Primary peak and chronic malaria infection levels are correlated in experimentally infected great reed warblers

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(*Received 24 August 2011; revised 30 November 2011, 2 February, 22 February and 26 February 2012; accepted 27 February 2012; first published online 1 May 2012*)

SUMMARY

Malaria parasites often manage to maintain an infection for several months or years in their vertebrate hosts. In humans, rodents and birds, most of the fitness costs associated with malaria infections are in the short initial primary (high parasitaemia) phase of the infection, whereas the chronic phase (low parasitaemia) is more benign to the host. In wild birds, malaria parasites have mainly been studied during the chronic phase of the infection. This is because the initial primary phase of infection is short in duration and infected birds with severe disease symptoms tend to hide in sheltered places and are thus rarely caught and sampled. We therefore wanted to investigate the relationship between the parasitaemia during the primary and chronic phases of the infection using an experimental infection approach. We found a significant positive correlation between parasitaemia in the primary peak and the subsequent chronic phase of infection when we experimentally infected great reed warblers (*Acrocephalus arundinaceus*) with *Plasmodium ashfordi*. The reason for this association remains to be understood, but might arise from individual variation in exoerythrocytic parasite reservoirs in hosts, parasite antigenic diversity and/or host genetics. Our results suggest that the chronic phase parasitaemia can be used to qualitatively infer the parasitaemia of the preceding and more severe primary phase, which is a very important finding for studies of avian malaria in wild populations.

Key words: *Acrocephalus arundinaceus*, *Plasmodium ashfordi*, parasitaemia, primary infections, chronic infections.

INTRODUCTION

Malaria parasites often manage to maintain an infection for long periods of time in their vertebrate hosts. Experimental infections with *Plasmodium* parasites in natural hosts (rodents and birds) result in lifelong infections and, in humans, *Plasmodium vivax* and *P. ovale* can relapse after up to several years (Krogstad, 1995; Calderaro *et al*. 2007). However, the general pattern for human malaria is that clinical episodes are unlikely to occur beyond 12 months after being infected (Snounou *et al*. 2000). When a malaria parasite infects a host there is first an initial primary (high parasitaemia) phase of the infection followed by a later chronic phase (low parasitaemia) that is more benign. A chronic, long-lasting infection provides a reservoir in times of no or low transmission, e.g. until vectors re-establish and conditions become more favourable for parasite transmission (Druilhe and Perignon, 1997). This parasite reservoir is probably an advantage to the malaria parasite which results in an increased probability of transmission to new hosts (Snounou *et al*. 2000).

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Primary (acute) phase *Plasmodium* infection intensities and their consequences are difficult to study in wild birds because this time-period is short (Valkiūnas, 2005). Individuals may suffer from parasite-induced mortality during the primary-phase infection, either directly due to the negative effect that the parasite has on the host (Atkinson and van Riper, 1991; Atkinson *et al*. 2000; Valkiūnas, 2005) or indirectly due to, for example, an increased risk of predation. In avian malaria a high primary parasitaemia is likely to restrict a bird's movements and ill individuals are not only more vulnerable but are also more difficult to catch and study by the commonly used mist netting technique (Valkiūnas, 2005).

An avian malaria infection is initiated when a vector injects malaria sporozoites into a host. The development of malaria can be divided into exoerythrocytic merogony, erythrocytic merogony and formation of gametocytes (Valkiūnas, 2005). Exoerythrocytic merogony can be further divided into primary and secondary exoerythrocytic merogony. Secondary exoerythrocytic merogony takes place in endothelial cells of capillaries in several different organs in the body including the brain. Here, several generations of meronts, which are called phanerozoites, are produced. Individuals that survive

Parasitology (2012), **139**, 1246–1252. © Cambridge University Press 2012
doi:10.1017/S0031182012000510

https://doi.org/10.1017/S0031182012000510 Published online by Cambridge University Press

the primary phase often develop long-term chronic infections (Snounou *et al*. 2000). During the chronic infection the parasite persists at a low density and is maintained by merozoites produced by phanerozoites together with erythrocytic meronts (Valkiūnas, 2005). The vast majority of wild-caught infected birds harbour chronic infections and not primary infection, and chronic infections typically have few or no measurable negative effects on the performance of the host phenotype (Valkiūnas, 2005; Woodworth *et al*. 2005; Valkiūnas *et al*. 2006; Bensch *et al*. 2007).

In studies of wild birds, it would be useful to know the relationship between the primary and chronic infections, as the former is likely to affect fitness and the latter is easier to study. Furthermore, such knowledge would aid to deduce past selective events during the primary phase infection. With the use of quantitative PCR (qPCR) in experimental studies such questions can be explored, as qPCR gives a more accurate estimate of parasitaemia in low-level (chronic) infections when compared with more traditional methods such as microscopy (Asghar *et al*. 2011). Moreover, an experimental setup provides an opportunity to sample highly infected individuals that are almost impossible to sample in the wild.

The avian malaria parasite *Plasmodium ashfordi* is probably one of the most pathogenic species of the subgenus *Novyella* (Iezhova *et al*. 2005; Valkiūnas, 2005). In experimental infections, the primary phase parasitaemia of this species can reach up to 85% of the red blood cells (RBCs) in great reed warblers (Zehtindjiev *et al*. 2008). *P. ashfordi* (cytochrome *b* lineage GRW2) is frequently found in birds sampled in Africa (both residents and intercontinental migrants), but in Europe it is found only in adults of long distance tropical migrants (i.e. in birds that have been in Africa at least once), strongly implying that there is no transmission in Europe (Bensch *et al*. 2000; Waldenström *et al*. 2002; Hellgren *et al*. 2007). The great reed warbler is a long distance migratory bird that breeds in Eurasia and winters in tropical Africa. It is commonly infected by several species of Haemosporidians (Bensch *et al*. 2007). Previous studies have shown that great reed warblers infected with GRW2 keep individual-specific chronic parasitaemia levels in both experimental (Zehtindjiev *et al*. 2008) and wild population studies (Asghar *et al*. 2011).

We conducted an experiment in great reed warblers by inoculating wild naïve juveniles before their first autumn migration with the avian malaria parasite, GRW2, to investigate whether there is an association between the parasitaemia of primary (peak) and chronic phase infections. We monitored the progress of the GRW2 infections for up to 10 months, thus covering the full infection episode (Fig. 1). This enabled us to investigate the association between the parasitaemia of primary (peak) and chronic phase infections.

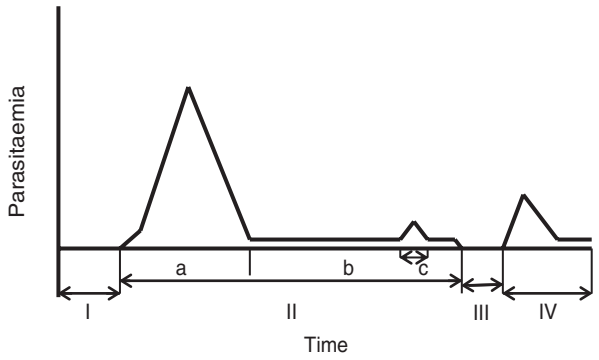

Fig. 1. Schematic illustration of GRW2 malaria in birds. (I) Pre-patent period (parasites develop in internal organ); (II) primary parasitaemia; (a) acute including the crisis; (b and c) chronic phase; (c) recrudescence. (III) Latent stage of infection (parasites are absent in the blood but persist in internal organs); (IV) secondary parasitaemia due to relapse. (Also see Valkiūnas, 2005.)

MATERIALS AND METHODS

The study was carried out at the Kalimok station, NE Bulgaria (44°00′N, 26°26′E) where juvenile great reed warblers (6 birds in 2005 and 23 birds in 2006) were experimentally infected with the lineage GRW2. Because it is only possible to investigate the relationship between parasitaemia of primary and chronic infections in the birds that were monitored long enough for chronic infections to be reached, our final sample size was reduced to 3 in 2005 and 5 in 2006, respectively.

We captured adult great reed warblers in the reed beds surrounding the Kalimok station. The captured birds were placed in captivity and screened for the presence of avian malaria parasite *P. ashfordi* (lineage GRW2) using molecular methods (see Zehtindjiev *et al*. 2008) and infected birds were kept as donors of infection for the coming experiments. In late July, juvenile birds (approximately 2 months old) were captured and placed in captivity for later use in the experimental infection of GRW2. As expected, the captive juveniles were proved to be free of any GRW2 infection before the start of the experiment. Body mass was measured in all birds before inoculation.

In 2006, 23 birds (3 birds on 18 July 2006, 8 birds on 2 August 2006, 7 birds on 3 August 2006 and 5 birds on 13 August 2006) were inoculated with GRW2 (for detail, see Supplementary Table 1, online version only). We obtained 0·2 ml of blood from the brachial vein of infected donor birds, mixed this with 0·8 ml of a 3·7% solution of trisodium citrate dehydrate ($C_6N_5Na_3O_7\_2H_2O$) and immediately (within 5 min) injected 0·3 ml of the mix into the pectoral muscle of experimental juvenile birds. Six control birds (2 in 2005 and 4 in 2006) were inoculated with uninfected blood in buffer, with 1 control for each time we did the inoculation. Full details of the infection experiment from 2005 have

https://doi.org/10.1017/S0031182012000510 Published online by Cambridge University Press

Table 1. Details about the 8 birds that survived the experimental infection and developed chronic malaria

(sex (F = female and M = male), body mass, pre-patent period (d = days), days post-infection (dpi) and parasitaemia (%) for peak and chronic phase)

ID	Sex	Body mass (g)	Pre-patent period (d)	Peak infection (dpi)	Primary peak parasitaemia (%)	Chronic phase (dpi)	Average chronic parasitaemia (%)	Range of chronic parasitaemia (%)
#228	F	36·0	35	85	85·419	130–235	0·081	0·0005–0·347
#227	F	37·3	15	30	0·271	75–145	0·008	0·001–0·0143
#299	M	43·0	35	110	0·023	155–180	0·0005	0·00005–0·0014
#048	M	31·1	25	65	10·151	110–280	0·313	0·07–0·616
#963	M	37·5	25	45	1·606	90–270	0·093	0·001–0·42
#996	F	29·3	10	55	91·346	100–275	0·528	0·210–1·005
#284	M	24·3	35	80	0·439	125–255	0·032	0·00005–0·067
#285	M	29·4	15	55	2·483	110–255	0·179	0·005–0·488

been reported by Zehtindjiev *et al*. (2008). We could not use mosquitoes to infect the birds because competent vectors for GRW2 remain to be identified. Instead we used standard methods of inoculating the birds with infected blood from a donor (Zehtindjiev *et al*. 2008; Palinauskas *et al*. 2010). Further information about the experimental setup, conditions for keeping the birds, handling the donors and experimental birds can be found in the paper by Zehtindjiev *et al*. (2008). Out of 29 inoculated birds (6 in 2005 and 23 in 2006), 13 were not kept long enough to study the chronic infection before being released, and 8 birds died during the primary infection phase. Only 8 birds out of 29 fulfilled the statistical criteria (at least 3 measurement points during the chronic phase, which was conservatively chosen as starting 45 days after the primary peak infection) for this study (for detail, see Table 1).

Because the bird's cages were not mosquito proof, all the captive individuals were potentially exposed to locally transmitted malaria parasites of the species *P. relictum* (lineages SGS1 and GRW11). These lineages only result in mild infections (maximum parasitaemia 1·2%) when infecting great reed warblers and will not be picked up by our lineage specific qPCR protocol (Zehtindjiev *et al*. 2008). The target parasite GRW2 was never recorded in any caged bird unless it was inoculated with blood from an infected donor, supporting the assumption that it does not have local transmission. The experiment was run over 10 months and blood samples were taken every 5th day during the primary phase infection until the peak parasitaemia level was reached. The chronic phase was conservatively set as starting 45 days after the primary peak infection, whereafter blood samples were taken on average every 10th day (Fig. 1 illustrates schematically the different phases of avian malaria infection). A previous study showed that parasitaemia in all experimentally infected birds had reached a stable low level 45 days after the peak primary parasitaemia (Zehtindjiev *et al*. 2008). The 8 birds that died during the primary phase did not have higher parasitaemia compared with some of the birds that survived this experiment. The highest parasitaemia was 6% among the dead birds. However, in almost all birds that died during the primary infection of GRW2, parasitaemia was increasing. Remarkably, 2 birds with very high GRW2 parasitaemia, 85% and 91% (confirmed with both microscopy and qPCR), survived the whole experiment. Control birds inoculated with uninfected blood survived throughout the course of the experiment and did not show any GRW2 infection.

Molecular analyses

DNA was extracted using standard phenol/chloroform methods (Sambrook *et al*. 1989) and diluted to a concentration of 1 ng μl^{-1}. We used real-time qPCR for quantification of *P. ashfordi* (GRW2) parasitaemia using lineage-specific primers (GRW2 8F 5′-CAAATTTTAACTGGTGTCTTATTAG CC-3′ and GRW2 9R 5′-AAAGCACCATCCGC TCCATAA-3′) to amplify a portion (101 bp) of the cytochrome *b* gene (Zehtindjiev *et al*. 2008). To obtain an accurate measurement of host DNA, a second reaction was carried out with host-specific primers, as described elsewhere (Asghar *et al*. 2011). These primers target a single copy nuclear sequence (114 bp) that is ultra-conserved across vertebrates (Bejerano *et al*. 2004). Each reaction of 25 μl included 5 μl (1 ng μl^{-1} DNA), 12·5 μl Supermix (Platinum SYBR-green q-PCR SuperMix-UDG, Invitrogen), 0·1 μl ROX, 0·5 μl (10 μM GRW2 primers) and 1 μl (10 μM SFSR3 primers) and ddH_2O. After the initial incubation at 50 °C for 2 min and 95 °C for 2 min, we ran 42 thermal cycles [95 °C for 15 s, 55 °C for 30 s (57 °C for SFSR3b) and 72 °C for 30 s]. Each DNA sample was run in duplicate and the average values were used for further analysis. Standard curves were produced for measuring parasite DNA by diluting samples with known parasitaemia with uninfected great reed warbler DNA and for quantification of host DNA by diluting great reed warbler DNA

https://doi.org/10.1017/S0031182012000510 Published online by Cambridge University Press

with ddH_2O in 5 step -5x dilutions (5, 1, 0·2, 0·04, and 0·008 ng ml^{-1}). We discarded and re-ran experiments producing standard curves that were steeper than −3·8 (80% qPCR efficiency), as this is an indication of inefficient amplification and errors in q-PCR estimations. We re-calculated the relative parasitaemia, after adjusting for the total DNA content in each reaction as described by Asghar *et al*. (2011).

Molecular sexing of the birds included in the analysis was done by PCR with the primers 0057 F (5′-CGTCAATTTCCATTTCAGGTAAG-3′) and 002 R (5′-TTATTGATCCATCAAGTCTC-3′) amplifying introns of the CHD1Z/W gene (Round *et al*. 2007).

Statistical analysis

Parasitaemia was $\log_{10}$-transformed ($\log_{10}$ (infection intensity + 1)) to meet the requirements of parametric tests in statistical analysis. Statistical analyses were performed in SPSS Inc., Chicago, IL, USA (version 18) and R 2.13.0. We used Pearson correlation to reveal the relationship between primary peak infection and average chronic infection. We set the primary peak of parasitaemia as day 1 and then defined the chronic phase as starting at day 45 after the primary peak of infection (see Table 1). To estimate the chronic infection intensities we took the average log value of the estimates from day 45 after primary peak to the end of the experiment (3–11 measuring points per individual). Some of the birds scored 0 in parasitaemia at 1–2 times during the chronic phase, and we replaced the 0 values (in between 2 positive values) with the lowest observed parasitaemia among all the birds (0·00005%), as these birds must have carried an infection, but at a level that had dropped below the q-PCR detection level. However, results did not differ when we did not replace the 0 values measured during the chronic phase in the statistical analysis. We used General Linear Mixed Model (GLMM) (individuals as random and time fitted as fixed factor) to test whether the parasitaemia within an individual was stable over time over the chronic phase (i.e. >45 days after the primary peak to the end of experiment). We also investigated the effects of body mass at time of inoculation, the pre-patent period (number of days between inoculation and measurable parasitaemia) and the identity of the donor bird on both primary peak and chronic phase parasitaemia by using the General Linear Mixed Model (GLMM) (donor fitted as random factor and body mass and pre-patent period as co-factors).

RESULTS

We found a significant positive correlation between the parasitaemia of the primary peak and the average chronic level ($N = 8$, r = 0·89, $P = 0·003$). As much as 91% of the red blood cells (RBCs) were infected in the most heavily infected bird during the primary peak, whereas in the chronic parasitaemia the infection was two orders of magnitude lower (Fig. 2, Table 1).

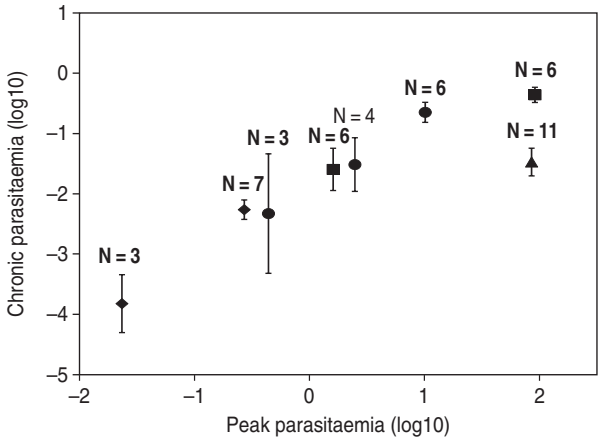

Fig. 2. Correlation between primary peak malaria infection and mean (± S.E.) chronic infection ($N = 8$, r = 0·892, $P = 0·003$). Different symbols represent the different donors [#043 (♦), #089 (●), #051 (■) and #968 (▲)]. N is the number of measurement points during the chronic phase of infection.

The parasitaemia within individuals did not change significantly over time during the chronic phase, (45–286 days after peak infection) (Estimate (S.E.) = −0·0009 (0·002), Z = −0·383, $P = 0·701$). By 45 days after peak parasitaemia there was no further decrease and it seemed that the parasitaemia had reached a stable level (see Supplementary Fig. 1, online version only). Despite generally low chronic parasitaemia, birds kept individual-specific parasitaemia levels ($F_7 = 7·502$, $P < 0·0001$) over the extended period when we sampled the chronic infections.

There was no significant effect of donors on parasitaemia neither for the primary peak ($F_{1,3} = 4·548$, $P = 0·186$) nor the chronic ($F_{1,3} = 1·586$, $P = 0·409$) phase. The duration of the pre-patent period varied among individuals from 10 to 35 days with a mean of 24·3 days (see Table 1), but the pre-patent period did not show any relationship either with primary peak ($F_{1,2} = 2·807$, $P = 0·236$) or chronic ($F_{1,2} = 3·060$, $P = 0·222$) parasitaemia. The body mass at the time of inoculation did not show any significant effect on either primary peak ($F_{1,2} = 0·053$, $P = 0·839$) or chronic ($F_{1,2} = 0·006$, $P = 0·944$) phase parasitaemia. Furthermore, sex (5 males and 3 females) did not show any significant effect either on peak or chronic phase parasitaemia (all $P > 0·214$).

DISCUSSION

In this experimental study, we show that parasitaemia during the primary peak phase of an avian malaria infection is positively correlated with

https://doi.org/10.1017/S0031182012000510 Published online by Cambridge University Press

parasitaemia during the subsequent chronic phase (i.e. 45–230 days after the primary phase; Fig. 2). It is nearly impossible to study the primary peak phase in natural populations of wild birds, whereas it is easy to monitor infections during the chronic phase. Our novel finding that the primary peak phase can be qualitatively inferred from the later chronic phase parasitaemia is therefore important for malaria studies in wild birds. However, because our sample size is rather small and the observed relationship is based on data from a single parasite-host system (*Plasmodium ashfordi* infecting great reed warblers) further analyses of a quantitative predictive model should await data from additional parasites and hosts.

In accordance with our results, a positive correlation between acute and chronic parasitaemia has previously been found in another host-parasite system. In this case laboratory mice that were experimentally inoculated with a higher dose of *Trypanosoma cruzi* had a higher level of parasitaemia when measured 1 year after first infection compared to mice exposed to lower inoculation doses (Marinho *et al*. 1999). In addition, Mackinnon and Read (2004) found that early and late parasitaemia was positively correlated in laboratory mice infected with malaria parasites. However, this study only monitored the infected individuals for three weeks, and it is thus possible that the true chronic phase of the infection had not been reached. In another malaria study in mice, where the relationship between primary and chronic malaria infection intensity was not the focus of the investigation, there appeared to be no relationship between parasitaemia of the primary and chronic phase of *P. chabaudi* parasites 5 weeks after inoculation (Bell *et al*. 2006).

A positive correlation between the primary peak and the chronic phase parasitaemia could result from at least 3 not mutually exclusive mechanisms. First, hosts with higher primary peak parasitaemia might have more exoerythrocytic replication sites (parasite reservoirs) in the host during the chronic phase. Second, more parasites during the acute phase might result in a higher diversity of antigenic variants in the parasite population. Such higher parasite diversity might be more challenging to control for the host`s immune system. Third, the efficiency of the host immune defence against particular parasites might vary between hosts.

The first explanation, proposing an input of parasite from the tissues into the blood, agrees with our results if the chronic parasitaemia is maintained not only by erythrocytic merogony but also by release of the parasites into the blood circulation from phanerozoites (located in host's organs, exoerythrocytic cycle) (Valkiūnas, 2005). Birds having high primary peak parasitaemia might have more parasite developmental sites in the endothelial cells of the capillaries and thereby be exposed to higher parasite input into the blood circulation during the chronic infection. This could also explain why there appears to be no relationship between primary and chronic phase parasitaemia in malaria in mice (Bell *et al*. 2006), because mammalian malaria parasites do not produce phanerozoites (secondary exoerythrocytic cycle) during the life cycle (Frevert *et al*. 2008).

The second explanation is based on the assumption that there is a correlation between peak parasitaemia and the diversity of antigenic variants during the chronic phase. Antigenic variation has been demonstrated for parasites in humans (*Plasmodium falciparum*; Gardner *et al*. 2002), simian [*Plasmodium fragile*; Handunnetti *et al*. 1987) and rodent [*Plasmodium chabaudi* (McLean *et al*. 1986)] malarias. In *P. falciparum* mutually exclusive transcriptional switching occurs between individual members of the nearly 60 different var genes. In *P. falciparum* these genes encode erythrocyte membrane protein 1 (PfEMP1) variant antigens, which through their ability adhere to a variety of host receptors (Gardner *et al*. 2002). However, no data are available to either reject or support a correlation between primary infection level and antigenic diversity in the chronic phase of malaria infection.

The third explanation for the positive correlation between the primary peak and the chronic infection levels proposes that there are phenotypic differences between host individuals, which affect both the acute and chronic parasitaemia. In humans, the outcome of a *P. falciparium* infection depends on a large number of individual-specific factors, including host age, nutrition, socio-economic factors, parasite genetics and host genetics (Mackinnon and Read, 2004). In the present study of captive birds we tried to control for most of these factors, and body mass at the time of inoculation did not show any significant effect on either the primary peak or the chronic phase parasitaemia. However, host genetics could not be controlled for and could hence potentially partly explain the different outcomes (parasitaemia) of the malaria infections in different individuals. Hill *et al*. (1991) found that humans with specific Major Histocompatibility Complex (MHC) alleles were less likely to develop severe cerebral malaria when infected with *P. falciparum*. Previous studies in great reed warblers have found associations between the presence of a specific MHC allele and GRW2 infection prevalence and parasitaemia. Great reed warblers with the MHC class I allele b4b had a higher prevalence of chronic GRW2 malaria but lower parasitaemia, implying that individuals carrying this allele were able to suppress, and survive, the parasitaemia during the primary infection (Westerdahl *et al*. 2005, 2011). Taken together, the data from humans, rodents and great reed warblers suggest that host genetics is a factor that influences the outcome of a malaria infection (Hill *et al*. 1991; Mackinnon and Read 2004; Westerdahl *et al*. 2011). At present, however, we cannot determine to what

https://doi.org/10.1017/S0031182012000510 Published online by Cambridge University Press

extent each of the three mechanisms (number of exoerythrocytic replication sites, diversity of antigenic variants in the parasite population, or efficiency of the host immune defence) contributes to the observed positive relationship between chronic and acute phase malaria.

In our study the parasitaemia within individuals did not change significantly during the chronic phase. Furthermore, the chronic parasitaemia showed significant repeatability within individuals demonstrating that the qPCR protocol is sensitive enough to pick up slight differences between individuals also at low levels of parasitaemia. This is in line with previous findings in both experimental (Zehtindjiev *et al*. 2008) and wild population studies (Asghar *et al*. 2011). In fact, great reed warblers seem to keep the chronic parasitaemia around an individual specific level even between years (Asghar *et al*. 2011).

One factor that must be remembered when applying findings from studies of captive animals to individuals infected in their natural environment is that birds in the wild with very high primary peak parasitaemia most likely die from the infection. Hence birds with the highest parasitaemia will never reach the chronic stage and will never be caught and sampled in the wild. So even if the chronic phase can give an estimate of the primary phase parasitaemia there will be a subset of individuals that never will be sampled because they died during the primary peak phase. A previous finding in great reed warblers could indirectly infer that individuals who are better able to suppress the parasitaemia during the primary infections are also more likely to survive the infection (Westerdahl *et al*. 2005, 2011).

To the best of our knowledge, this is the first study to investigate the association between primary and chronic infection intensities of an avian malaria parasite. We found a significant positive correlation between primary and chronic infection intensities. This result has important implications for research on avian malaria in wild host systems that to date mainly work with chronic phase infections, as it will allow for qualitative inference of the parasitaemia of the preceding more severe primary phase infection. However, more studies are needed before we can develop quantitative models applicable to other host and malaria parasite species. The relationship we have found between the acute and chronic phases of malaria infections may allow us to study malaria-specific immune responses and relate these to parasitaemia levels in order to better understand disease dynamics and fitness consequences of avian malaria in wild birds.

ACKNOWLEDGMENTS

The experiment was carried out with the permission from the Bulgarian Ministry of Environment and Waters. We thank L. Råberg, M. Andersson and M.R. Lima for the discussions and comments on the manuscript.

FINANCIAL SUPPORT

This work was supported by grants from the Swedish Research Council (VR, 621-2007-5193 to S.B.), (VR, 621-2007-5385 to D.H.) and (VR, 621-2006-4551 to H.W.) and to P.Z. from the FP7 Capacities project WETLANET and from Higher Education Commission of Pakistan (HEC) to M.A. This study was partly funded by the Centre for Animal Movement Research (CAnMove) financed by a Linnaeus excellence research grant (349-2007-8690) from the Swedish Research Council and Lund University.

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