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TECHNICAL NOTES

A real-time PCR protocol for simple and fast quantification of blood parasite infections in evolutionary and ecological studies and some data on intensities of blood parasite infections in a subtropical weaverbird

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Abstract In several fields of research, such as immunoecology, evolutionary ecology, sexual selection, parasitology or host-parasite coevolution, a reliable quantitative assessment of blood parasite infections is necessary for testing specific predictions regarding relationships between the degree of infections and various parameters of interest. Here, we present a relatively simple, fast and reliable protocol based on quantitative real-time PCR to determine the intensity of infections with blood parasites of the genus *Plasmodium* and/or *Haemoproteus* in blood samples of birds, using male Red Bishops (*Euplectes orix*; Ploceidae, Passeriformes) as example. The intensity of infections is assessed by amplification of a specific 85-bp fragment within the plastid-like large subunit ribosomal-RNA (LSU-rRNA) gene, which is conservative across a range of *Plasmodium* and *Haemoproteus* species. By measuring the accumulation of the product during the PCR (in real-time) using a fluorescent labelled oligonucleotide probe, a threshold can be determined at which the fluorescence of the product raises above background level. The starting quantity of blood parasites in the investigated blood samples is then calculated by comparison with thresholds determined for standards of known quantity (clones of a 594-bp fragment within the LSU-rRNA gene from *Plasmodium falciparum* including the target sequence) in the same PCR reaction. With this method, blood parasites were detected in 123 out of 127 samples from male Red Bishops, with a median of 0.059 blood parasites per 100 blood cells (range 0–19.2 blood parasites per 100 blood cells). The method described here produces consistent and reproducible data, can easily be modified and extended to detect and quantify blood parasites at different systematic levels, and thus has broad application to many researchers in the field of evolutionary and behavioral ecology.

Keywords Avian haemosporidians · *Haemoproteus* · Intensity of infections · *Plasmodium* · Quantitative real-time PCR

Zusammenfassung

Ein Real-Time PCR Protokoll zur einfachen und schnellen Quantifizierung von Blutparasitenbefall in evolutionsbiologischen und ökologischen Studien, und einige Daten zu Intensitäten von Blutparasitenbefall bei subtropischen Webervögeln

In vielen Forschungsfeldern wie Immunoökologie, Evolutionsökologie, sexuelle Selektion, Parasitologie und Parasit-Wirts-Koevolution ist eine verlässliche Quantifizierung von Blutparasitenbefall notwendig, um spezifische Vorhersagen zum Zusammenhang zwischen der Intensität von Blutparasitenbefall und verschiedenen Parametern von Interesse zu testen. Hier stellen wir am Beispiel von Männchen des Oryxwebers (*Euplectes orix*; Ploceidae, Passeriformes) ein relativ einfaches, schnelles und verlässliches, auf quantitativer Real-Time PCR beruhendes Protokoll vor, mit dem die Intensität des Befalls mit Blutparasiten der Gattungen *Plasmodium* und/oder *Haemoproteus* bestimmt werden

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kann. Die Intensität des Befalls wird mittels Amplifikation eines 85 bp Fragments des Gens der ribosomalen RNA aus der großen Untereinheit des Ribosoms (LSU-rRNA) ermittelt, welches spezifisch für die beiden Gattungen *Plasmodium* und *Haemoproteus* ist. Durch die Messung der Zunahme des PCR-Produkts mittels einer mit einem Fluoreszenzfarbstoff markierten Oligonukleotidsonde in Echtzeit kann eine Schwelle bestimmt werden, bei der die durch die Produktamplifikation bedingte Fluoreszenz über die Hintergrundfluoreszenz ansteigt. Die Menge an Blutparasiten in den untersuchten Blutproben wird dann durch den Vergleich mit in derselben PCR-Reaktion ermittelten Schwellenwerten von Standards mit bekannter Menge an Blutparasiten-DNA (Klone eines 594 bp Fragments des LSU-rRNA Gens von *Plasmodium falciparum*, welches die Zielsequenz beinhaltet) bestimmt. Mit dieser Methode wurden Blutparasiten in 123 von 127 Blutproben von Oryxwebermännchen mit einem Median von 0.059 Blutparasiten pro 100 Blutzellen (Bereich 0 bis 19.2 Blutparasiten pro 100 Blutzellen) detektiert. Die hier beschriebene Methode ergibt konsistente und reproduzierbare Daten, kann leicht für die Detektion und Quantifizierung von Blutparasiten verschiedener taxonomischer Ebenen modifiziert und erweitert werden, und ist demzufolge von großem Interesse für viele Forscher auf den Gebieten Evolutionsbiologie und Verhaltensökologie.

Introduction

The detection and quantification of parasitism is an important component of the work in many research fields, such as studies on the pathology and morphology of blood parasites (e.g. Bennett et al. 1993; Merino et al. 2000), on the relationship between parasites and the expression of sexually selected ornaments (e.g. Hamilton and Zuk 1982; Schall and Staats 1997; Merilä et al. 1999), or on relationships between parasites, immune status, and reproduction in the context of life-history trade-offs or immunoecology (e.g. Sheldon and Verhulst 1996; Norris and Evans 2000). For decades, blood parasite prevalence (and to some extent also intensity) has been studied predominantly by scanning blood films under a microscope. In recent years, molecular PCR-based techniques have increasingly been used to assess blood parasite prevalence, more and more replacing the time-consuming microscopy. Several PCR protocols have been developed to assess blood parasite prevalence in birds (e.g. Feldman et al. 1995; Bensch et al. 2000; Fallon et al. 2003; Richard et al. 2002; Hellgren et al. 2004; Waldenström et al. 2004). Some studies have specifically compared the sensitivity in detecting haemosporidian infections in birds between molecular PCR-based methods and microscopy. Valkiunas et al. (2008) have found microscopy to yield similar results as PCR diagnostics with regard to the determination of blood parasite prevalence in birds when blood films of good quality are examined by experienced and skilled investigators. Likewise, Krone et al. (2008) reported that microscopical examinations of blood smears and PCR-based amplifications of parasite mtDNA resulted in similar detection rates of haemosporidian blood parasites in birds. In contrast, several other studies found microscopy to be less sensitive and reliable than PCR-based techniques (e.g. Jarvi et al. 2002; Richard et al. 2002; Fallon et al. 2003; Durrant et al. 2006; Fallon and Ricklefs 2008).

While these PCR-based techniques now enable researchers that are not specialized in parasitology to evaluate the presence or absence of blood parasites in the context of ecological studies, there is still a lack of an easy and quick method to reliably quantify the intensity of blood parasite infections. However, many research questions require a quantification of infections by blood parasites rather than just a determination of whether or not an individual is infected. For example, a recent meta-analysis investigating the relationship between experimentally altered reproductive effort and infections with blood parasites in birds (Knowles et al. 2009) found that the effect of reproductive effort on blood parasite infections was three times larger when quantitative measures of infections with blood parasites were used rather than blood parasite prevalence (presence or absence of infections). In general, the effects of blood parasites on host immunology and overall fitness often strongly depend on the level of infection, with chronic low levels of infection with haemosporidian blood parasites often being rather harmless to hosts, while acute high levels of infections can cause haemolytic anaemia, decrease in haematocrit values, elevated plasma gammaglobulin levels, increased heterophil to lymphocyte ratio, hypertrophy of spleen and liver, and even death (Beier et al. 1981; Hayworth et al. 1987; Campbell 1995; Ots and Horak 1998; Samour 2000; Edler et al. 2004; Palinauskas et al. 2008).

Here, we present a quick and relatively easy molecular method for a reliable and accurate quantitative assessment of blood parasite infections from small blood samples. This method is based on the ability to monitor amplifications of target sequences in polymerase chain reactions in real-time using specific fluorescent oligonucleotide probes (real-time PCR), making it possible to accurately quantify starting amounts of DNA. Out of several different probe strategies developed (see, for example, Dorak 2006), the so-called TaqMan assay based on the 5′ exonuclease activity of *Taq* polymerase (Holland et al. 1991) is in our opinion the one which is best suited for quantifying small amounts of blood parasite-specific DNA in the presence of a great excess of

host DNA as is the case in blood samples of birds. In a TaqMan assay, a target-specific oligonucleotide hybridisation probe is labeled at the 5′ end with a fluorescent reporter molecule and at the 3′ end with another fluorescent molecule that acts as a quencher for the reporter molecule (Heid et al. 1996). When the reporter fluorophore is excited by an outside shortwave light source, the fluorescence of the reporter molecule is absorbed by the quencher as long as both are bound to the hybridisation probe. However, when the *Taq*-polymerase encounters the hybridisation probe during extension from one of the primers, the hybridisation probe is degraded by the 5′ exonuclease activity of the *Taq*-polymerase, releasing the reporter from the vicinity of the quencher and generating a signal which can be detected and measured. While the fluorescence of the reporter molecule increases with increasing product accumulation with each successive amplification cycle, the change in fluorescence is usually undetectable in the early phases of the PCR. At some cycle during amplification, however, the fluorescence caused by increased product accumulation rises clearly above background levels, the point of which is termed the threshold cycle. Since there is a linear relationship between the logarithm of the starting amount of a template and its threshold cycle, the starting amount of the target sequence in each unknown sample can be determined using a standard curve calculated for templates with a known starting amount of the target sequence.

Recently, Zehtindjiev et al. (2008) have used quantitative real-time PCR to determine intensities of parasite infections with two lineages of *Plasmodium ashfordi* and *P. relictum* in captive Great Reed Warblers (*Acrocephalus arundinaceus*). However, the general applicability of the protocol presented by Zehtindjiev et al. (2008) is limited by the fact that they used DNA samples of birds with levels of parasitemia assessed from blood smears by experienced parasitologists for producing the standard curve. That is, this method requires both samples of already infected birds to be used as standard and expertise in identifying and quantifying blood parasites in blood smears. Furthermore, SYBR-green-based detection as used in the protocol described by Zehtindjiev et al. (2008) might be problematic in the presence of a great excess of host DNA, since SYBR-green is an intercalating dye which binds to all double-stranded DNA products. Therefore, nonspecific binding as well as primer dimers could potentially confound the quantitative data on blood-parasite-infection intensities and have to be accounted for after the PCR when calculating blood parasite intensities.

The protocol described here is based on real-time PCR using a TaqMan assay to quantify blood parasite infections with parasites of the genus *Plasmodium* and *Haemoproteus* using blood samples from birds. As target sequence, we used an 85-bp fragment of the plastid-like large subunit ribosomal-RNA (LSU-rRNA) gene (Tan et al. 1997), which is conservative across a range of *Plasmodium* and *Haemoproteus* species. The standard curve was calculated based on DNA samples from *Plasmodium falciparum* with known copy numbers, enabling us to accurately quantify the number of blood parasites found in a sample of bird DNA without the need to use DNA samples of infected birds with infection levels assessed by prior microscopy. Our protocol was developed and tested using blood samples obtained from adult males of the Red Bishop (*Euplectes orix*), a weaverbird species of the family Ploceidae which is common in Sub-Saharan Africa (MacLean 1993) and has been reported to be parasitised by different haematozoa species of the genus *Plasmodium* and *Haemoproteus* (Bennett et al. 1992).

Methods

Study objects and study site

Blood samples were collected from adult male Red Bishops as part of a long-term study on sexual selection, reproductive biology and life-history evolution in this species. Red Bishops breed in dense colonies in reed beds or bulrush stands around water, where males in a brilliant orange and black breeding plumage establish and defend territories and build several nests to which they try to attract females. The blood samples were collected in five consecutive breeding seasons (approximately from October to March) between 1994 and 1999 at our study site in the Addo Elephant National Park, Eastern Cape Province, South Africa (33°26′S, 25°45′E). A detailed description of Red Bishop breeding behaviour is given in Friedl (2004). The study was conducted under licence from the Provincial Administration of the Cape of Good Hope, Chief Directorate Nature and Environmental Conservation (Licence numbers: 653/94, 144/95, 92/96, 1/97) and with permission from the South African National Parks Board.

Blood sample collection and DNA extraction

Adult Red Bishops were caught in walk-in traps baited with commercially available mixed bird seeds or with mist nets set up close to the border of the colony. Each individual was leg-banded with a unique combination of four colour rings and a numbered stainless steel ring provided by the South African Bird Ringing Unit. Blood samples of adult birds were obtained by puncturing the brachial vein with a sterile 25-gauge needle and collecting about 20–80 µl of blood with a heparinised microcapillary tube. The blood samples were transferred into sterile 1.5-ml microfuge tubes containing about 0.7 ml of the nonlytic buffer PBS (phosphate-buffered saline: 3 mM KCl, 8 mM

Na_2HPO_4, 2 mM KH_2PO_4, 0.14 M NaCl, 6 mM EDTA, 0.2% sodium azide), and the tubes were inverted several times to bring the cells into suspension. After the cells had settled at the bottom of the tube, the supernatant was removed, replaced with 500 µl of fresh PBS solution, and the cells were resuspended by inverting the tubes several times. After the cells had settled again, this rinsing procedure was repeated one more time and then the blood samples were stored in the refrigerator at 4°C until further processing. DNA was extracted from blood samples about 1–3 months after the end of the breeding season in which the sample was collected using standard phenol–chloroform extraction, followed by dialysis against TNE_2 (10 mM Tris, pH 8.0; 10 mM NaCl; 2 mM EDTA) using dialysis tubes with a molecular weight cut off around 12,000–14,000 Dalton to remove remaining proteins, phenol, and potential inhibitors of PCR reactions such as haemoglobin. We determined the concentrations of the extracted DNA by examining UV absorbance with a spectrophotometer (Hitachi U-1100 or Eppendorf BioPhotometer) at a wavelength of 260 nm. Concentration estimates for our samples ranged from about 0.05 to 0.8 µg DNA/µl, resulting in yields of extracted DNA of about 30–600 µg DNA per sample (depending on the total amount of each sample), with most samples having concentrations of more than 0.5 µg DNA/µl. For details regarding DNA extraction procedures and purity of extracted DNA see Friedl and Klump (1999). Overall, we collected 170 blood samples from 90 individual adult males. For 53 of these males we had more than one blood sample available (range 2–5 samples per male).

Target sequence and performance of real-time PCR

Our search for a suitable target sequence for the detection of *Plasmodium* and *Haemoproteus* in blood samples from birds was based on the sequence of a 595-bp fragment of the plastid-like large subunit ribosomal-RNA (LSU-rRNA) gene of *Plasmodium falciparum* published by Tan et al. (1997). After experiments with several different primer–probe combinations (designed with Beacon Designer 2.06 software; Premier Biosoft International) and different PCR protocols, we found the following combination to yield the best results in real-time PCR. DNA extracted from blood samples of adult male Red Bishops was amplified in 4 replicate reactions with the primers Plasmo474for (5′-AGGCTAATCTTTTCCGAGAGTCC-3′), Plasmo558rev (5′-ACATACTACTGCTTTAGGATGCGA-3′), and the dual-labeled hybridisation probe (5′-AAGCCGACATCGAGGTGCCAAAAC-3′) labeled with 6-FAM as a reporter molecule at the 5′-end and with BHQ (Black Hole Quencher) at the 3′-end, resulting in an amplification product of 85 bp (including primers). The 25-µl reaction mixes contained 5,000 ng of extracted DNA, 12.5 µl iQ-Supermix containing i*Taq* DNA polymerase and dNTPs (Bio-Rad), 0.5 µl of Plasmo474for, 0.75 µl of Plasmo558rev, and 0.5 µl of the dual-labeled hybridisation probe. PCR was performed using 96-well plates on a iCycler iQ Real-Time Detection System (Bio-Rad), with 8 min at 95°C for denaturation and activation of the antibody-mediated hot-start i*Taq* DNA polymerase, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

Production of a *Plasmodium* standard

For every real-time PCR, standards of the target sequence of known quantity are needed to be able to calculate the standard curve necessary to determine starting quantities of unknown samples. As our target sequence was an 85-bp fragment (including primers) of the plastid-like large subunit ribosomal-RNA (LSU-rRNA) gene of *Plasmodium falciparum* (see above), we made clones of the larger 595-bp fragment described by Tan et al. (1997) to be used as our standards. First, 32 ng of DNA from *Plasmodium falciparum* (kindly provided by Thomas Jacobs from the Bernhard-Nocht-Institut für Tropenmedizin) were amplified with 12.5 µl iQ-Supermix (Bio-Rad) and 2 µl each (10 pmol/µl) for the primers L1 (5′-GAC CTG CAT GAA AGA TG-3′) and L2 (5′-GTA TCG CTT TAA TAG GCG-3′) (Tan et al. 1997) in several 25-µl reactions performed in a UNO Thermoblock Cycler (Biometra). A initial step of 5 min at 95°C for denaturation was followed by 40 cycles with 1 min at 95°C, 2 min at 52°C for annealing and 3 min primer extension at 72°C, and a final extension step for 5 min at 72°C. After PCR products were analysed on a 1.5% agarose gel under UV light, primers and unincorporated dNTPs were removed (Montage PCR Centrifugal Filter Devices, Millipore) prior to the cloning in the pGEM-T Vector System (Promega). Plasmid DNA of positive clones was obtained by the use of the Perfectprep Plasmid Mini Kit (Eppendorf) and then sequenced in both directions by MWG Ag Biotech. Sequence alignment was carried out with the program CLUSTAL W (http://www.ebi.ac.uk/cgi). Clone plL1/L2-2 was tested for the presence and function of recognition sites for the primers Plasmo474for and Plasmo558rev as well as for the dual-labeled hybridisation probe to amplify the 85-bp target sequence in quantitative real-time PCR using the iCycler IQ Real-Time Detection System (Bio-Rad).

When performing the real-time PCR for quantification of *Plasmodium* and *Haemoproteus* in DNA samples of Red Bishop males with unknown amounts of blood parasites, two replicates each of a 10-fold dilution series of clone plL1/L2-2 plasmid DNA ranging from 1 ng (standard 1) to 10^{-5} ng (standard 6) were added as standards to each 96-well plate for the calculation of the standard curve.

Standardisation and criteria for obtaining valid data

Two kinds of standardisation are necessary for obtaining reliable and meaningful results. First, the amount of bird DNA has to be equal in all samples to facilitate the calculation of infection levels with *Plasmodium* and *Haemoproteus* in terms of number of blood parasites per 100 blood cells. We used the relatively large amount of 5,000 ng bird DNA for each 25-µl reaction to be able to quantify low infection levels (see below). As a consequence, only samples with concentrations higher than about 500 ng DNA/µl could be used for the 25-µl reactions (most of our samples had concentrations above this value). Second, PCR reactions have to be normalised across different runs of real-time PCR to account for possible differences with regard to overall amplification efficiency. Prior to data analyses, we therefore manually set the threshold cycle for standard 4 (equivalent to 10^{-3} ng) to 24 for all 11 real-time PCR runs. Online Resource 1 shows an exemplary amplification process and Online Resource 2 shows an example of a standard curve.

We applied two very conservative and strict criteria for accepting data on *Plasmodium* and/or *Haemoproteus* infection levels as valid. First, we only accepted PCR runs were the correlation coefficient for the standard curve (log of the starting amounts of the six standards with two replicates each plotted against their threshold cycles) was higher than 0.99, since lower correlation coefficients might indicate problems with the amplification of the standards. If this criterion was not fulfilled, all data for the run (i.e. all 96 single PCR reactions) were discarded. Out of 11 runs, only 1 had to be completely excluded from further analysis because of a failure to fulfil this criterion (correlation coefficient 0.962); all other runs showed correlation coefficients for the standard curve of above 0.99. Once a PCR run was accepted as valid, we applied our second criteria at the level of each quadruplicate of individual bird DNA samples. Quantification of the infection with *Plasmodium* and/or *Haemoproteus* of each unknown sample was regarded as valid only if the standard deviation of the threshold cycles calculated from the four replicates was less than 1.0, or when all four replicates of an unknown sample showed no amplification of the target sequence at all, indicating absence of *Plasmodium* and *Haemoproteus* in this sample. Out of 170 samples investigated, we obtained a valid quantification of infection levels with *Plasmodium* and/or *Haemoproteus* for 123 samples (i.e. 72%) in the first PCR run. Twenty of the remaining 47 samples were run a second time and we obtained a valid quantification for 4 of these 20 samples, resulting in a total of 127 valid data on *Plasmodium* and/or *Haemoproteus* infection levels in 77 individual Red Bishop males available for further analyses.

Calculation of infection levels with Plasmodium and/or Haemoproteus

Before interpreting our quantitative data on *Plasmodium* and/or *Haemoproteus* infections, we had to convert the rather abstract values of amount of blood parasite DNA per sample in a more meaningful measure for the level of infection. Our standards consisted of clones of the 595-bp fragment of the plastid-like large subunit ribosomal-RNA (LSU-rRNA) gene of *Plasmodium falciparum* with the total size (vector plus clone) being 3,610 bp, which is equivalent to 3.836×10^{-6} pg (assuming that the average weight of a base pair is 650 Daltons). Thus, our standard 1 with 1 ng DNA corresponds to about 260×10^{6} units of the standard (vector plus clone), with each unit containing one copy of our target sequence, our standard 4 with 10^{-3} ng DNA corresponds to 260,000 units of the standard, and our standard 6 with 10^{-5} ng DNA corresponds to 2,600 units of the standard. The fluorescence measured in real-time PCR for one unit of the standard is equal to the fluorescence measured for a sample of bird DNA with one *Plasmodium* or *Haemoproteus*, because both contain exactly one copy of the target sequence. Thus, the total number of *Plasmodium* and/or *Haemoproteus* detected in a sample of bird DNA can easily be determined by multiplying the starting amount of DNA in the bird sample in pg calculated based on the standard curve by 260,000. Based on the fact that the average genome size of a passerine bird is 2.8 pg (e.g. Tiersch and Wachtel 1991), the amount of 5,000 ng bird DNA per sample is equivalent to about 1,786,000 blood cells. Infection levels with *Plasmodium* and/or *Haemoproteus* in terms of number of blood parasites per 100 blood cells can therefore be calculated by dividing the total number of blood parasites detected in a sample by 1,786,000 and multiplying with 100. Please note that the number of blood parasites per 100 blood cells is not necessarily equivalent to percentage of blood cells infected, since due to schizogony a single blood cell may contain many merozoites.

Statistical analysis

Statistics were performed using the software package SPSS 16.0, and all *P* values reported here are for two-tailed tests.

Results

Infection levels

The infection levels with *Plasmodium* and *Haemoproteus* as determined with real-time PCR for all 127 samples (from 77 individual Red Bishop males) with valid data averaged 0.53 ± 1.97 blood parasites per 100 blood cells

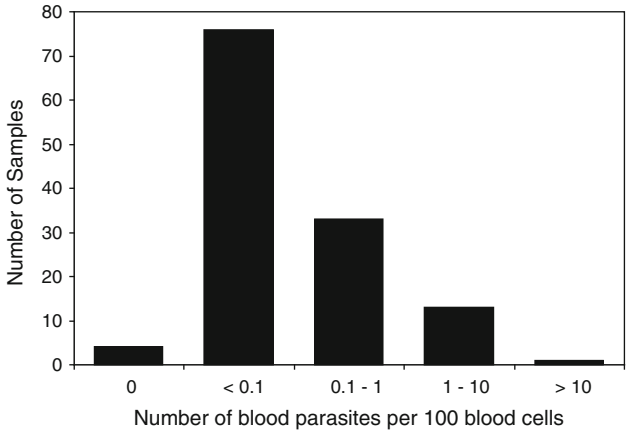

Fig. 1 Frequencies of infections of adult Red Bishop (*Euplectes orix*) males with blood parasites of the genus *Plasmodium* and *Haemoproteus* given as number of blood parasites per 100 blood cells ($n = 127$ samples from 77 males)

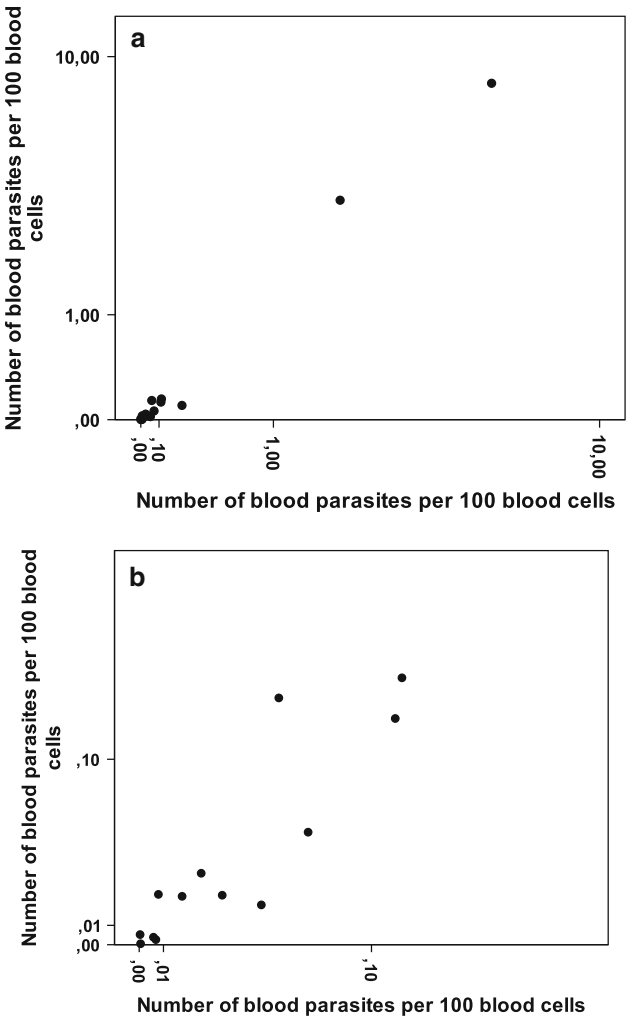

Fig. 2 Relationship between the detected infection levels (number of blood parasites per 100 blood cells) for two samples each collected at the same time from 16 adult Red Bishop males. For better visualisation, two graphs with different scales are shown, with all data points ($n = 16$) presented in (**a**), and a magnification of the part with data points with infection levels lower than 0.2 blood parasites per 100 blood cells ($n = 13$) presented in (**b**)

and ranged from no blood parasites up to 19.2 blood parasites per 100 blood cells. However, the distribution of infection levels was heavily biased towards low to very low infection levels (median 0.059 blood parasites per 100 blood cells), and, consequently, was significantly different from a normal distribution (Shapiro–Wilk test, $P < 0.001$). There was only one case with an infection level higher than 10 blood parasites per 100 blood cells, 13 cases with infection levels between 1 and 10 blood parasites per 100 blood cells, 33 cases with infection levels between 0.1 and 1 blood parasites per 100 blood cells, 76 cases with infection levels lower than 0.1 blood parasites per 100 blood cells, and only 4 cases without any sign of infection (see Fig. 1).

Repeatability of infection levels for blood samples of individual males taken at the same time

In 16 cases, we took two blood samples from an adult male Red Bishop at the same time. The samples were extracted separately and we ran four replicates for each of the two samples. Since the blood samples were taken at the same time, the infection level detected in the two samples should be the same or at least very similar. A correlation analysis revealed a very high repeatability of infection levels across these samples ($n = 16$, $r_s = 0.906$, $P < 0.001$), demonstrating the reliability of our method (see Fig. 2).

Infection levels of individual males across years

For 18 males, data on the level of infection with blood parasites were available for more than one season. Since data on infection levels with blood parasites collected for individual passerine birds over a time span of several years are very scarce, we present these data in detail in Table 1. There was a significant positive correlation between the two samples of individual males collected in different seasons ($n = 18$, $r_s = 0.478$, $P = 0.045$), indicating that males with high or low levels of blood parasites in one season also had high or low levels, respectively, in the following seasons. Furthermore, individual infection levels in the first season for which a sample of a Red Bishop male was available did not differ significantly from infection levels in the second season (2–4 years later) for which a sample of this male was available (Wilcoxon matched pairs signed ranks test, $Z = -0.98$, $P = 0.327$), showing that there was no clear tendency for individual infection levels to increase or decrease with age. A similar pattern was

Table 1 Infection levels with *Plasmodium* and/or *Haemoproteus* blood parasites (number of blood parasites per 100 blood cells) for 18 Red Bishop (*Euplectes orix*) males for which quantitative data on blood parasites were available for two seasons

Male ID	Season first sample collected	Number of blood parasites per 100 blood cells	Season second sample collected	Number of blood parasites per 100 blood cells
13	1993/94	0.09288	1994/95	0.06566
14	1993/94	0.01587	1995/96	0.09593
19	1993/94	0.02125	1994/95	0.00182
41	1993/94	0.11704	1994/95	1.12822
95	1993/94	0.00010	1994/95	0.00021
97	1993/94	0.00280	1994/95	0.01280
101	1993/94	1.32475	1994/95	3.81411
122	1994/95	0.00562	1995/96	0.12505
137	1993/94	0.25621	1995/96	0.43964
142	1993/94	0.02147	1995/96	0.00033
296	1994/95	0.00053	1995/96	0.00026
320	1994/95	0.00052	1996/97	0.17178
362	1994/95	3.15901	1995/96	0.52408
450	1995/96	0.93315	1996/97	0.01374
475	1994/95	0.01179	1998/99	0.01747
488	1994/95	0.00316	1996/97	0.09710
763	1995/96	0.01705	1996/97	0.00763
797	1995/96	0.00441	1996/97	0.01951

found for the three males for which data on blood parasites were available for three seasons. In none of these three cases was there a consistent increase or decrease of blood parasite numbers over the three seasons.

Discussion

Infection levels of Red Bishop males

While there is a large amount of data on prevalence of different blood parasites in a wide range of bird species, quantitative data on intensities of blood-parasite infections (parasitemia) in free-living passerine birds are rather scarce. Here, we presented a quantitative analysis of blood parasite infections in a large number of adult Red Bishops as found during the breeding season. Blood-parasite prevalence was very high, with only 4 out of 127 samples (i.e. 3.1%) showing no sign of infection. This high prevalence indicates that in Red Bishops most adult males were exposed to blood-parasite transmitting insects (for *Plasmodium* and *Haemoproteus*, these are mainly mosquitoes and louse flies, respectively) at least once during their life time. The levels of infection with blood parasites of the genus *Plasmodium* and *Haemoproteus* are generally rather low, with about 90% of all samples showing less than 1 blood parasite per 100 blood cells, and the highest level of infection being 19 blood parasites per 100 blood cells. Edler et al. (2004) reported quantitative data on blood parasite infections in Red Bishop males obtained by microscopy and found 3–141 red blood cells infected with *Plasmodium* during 20 min of scanning at ×1,000 magnification in 54 out of 131 blood smears investigated, which corresponds approximately to 0.02–0.9 blood parasites per 100 blood cells (given that about 15,000 red blood cells were counted per sample; R. Edler, personal communication). Zehtindjiev et al. (2008) reported a maximum parasitemia (determined by quantitative PCR) of *Plasmodium* in adult Great Reed Warblers caught in the wild of 37.6%, while another study on adult Great Reed Warblers found intensities of infections with *Haemoproteus* (as assesses by microscopy) ranging from 0.011 to 8.4% (mean 0.58%, median 0.18%) infected red blood cells (Hasselquist et al. 2007). A likely explanation for the low infection intensities with blood parasites as found in this and other studies would be that infections rapidly eliminate heavily parasitised individuals from the population, leaving only non-infected individuals or individuals with a chronic non-pathogenic level of infection. In addition, males with a heavy acute infection of avian malaria might be weakened to an extent that they are unable to take part in breeding activities and to compete with other males for breeding territories. Such males would be less likely to be caught with mist nets, biasing the sample towards males less affected by blood parasites (Yorinks and Atkinson 2000; Valkiunas 2001).

There are only a few studies that have investigated variation in parasitemia of individual wild birds within or

across seasons. Similar to our results on across-season variability in infection level of Red Bishops, Dale et al. (1996) found a significant positive correlation and no significant difference between infection levels of individual Pied Flycatcher (*Ficedula hypoleuca*) males with *Haemoproteus* (assessed by microscopy) caught twice within a breeding season. Likewise, Zehtindjiev et al. (2008) found high and significant repeatabilities of infection levels with different lineages of *Plasmodium* in experimentally infected Great Reed Warblers over a time span of several months. These results indicate that the intensities of blood parasite infections seem to be kept around individual-specific levels, at least in the stage of chronic infections. However, even if an infection is kept around a certain level for some time reaching a steady state, a burst of physiological or social stress can always trigger a sudden and pronounced increase in the parasitemia.

Application of the developed protocol for quantitative determination of blood parasite infection in birds

The protocol for a quantitative assessment of blood parasite infections by real-time PCR presented here proved to produce fast, repeatable and accurate data regarding levels of infections with blood parasites of the genus *Plasmodium* and *Haemoproteus* in birds. There are several advantages of our approach, which make it suitable for a wide range of research questions in ecology, parasitology, and evolution where an exact quantification of blood parasite infection is required. First, the fact that our standard consists of clones of a DNA fragment obtained from *Plasmodium falciparum* means that there is no need for experienced parasitologists to assess parasitemia in blood samples of infected birds via microscopy to be able to produce a standard (as is the case in the protocol described by Zehtindjiev et al. 2008). Moreover, since we can calculate the copy number used in the standards, we can determine the number of blood parasites present in a given unknown sample, enabling us to calculate the intensity of blood parasite infections not only in relative, but also in absolute terms.

The method can easily be modified and extended to detect and quantify blood parasites at different taxonomic levels, for example by using target sequences specific for certain *Plasmodium* or *Haemoproteus* species that are of special interest. Such species-specific quantification could also be very useful when several different but closely related blood parasite species should be investigated that are very difficult to distinguish on blood smears, at least in certain developmental stages. By using multiplex real-time PCR, it would even be possible to detect and quantify two or more different blood parasite species within one PCR run.

Since small blood samples can easily be obtained, and in fact might often be already available in ecological studies because they are used for determining phylogenetic relationships, evaluating paternity, or determining sex, our method should be applicable to a wide range of research projects where blood samples can be collected on a routine basis.

Overall, the method for quantification of blood parasite infections based on real-time PCR presented here is time-saving and proved to produce consistent and reproducible results without the need of acquiring expertise in the difficult task of identifying and counting blood parasites in blood smears. It can easily be modified and extended to be applied in a wide range of research questions that require an accurate and reliable quantification of blood parasites at different systematic levels, and thus has broad application to many researchers in the fields of parasitology, behavioural ecology, evolutionary biology, and many others.

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