; Atkinson 2008a, b). As a consequence, some of the interactions between the parasites and their wild hosts will remain undetected, and potentially important stages of the parasitic life cycle will be underestimated in studies of host–parasite interactions.

Haemosporidians from the *Plasmodium* and *Haemoproteus* genera infect a wide range of bird families and are geographically widespread (Valkiūnas 2005). These parasites are transmitted among birds by vectors such as mosquitoes (for *Plasmodium* sp.) and biting midges and hippoboscid flies (for *Haemoproteus* sp.). In general terms, the infection cycle in a vertebrate host comprises the following phases: (1) sporozoites injected in the bloodstream by an infected vector enter a tissue and start producing asexually-reproducing meronts; (2) cells resulting from these meronts produce other meronts or differentiate to enter the bloodstream where they penetrate red blood cells to mature into gametocytes (a stage called "parasitemia"). This is a phase when parasites show an exponential increase in numbers in the bloodstream, followed by a decrease when very few parasites remain in the blood, and (3)

if the infection cannot be fully cleared, it will sometimes turn latent with parasites retreating to organs. Parasites can then reappear in the blood seasonally, or if there is a weakening of the host immune system, even in the absence of vectors in the environment, a phenomenon known as "relapse" (Valkiūnas 2005).

The length of the "prepatent" phase (before parasites are visible in the blood) varies from five days in the case of the *Plasmodium relictum* (Valkiūnas 2005) to around two weeks in the case of several *Haemoproteus* species (Atkinson 2008b). Latent infection occurs later in the infection process, when the parasite disappears from the blood stream and enters a dormant stage in the internal organs (Valkiūnas 2005). Most of the avian lineages from which the prepatent period is known take between two to three weeks to appear in the blood (Atkinson et al. 2000; Palinauskas et al. 2008, 2009; Zehtindjiev et al. 2008. Transmission can only occur during the parasitemia stage and when capable vectors are present in the environment. In Southwest Europe, this corresponds to summer and early autumn (Ventim et al. 2012).

Noncoastal shorebirds tend to use habitats with high densities of mosquitoes and winter in geographical areas with a high risk of infectious pathogens (Piersma 1997, 2003; Mendes et al. 2005). Ruffs (*Philomachus pugnax*) are long-distance migratory birds that breed across the Northern Palearctic and winter across a broad array of inland areas that range from Western Europe to South Africa (van Rhijn 1991; Rakhimberdiev et al. 2011; Verkuil et al. 2012a). Ruffs seem to be more affected by *Plasmodium* or *Haemoproteus* than other shorebirds, with infections being frequently detected in the African wintering areas. However, blood analyses suggest that Ruffs are free of parasites when tested in an important stopover area in The Netherlands (Mendes et al. 2005). Although this would suggest that transmission mainly occurs in Sub-Saharan Africa, the question remains whether Ruffs are able to clear their African parasites, thereby eliminating parasitic impacts during migration and breeding, or instead manage to keep their parasites in latent form in their inner organs.

In this study, we used a combination of blood and organ samples from Ruffs collected both in the wintering areas of Mali and the staging areas of The Netherlands to: (1) confirm if they were infected with *Plasmodium* and *Haemoproteus*, (2) detect if they traveled with organ infections, (3) characterize the type of parasites that infected them, and (4) identify the main transmission areas. In addition, we also aimed to verify whether haemosporidians influenced the health stage of individuals caught during autumn. In general, the findings of this work contribute to a better understanding not only of the elusive shorebird–haemosporidian relationship, but also of the host–haemosporidian association as a whole.

Materials and methods

Study system

Recent studies have shown that Ruffs migrating through The Netherlands in spring spend the winter mostly in West Africa (with some individuals staying behind in Northwest Europe) and breed in northern Europe and northwestern Siberia (Rakhimberdiev et al. 2011; Verkuil et al. 2012a, b). In the Frisian study area, northern Netherlands, Ruffs occur from mid-March to mid-May to fuel up in order to continue migration to the northern breeding areas (Verkuil et al. 2012b).

We screened 53 migrating adult Ruffs (40 males and 13 females) killed accidentally during the capture of over 8,000 individuals with "wilsternets" (Jukema et al. 2001) for ringing in Friesland in 1997–2005. Another 18 Ruffs (15 males and 3 females) were collected in July and August 2005 at a shoreline roost site called Kreupel in the west of Lake IJsselmeer (2°47′55″ N, 5°13′41″E). These were weakened and sick birds that were caught by hand and soon died. Finally, we used a set of blood samples from 53 birds caught with mist nets in the Inner Niger Delta, Mali in January 2005. This work was covered by a permit to catch ruffs (Vogelvergunning F) of the Netherlands Ministry of Agriculture, Fisheries, and Food Safety to Theunis Piersma.

Dissection techniques

Within a maximum of eight hours after death, bird carcasses were stored in a freezer at −20 °C. Dissections were carried out at the NIOZ Royal Netherlands Institute for Sea Research. The evening before dissection, the birds were thawed at approximately 4 °C. We followed the methods detailed in Piersma et al. (1999). Before dissection, we disinfected the working area with 70 % ethanol to avoid bacterial proliferation. We then proceeded by separating the tarsus from the flesh, by slicing a bit of skin from the belly, and by excising the pectoralis major flight muscles and cutting a piece. We then proceeded to separate the brain, spleen, liver, kidneys, heart, and lungs from the rest of the body. We placed small pieces of all these tissues in 90 % ethanol filled tubes. To destroy every trace of DNA and avoid contamination between organs, we used a single razor blade for each bird which was sterilized using a Bunsen burner every time we changed organ. The storage tubes were kept at −80 °C until further analysis. The rest of the body, with skull and feathers attached, was kept intact for museum collections.

Molecular analysis

Total genomic DNA from avian blood and tissue was: (1) extracted by standard ammonium acetate protocol in which ammonium acetate was used to separate the proteins, and DNA was precipitated from the resulting supernatant with ethanol; (2) quantified to 25 ng in NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) to achieve concentration standardization; and (3) diluted to 25 ng/μL. To check the quality of the DNA, we then proceeded to molecularly sex our samples (Schroeder et al. 2010). If the polymerase chain reaction (PCR) delivered no positive result, we would reextract the blood samples until a sex ID was obtained. Only then did we proceed with the screening of haemosporidians using a nested PCR method described by Waldenström et al. (2004). Using this method first, a PCR is carried out that aims to amplify a 580 bp fragment of mtDNA *cytb* gene of *Plasmodium* and *Haemoproteus* parasites. This occurs in a ×20 amplification cycle and uses the HaemNF and HaemNR2 primers. At least one positive and one negative control were used in all PCRs amplifications; no negative controls amplified. The second amplification uses as a template the PCR product from the first amplification and is designed to obtain a 479 bp inside the original fragment by using the HaemF and HaemR2 primers, in a ×35 cycle. For further details, please see Waldenström et al. (2004). We always used negative controls from the extraction and also negative PCR controls, all of which behaved as expected. Positive samples were double checked from the extraction, which confirmed parasite infections.

Afterwards, 3 μL samples of the PCR products were run on 2 % agarose gel stained with ethidium bromide and TAE buffer. Samples that showed amplification were precipitated and sent for sequencing with an ABI Prism 3730 capillary robot (Applied Biosystems, Foster City, USA). The output sequence files were visualized and edited on FinchTV and aligned using ClustalW in BioEdit (Hall 1999) and MEGA4 (Tamura et al. 2007) and then blasted at the GenBank International Nucleotide Sequence Database (NCBI) and the MalAvi database (Bensch et al. 2009) to identify the parasite lineage. Lineage names are referred to according to the MalAvi database.

We assumed that our PCR protocol had the same detection sensitivity for blood and for organs. Although we did not test this assumption ourselves, other authors have obtained similar proportions of positive results from blood tissue and the tissue of organs and muscles (Ishtiaq et al. 2007; Pagenkopp et al. 2008).

Results

Of the 53 individuals caught in the Inner Niger Delta, blood samples from two were found to be infected with haemosporideans. From the group of 53 Ruffs sampled at Friesland during their northward migration, haemosporideans were detected in the organs of four individuals. And lastly, from the group of 18 birds found sick during southward staging at Kreupel, two were found to have infected organs (Table 1). Three out of the four infected individuals from Friesland had a single infected organ, and one had two infected organs. This observation of parasite infection in apparently healthy birds contrasted sharply with the extent of infection in the two sick individuals from Kreupel, where haemosporidian DNA was detected in five and six of the eight examined organs, respectively. All parasite lineages detected in the subsample tissue set were detected in the pectoral muscle (Table 1). Infections in the liver and spleen were only seen in the two sick individuals from Kreupel. *Haemoproteus* was the only parasite whose DNA was found in the lung of a female from Friesland.

Six different parasite *cytb* lineages were encountered; five of which were *Plasmodium*: GRW11, Turdus1, TERUF02, GRW02, and PLASCOQ8. The sixth lineage, MW1, belonged to the genus *Haemoproteus* and was found in two healthy northward-migrating individuals. DNA from the PLASCOQ8 *Plasmodium* lineage was detected in the heart and pectoral muscle of an individual from Friesland and in the blood of one bird from the Inner Niger Delta. Except for GRW02, which was only found in the blood of one individual wintering in Africa, all other lineages were found in the organs of migrating individuals during spring (at Friesland) and summer (at Kreupel) in The Netherlands (Table 1). Some of the detected lineages belong to the known haemosporidian species: GRW02 is one of the lineages from *Plasmodium ashfordi*; GRW11 is from *P. relictum*; TURDUS1 is from *P. circumflexum*; and MW1 is a haplotype from *Haemoproteus belopolskyi* (Table 2). Some of these lineages are likely to be transmitted in Africa (*P.* TERUF02, *P.* GRW02, *H.* MW1, and *P.* PLASCOQ8) and others in Europe (*P.* GRW11 and *P.* TURDUS1) (Table 2).

Discussion

To the best of our knowledge, this is the first time that haemosporidians have been found in the tissues of free-living, naturally infected shorebirds. This is important for our understanding of shorebird–haemosporidian relationships; a system that is difficult to study because of low infection rates (Mendes et al. 2005; Yohannes et al. 2009). There are probably several ecological factors that contribute to this low prevalence, but there are also methodological limitations. Many of the shorebirds in the Palearctic are migrants that spend the winter in African wintering grounds, and Africa may be the main transmission area in this host–parasite system (Mendes et al. 2005; Yohannes et al. 2009).

Table 1 Parasite lineages encountered in the organs and blood of Ruffs

Organs/ tissues		Spleen	Liver	Kidneys	Lungs	Heart	Pectoralis	Skin	Brain	Blood
	Netherlands									
	Spring									
# 1 ♂							*P.* GRW11			
# 2 ♂						*P.* PLASCOQ8	*P.* PLASCOQ8			
# 3 ♀							*H.* MW1			
# 4 ♀					*H.* MW1					
Total		0/52	0/51	0/53	1/53	1/53	3/52	0/52	0/53	-
	Autumn									
# 5 ♂		*P.* TURDUS1	*P.* TURDUS1			*P.* TURDUS1	*P.* TURDUS1	*P.* TURDUS1	*P.* TURDUS1	
# 6 ♂		*P.* TERUF02	*P.* TERUF02	*P.* TERUF02		*P.* TERUF02	*P.* TERUF02			
Total		2/18	2/18	1/18	0/18	2/18	2/18	1/18	1/18	-
	Mali									
	Winter									
#7 ♀										*P.* PLASCOQ8
#8 ♀										*P.* GRW02
Total		-	-	-	-	-	-	-	-	2/53

Parasite lineages encountered in the organs of 71 Ruffs caught in The Netherlands (53 caught during spring migration and 18 caught after the breeding season) and in the blood of 53 Ruffs caught in the wintering grounds of the Inner Niger Delta, Mali

Table 2 List of parasite lineages encountered in the organs and in the blood of 124 Ruffs caught in The Netherlands and in the Inner Niger Delta, Mali

Parasite lineage	Morphological species	GenBank accession number	Putative area of transmission
GRW02	*Plasmodium ashfordi*	AF254962	Africa
GRW11	*Plasmodium relictum*	AY831748	Europe
PLASCOQ8	*Plasmodium* sp.	HM179149	Africa
TURDUS1	*Plasmodium circumflexum*	AF495576	Europe
TERUF02	*Plasmodium* sp.	EU810618	Africa
MW1	*Haemoproteus belopolskyi*	AF254969	Africa

When the birds migrate through Europe (where most studies have been carried out), or breed there, the infections may have developed into the latent phase. The more resistant the host, the lower the likelihood of finding haemosporidians in the blood. Indeed, most resistant hosts, like the Chaffinch (*Fringilla coelebs*) or House Sparrow (*Passer domesticus*) have longer prepatent periods, shorter durations of parasitemia (around two weeks), and lower intensities of infection than less resistant hosts like the Common Crossbill (*Loxia curvirostra*) or the Siskin (*Spinus spinus*) (Palinauskas et al. 2008). At an extreme, the parasitemia period may be so short as to make it very difficult to detect any active blood infection, or the intensity of infection may be very low, rendering parasites undetectable by microscopy. If recognizing nonviable sporozoites (parasite cells injected in the blood by an infected dipteran) in a host may lead to false positives, ignoring evidence other than the detection of gametocytes or erythrocytic meronts, as suggested by (Valkiūnas et al. 2009), may equally lead to false negatives. The rate of false negatives will be higher in more resistant species.

Our technique was based on the detection of parasite DNA. In this way, we cannot distinguish between stages of the life cycle of the parasite and whether a parasite is alive or dead, e.g., in the form of nonviable sporozoites (Valkiūnas et al. 2009). Only a combination of blood and organs assays would allow a decisive identification of the parasite stage. However, that many of the spring infections were detected in just one (or two) organs is a strong indication that the parasite was not in the blood and strongly suggests that it had been able to develop further than the initial sporozoite stage. Therefore, we suggest that lineages that were detected during spring migration in The Netherlands (*P.* GRW11, *P.* Plascoq8, and *H.* MW1) were able to infect Ruffs. Moreover, *P.* TERUF02 is an African transmitted lineage that has been detected in several organs of an adult male caught in The Netherlands five months after leaving the African wintering grounds, clearly showing that *P.* TERUF02 is capable of infecting Ruffs. We cannot make any conclusions regarding the *P.* GRW02 lineage detected in the blood of an African wintering bird, or TURDUS1 detected in several organs of a juvenile caught after the breeding season in the Netherlands, but given that generalist lineages are the most commonly reported in shorebirds, e.g., *Haemoproteus* SW5 or *Plasmodium* GRW4 (Mendes et al. 2005; Beadell et al. 2006; D'Amico and Baker 2010; Yohannes et al. 2009), it would not be surprising if these generalist lineages too were able to thrive in Ruffs.

Some of the lineages detected Ruffs are generalist. The European lineages of *Plasmodium* GRW11 and TURDUS1 were found in as much as 20 different host species (MalAvi—July 2011 version) (Bensch et al. 2009). The African lineage GRW02 (*Plasmodium* spp.) was found in seven passerine species. Most studies are biased towards passerine birds caught in temperate areas, so it is possible that these African lineages are more widespread than currently known. The same could be true for the *Plasmodium* lineage TERUF02, which was previously found in the Black-headed Paradise Flycatcher (*Terpsiphone rufiventer*) in Gabon (Beadell et al. 2009), as well as PLASCOQ8, which was formerly detected in a mosquito from the *Culex* genus in the lowland forests of Cameroon (Njabo et al. 2011). *P.* PLASCOQ8 may be one of the most prevalent lineages in this species (Table 1). The detection of the lineage *H.* MW1 in Ruffs was a surprise. This lineage was previously detected only in songbirds (Bensch et al. 2009), and the genus *Haemoproteus* is known to be more specific than *Plasmodium* (Cumming et al. 2012).

Similar to other migrant birds (Waldenström et al. 2002), noncoastal shorebirds can acquire parasites in at least two different areas: their sub-Saharan wintering quarters and in Europe. The likelihood of fresh infections in early spring in The Netherlands should be extremely low due to the inactivity of capable vectors. However, after the breeding season, transmission rates should increase as the numbers of active vectors in the environment increases. In temperate areas, mosquito development does not occur below a threshold of 17 °C (Trawinski and Mackay 2008), and populations tend to peak only after wet and warm conditions (DeGaetano 2005), i.e., in late spring/early summer. Moreover, parasite development within the vector also seems to be impaired by low environmental temperature. Experiments with *Plasmodium relictum* have shown that in Hawaii, this species cannot develop in the vector under a temperature of 13 °C (LaPointe et al. 2009). In Africa, the situation should be different because rainfall, rather than temperature, seems to be the limiting factor, at least in natural habitats (Minakawa et al. 2002). Two of the major wintering areas for Ruffs, the Doujdj National Park in Senegal and the Inner Niger Delta in Mali, are located in the Sahel. In this area, transmission is thought to be higher during and just after the rainy season (Fontenille et al. 1997), which occurs between June and October (Zwarts et al. 2009). This seasonal

pattern can be disturbed if the birds use local rice fields, where rainfall is irrelevant for mosquito reproduction and the main factor driving vector abundance is water filled paddies (Muturi et al. 2008). Rice fields are the main habitat for Ruffs in West Africa, and where the paddies are wet throughout the dry season (Bos et al. 2006). As a consequence, the opportunities for transmission will be extended.

Our results also show that part of the haemosporidian infection process will be missed when one only examines the blood stage of the infection. The percentage of missed infections remains unknown, but in shorebirds latent infections may be the most prevalent parasite stage during migration. Indeed, while studies that only examined haemosporidians in blood detected levels of infection lower than 0.5 % in actively migrating shorebirds (Mendes et al. 2005; D'Amico and Baker 2010), the infection level detected in the organs of Ruffs was considerably higher (7.5 %) during spring migration. For hosts, the "latency" phase would not pose a strong cost, as the parasite is not actively dividing or causing important damage (e.g., hemolytic anemia), and Ruffs seem to be able to cope with this infection stage, even during the energetically demanding migration period. It is also possible that Ruffs are able to resist the majority of lineages and/or clear the infection at a faster pace than songbirds. We have no information on how susceptible Ruffs are to haemosporidians, or to which haemosporidian lineages, but the fact that it is difficult to find parasites in blood smears of infected individuals, even when they are caught in high prevalence areas, suggests that infections are usually of low intensity (Mendes et al. 2005).

There seems to be considerable variation between years in infection rates of ruffs in the Inner Niger Delta. Although, the current results are not directly comparable with the results from Mendes et al. (2005), as two different PCR protocols were used, the difference in prevalence between 2001 and 2005 was considerable. In 2001, 10 out 16 ruffs had haemosporidians in their blood and in 2005 only 2 out of 53 birds showed blood infections. This difference may be related not only with a different sensitivity of the PCR method used, but also with local year variations in water level and flooded area. Normally, in the Inner Niger Delta, lower-level floods cover the floodplains for about 4 months (October–February), but if there is a period with consecutive wet years, the flood plains can be inundated for as 8 months (September–April) (Zwarts et al. 2009), therefore extending the breeding conditions for vectors and potentially increasing transmission.

Finally, our data suggest that the most vulnerable period for ruffs in Europe is the summer. Not only is the infection rate higher than in the spring, but ruffs that return from the breeding grounds also seem to have poor body condition (male body mass during the spring was 220 g (n=40) and in the summer 139 g (n=15), female numbers were too small for a comparison), probably as a result of the demanding breeding season. This is also the period when potential vectors are abundant in the environment (Ventim et al. 2012) and when naïve hosts enter the population (juveniles). It is likely that the combination of these factors facilitates pathogen transmission at a time when hosts may be less equipped to control the infections properly.

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