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EXPERIMENTAL INVESTIGATION OF AVIAN MALARIA PARASITES (*PLASMODIUM*, HAEMOSPORIDA): LINKAGE OF TRADITIONAL AND MOLECULAR DATA

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INTRODUCTION

Relevance of the study. Malaria parasites (*Plasmodium,* Plasmodiidae) parasitise and cause disease, which is well known as malaria, in a wide range of vertebrate hosts: amphibians, reptiles, birds and mammals including humans (Valkiūnas, 2005; Martinsen et al., 2008). A shortcoming of recent malaria research is that the great majority of studies on general malariology deal with a handful of *Plasmodium* species infecting humans, some other primates and mice (Carlton et al., 2002; Sherman, 2005; Pain et al., 2008). Zoological studies, particularly experimental ones, on malaria parasites infecting reptiles and birds remain scarce. Moreover, fragmentary information is available about relationships between bird haemosporidians and their vectors (Valkiūnas, 2005; Ishtiaq et al., 2008; Kimura et al., 2009). These are obstacles for developing general models of *Plasmodium* parasites evolution and for understanding the evolutionary biology of this large and diverse group of parasites, particularly from the perspectives of zoology and evolutionary biology.

Avian malaria parasites are responsible for severe diseases in some domestic and wild birds (Valkiūnas, 2005). These parasites are cosmopolitan in distribution; they are widespread in Europe, including the Baltic region (Waldenström et al., 2002; Križanauskienė et al., 2006; Hellgren et al., 2007a), so are easily accessible for research. A peculiarity of current studies of avian *Plasmodium* species is that information about ecology, distribution, prevalence and other aspects of their biology has been accumulated using free-living birds caught in mist nets or different types of traps. The main principle of catching using these methods is that birds actively enter the nets and traps themselves. Unfortunately, such studies provide incomplete information about the severity of malaria infections for avian hosts because heavily infected individuals are frequently immobile, thus the effects of parasites can be underestimated using such sampling methods (Valkiūnas, 2001). Experimental information about *Plasmodium* spp. virulence, specificity and dynamics of parasitemia in different avian hosts under controlled laboratory conditions is crucial to elucidate the

significance of malaria infections and their impact on host fitness, behaviour, sexual selection and parasite-host co-evolution. Unfortunately such studies remain uncommon.

During the last 15 years, numerous polymerase chain reaction (PCR)-based methods have been developed and broadly applied to diagnose malaria and other haemosporidian infections (Feldman et al., 1995; Li et al., 1995; Bensch et al., 2000; Fallon et al., 2003b; Hellgren et al., 2004; Waldenström et al., 2004). The development of molecular markers, and precise comparison of results collected using such markers with data based on traditional parasitology methods can be helpful for understanding many questions of evolutionary biology such as the mechanisms maintaining parasite genetic diversity, specificity, pathogenicity, phylogeny and phylogeography. Accumulation of information on these subjects are important to further our understanding of the origins of malaria parasites, epidemiology of this disease in wildlife, and the mechanisms of parasite expansion to new hosts and regions.

Objective and main tasks of the study:

The objective of this study was to obtain new field and laboratory experimental data about the biology of avian malaria parasites and to link PCR-based information with data from traditional parasitology.

The following tasks were set to achieve this objective:

1. To verify the sensitivity of microscopy in studies of avian malaria parasites and closely related haematozoa.
2. To develop molecular identification for avian malaria parasite species that are widespread in Europe.
3. To develop a new method of DNA extraction and amplification from dissected single cells of avian malaria parasites and closely related blood parasites.
4. To determine vertebrate host specificity of *Plasmodium relictum* (lineage SGS1) and its effects on experimentally infected birds.

5. To determine vertebrate host specificity of *Plasmodium ashfordi* (lineage GRW2) and the effects of *P. relictum* (SGS1) and *P. ashfordi* (GRW2) simultaneous infection on experimentally infected birds.

6. To estimate the efficacy of the antimalarial drugs Malarone™ and primaquine for treatment of avian malaria.

7. To investigate the geographic distribution of *Plasmodium relictum* lineages in Europe using the house sparrow *Passer domesticus*, as a model host.

Statements being defended:

1. Microscopy is a reliable method for determining distribution patterns of haemosporidian parasites if blood films of good quality are examined properly by skilled investigators.

2. Mitochondrial cytochrome *b* gene lineages SGS1 and TURDUS1 belong to the morphospecies*Plasmodium relictum* and *P. circumflexum,* respectively; these sequences can be used for molecular diagnostics of malaria caused by these parasites.

3. Laser microdissection microscopy is a suitable method for dissecting of single cells of malaria parasites and other haemosporidians. This method can be used for collection of purified material for DNA extraction and PCR-based studies of these parasites.

4. Susceptibility of different passeriform birds to infection with the same lineage of *Plasmodium relictum* (lineage SGS1) is markedly variable; this infection causes disease of different severity in different avian hosts.

5. Different passeriform species differ in their susceptibility to infection with *Plasmodium ashfordi* (lineage GRW2). This parasite has a broad range of avian hosts.

6. The effect of simultaneous infection with *Plasmodium ashfordi* (lineage GRW2) and *P. relictum* (SGS1) varies in severity across different experimentally infected bird species. During simultaneous infection, these parasites act synergistically and cause death of some infected birds.

7. Treatment with Malarone™ is efficient for blood stages but not for tissue stages of *Plasmodium relictum* (lineage SGS1) and *P. ashfordi* (GRW2).

8. Two mitochondrial cytochrome *b* gene lineages of *Plasmodium relictum* (SGS1 and GRW11) have spread to ecosystems in the Baltic region from southern Europe. The lineage SGS1 is transmitted up to the North Polar Circle.

Novelty of the study:

1. It was demonstrated that prevalence of avian malaria and other haemosporidian parasites is estimated equally well by microscopy and currently used nested PCR-based methods. Both methods have advantages and disadvantages in diagnostics of haemosporidian parasites in wildlife, so we encourage using both these tools in parallel during studies of haemosporidians.
2. Lineages for molecular identification of *Plasmodium relictum* (lineage SGS1) and *P. circumflexum* (TURDUS1) were determined; these lineages are recommended for diagnostics of avian malaria caused by these widespread parasites.
3. New methods of single cell dissection, DNA extraction and PCR-based analysis of avian malaria and closely related blood parasites were developed.
4. It was shown experimentally that the susceptibility of different passerine birds to the same lineage of malaria parasites is markedly different, and that the same parasite lineage can cause malaria of different severity even in phylogenetically closely related bird species. Studies using avian malaria virulence data should take this into consideration.
5. The antimalarial drugs Malarone™ and primaquine were tested against avian malaria for the first time. Malarone™ is non-toxic for birds and is effective against blood stages of avian *Plasmodium* spp.
6. The geographic distribution of *Plasmodium relictum* lineages SGS1 and GRW11 in the house sparrow was determined. The lineage SGS1 has a particularly large range of transmission in Europe.

Scientific and practical significance:

1. The conclusion that microscopy is a reliable method for determining patterns of distribution of avian malaria parasites is particularly important for researchers and veterinarians in developing countries, where the use of molecular

techniques in field and laboratory studies is limited due to high costs of molecular reagents and equipment.

2. Molecular identification of *Plasmodium relictum* and *P. circumflexum* was developed; this will aid field and laboratory studies of these parasites and contribute to phylogenetic analyses based on positively identified morphospecies of *Plasmodium* spp.

3. New single cell dissection, DNA extraction and PCR-based methods can be used for a) identification of malaria parasites and other related haematozoa during simultaneous infections, b) collection of pure material for development of new nuclear genetic markers, c) deciphering mechanisms behind apparent reproductive isolation between parasite lineages in hybridization experiments, as well as many other fields of research where DNA from single cells is needed.

4. New data on the effects of avian malaria on different bird hosts will contribute towards understanding the epidemiology and ecology of avian *Plasmodium* spp. in wild populations.

5. Knowledge about malaria treatment is important both for laboratory and field experimental studies in avian malariology; it is also useful for treatment of birds in captivity and in conservation biology projects to protect vulnerable individuals from heavy parasitemia.

6. New data on the geographic distribution of *Plasmodium relictum* lineages in house sparrows has implications for understanding the phylogeographic history of this parasite in Europe. It provides baseline information for evaluating the likelihood of malaria expansion to new regions, and may help to determine potential emerging avian malaria parasites in northern Europe.

7. As result of experimental infections and the ability to collect blood with heavy parasitemia, CryoBank containing 60 samples of 4 gene lineages of avian malaria parasites was established and is available in the Institute of Ecology of Vilnius University.

Approbation of results. The results of this study have been published in 10 publications: among them 4 full articles and 6 abstracts of scientific conference reports. Nine reports on dissertation topics were made at the Ist and

II[nd] Workshops of Avian Malaria Parasites (Lund, Sweden, 2006, March, December), the II[nd] Symposium of the Scandinavian – Baltic Society for Parasitology (Rovaniemi, Finland, 2007), the II[nd] International Eurasian Ornithology Congress (Antalya, Turkey, 2007), the X[th] European Multicolloqium of Parasitology (Paris, France, 2008), the IV[th] Congress of the Russian Society of Parasitologists – Russian Academy of Sciences (Saint Petersburg, Russia, 2008), the IV[th] Workshop of Avian Malaria Parasites (Badajoz, Spain), the III[rd] Symposium of the Scandinavian-Baltic Society for Parasitology (Riga, Latvia, 2009).

This dissertation work has been discussed and approved at annual meetings of PhD students at the Institute of Ecology of Vilnius University (Vilnius, Lithuania, 2006, 2007, 2008).

Structure of the study. The dissertation is presented in the following chapters: *Introduction*, *Literature review*, *Material and Methods*, *Results and Discussion*, *Generalisation*, *Conclusions*, *Acknowledgements*, *References* and *List of author's publications*. References include 170 sources. The dissertation contains 138 pages, 6 tables and 25 figures. The text of the dissertation is in English with a summary in Lithuanian.

1. LITERATURE REVIEW

1.1. Brief outline of the life cycle of *Plasmodium* parasites

The main knowledge of the life cycle of avian malaria parasites was accumulated in the 1930 to 1950's when these protists were widely used as experimental laboratory models to study human malaria (Garnham, 1966). Complete life cycles of many species of bird malaria parasites remain unknown or have been investigated fragmentarily. This refers primarily to the species of the subgenera *Novyella* and *Giovannolaia*. The development of species of the subgenus *Haemamoeba* is the most well studied, so we use these parasites to describe the general scheme of life cycle of avian malaria parasites. *Plasmodium relictum* is used as an example (Fig. 1).

Blood sucking mosquitoes (Diptera: Culicidae) are vectors of malaria parasites of birds. The great majority of vector species belongs to the genera *Culex*, *Aedes*, *Culiseta* and *Anopheles* (Garnham, 1966). Mosquitoes of the genus *Coquillettidia* were recently also identified as vectors of some avian *Plasmodium* spp. (Njabo et al., 2009).

Sporozoites injected by the vector into birds give rise to the first generation of primary exoerythrocytic meronts (cryptozoites) (Fig. 1., a). They develop predominantly in the reticular cells of many organs and tissues, including skin. The merozoites developing in cryptozoites induce the second generation of primary exoerythrocytic meronts (metacryptozoites), which develop in macrophages in many organs and tissues (Fig. 1., b). Merozoites, which develop in metacryptozoites, are able to infect the cells of the erythrocytic series (Fig. 1., c) or induce the next generation of metacryptozoites and phanerozoites (Fig. 1., d). The time from inoculation of sporozoites into birds until the maturation of the first generation of metacryptozoites is called a prepatent period of development.

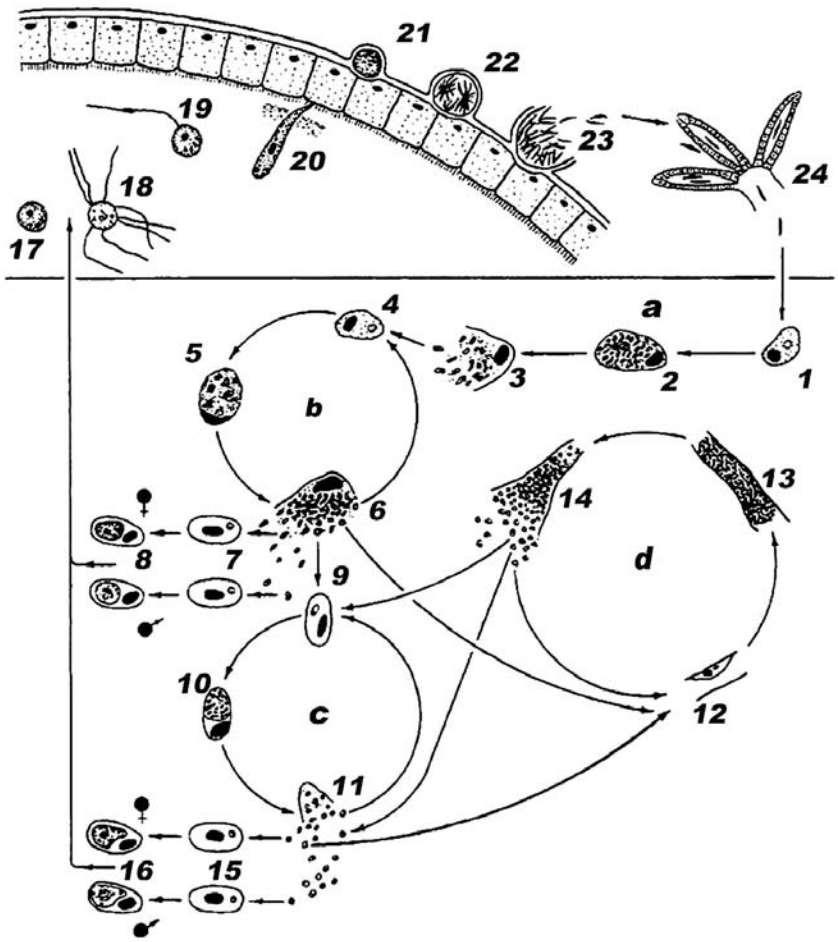

FIGURE 1. Diagrammatic representation of the life cycle of bird malaria parasites:
Upper part, in vector; lower part, in bird: a, b - primary exoerythrocytic merogony; c - erythrocytic merogony; d - secondary exoerythrocytic merogony; 1 - sporozoite in reticuloendothelial cell; 2, 3 - cryptozoites; 4 - merozoites in macrophage; 5, 6 - metacryptozoites; 7 - merozoites in erythrocytes; 8 - gametocytes; 9 - merozoites in erythrocyte; 10, 11 - erythrocytic meronts; 12 - merozoite in endothelial cell of capillaries; 13, 14 - phanerozoites; 15 - merozoites in erythrocytes; 16 - gametocytes; 17 - macrogamete; 18 - exflagellation of microgametes; 19 - fertilization of macrogamete; 20 - ookinetes penetrating the peritrophic membrane; 21 - young oocyst; 22, 23 - sporogony; 24 - sporozoites in the salivary glands of vector (according to Valkiūnas, 2005).

After penetration of merozoites into young and (or) mature erythrocytes, they becomes roundish and give rise to the growing nonfissionable parasites,

which are called trophozoites. From the moment of the first nucleus division, the parasite develops into a stage called erythrocytic meront, which is a characteristic stage of development of parasites of the genus *Plasmodium*, but also is present in species of the Garniidae. Due to presence of merogony in erythrocytes, the infection of vertebrate hosts can be easily achieved by subinoculation of infected blood. Uninuclear merozoites are formed in erythrocytic meronts after asexual division. The cycle of erythrocytic merogony in the majority of parasite species terminates after 24 to 36 h. A part of merozoites formed in the erythrocytic meronts induces the next cycles of erythrocytic merogony and gives rise to gametocytes, while the other part penetrates the endothelial cells of the capillaries of many organs including the brain, initiating secondary exoerythrocytic merogony (phanerozoites). Phanerozoites are responsible for relapses.

Several minutes after feeding on infected birds, mature gametocytes in the midgut of mosquitoes round up and escape from the erythrocytes. Gametes are formed and fertilization occurs, and then motile ookinete develops. Ookinetes move toward the epithelial cells of the midgut, reach the basal lamina, become round and transform into the oocysts surrounded by a capsule-like wall. After several germinative centers are formed many hundreds of sporozoites develop during the sporogony. When mature oocysts rupture, the sporozoites get into the haemocoele and penetrate into the salivary glands. The sporogony of *P. relictum* at the optimal temperature 24°C is completed in seven days after ingestion of mature gametocytes. Infection of new hosts occurs by means of injection during a blood meal of infected vectors.

1.2. Brief review of studies of avian malaria parasites

Since V. Ya. Danilewsky, professor of Kharkov University, published the article ‘About Blood Parasites (Haematozoa)’ in the Russian Medicine journal in 1884, marked development of this field of parasitology has been started. Already in 1885, E. Marchiafava and A. Celli established the first genus of haemosporidians (*Plasmodium*), and attributed human malaria parasites to this

genera. Six years later, Celli and Sanfelice (1891) for the first time managed to infect birds with malaria by subinoculation of infected blood. This was the basis for further experimental investigation of malaria; the same method was used in our experimental studies.

Investigation of avian malaria parasites contributed significantly to the knowledge on biology and ecology of malaria parasites of other vertebrates, including human malaria. The peak achievement of discoveries in avian malaria was the discovery and description of the development of *Plasmodium relictum* (Grassi and Feletti, 1891) in 'grey' mosquito (genus *Culex*) and the confirmation of the transmissive way of spreading of malaria by R. Ross in 1898. Roland Ross was awarded the Nobel Prize in Medicine in 1902 for this discovery (Garnham, 1966).

Since the discovery by R. Ross, bird malaria parasites began to attract the attention of scholars as an experimental model for the investigation of human malaria; they were used for this purpose in many laboratories till the discovery of the rodent malaria parasites in 1948 (Killick-Kendrick, 1974) and successful infection of the *Aotus trivirgatus* monkey with human malaria in 1966 (Young et al., 1966). These parasites are more close to human malaria by numerous their biological features. After that, bird haemosporidians have lost attractiveness in human malaria research. The number of experimental studies with avian *Plasmodium* spp. decreased remarkably after that. In spite of that fact, they remain convenient model organisms for further investigations on general biology of *Plasmodium* spp. and their close relatives, including questions of general evolution of haemosporidians, including human malaria parasites (Martinsen et al., 2008). However, the list of discoveries and achievements made with the use of bird haemosporidian models is impressive. These are discoveries of new antimalarial drugs (Davey, 1951; Coatney et al., 1953), the first cultivation methods of the tissue and erythrocytic stages *in vitro* (Trager, 1950; Ball and Chao, 1961), and first steps in the development of antimalarial vaccines (McGhee et al., 1977). Until now over 40 morphospecies of avian

malaria parasites of the genus *Plasmodium* have been described (Valkiūnas, 2005; Valkiūnas et al., 2007, 2009).

1.2.1. Investigation of avian malaria parasites using traditional methods

For over 100 years, the haemosporidians have been studied predominantly by microscopic examination of Giemsa-stained blood and using other traditional methods. The current knowledge on the basic life history strategies of haemosporidians, their geographical distribution and distribution by hosts, vertebrate hosts and vector specificity, seasonal changes of infection, and other aspects of ecology of these parasites have been accumulated primarily by microscopy (Garnham, 1966; Atkinson and van Riper 1991; Forrester and Spalding, 2003; Valkiūnas, 2005). It is important to note that researchers using traditional methods faced some of the problems. The identification of avian malaria species from the smears often is impossible. Even when recorded, most of chronic infections of *Plasmodium* spp. couldn't always be identified to the specific level, even by experts, due to low intensities and common mixed infections (Valkiūnas, 2005).

Five subgenera of avian *Plasmodium* have been described using traditional methods. These are *Haemamoeba*, *Giovannolaia*, *Novyella*, *Huffia*, and *Bennettinia* (Corradetti et al., 1963; Garnham, 1966; Valkiūnas, 2005). After Grassi and Feletti (1891) described the first two species of avian malaria parasites, the "similarity species concept", which is based on morphological and some other biological characters, has traditionally been used in taxonomy of species of *Plasmodium* and other related blood haemosporidian parasites (the order Haemosporida). In systematics of avian malaria parasites, it is generally accepted that any new species should only be established if supported (at least) by the full range of blood stages (Garnham, 1966; Valkiūnas, 2005). That provides opportunities for detailed comparison of morphology of similar parasites and identification of their species.

Phylogenetic relationships between species of *Plasmodium* remained insufficiently investigated.

General conclusions of studies using traditional methods (microscopy) have been supported by recent molecular investigations, which added new and innovative aspects to the knowledge of the biology of haemosporidians, especially to their genetic diversity, phylogeography, phylogeny and vertebrate-host specificity (Perkins and Schall, 2002; Ricklefs et al., 2004; Sehgal et al., 2005; Szymanski and Lovette, 2005; Kimura et al., 2006; Križanauskienė et al., 2006; Martinsen et al., 2006; Bensch et al., 2007; Palinauskas et al., 2007; Perkins et al., 2007; Krone et al., 2008). For instance, a molecular study by Martinsen et al. (2007) supported validity of the subgenera *Haemamoeba*, *Huffia*, and *Bennettinia*, but questioned the validity of the *Giovannolaia* and *Novyella*. This study raised some questions about the reliability of previously used methods and about subgeneric classification of avian malaria parasites. This requires clarification. One of the ways to clarify this contradiction between traditional parasitology and recent molecular data is to increase number of linkages between DNA sequences and positively identified morphospecies in phylogenetic trees.

The term 'morphospecies' (morphological species) is used in this dissertation when we intend to point out that discussion, particularly on practical taxonomy issues, is dealing mainly with information based on morphological parasites criteria. Each morphospecies is defined on a set of morphological characteristics that vary within well-defined limits; this set of characters is sufficient to distinguish the morphospecies from all other morphospecies. The morphospecies concept currently is frequently used in evolutionary biology studies of lower eukaryotes. For instance, in the eighth edition of the Dictionary of the Fungi (Hawksworth et al., 1995), the concept of morphospecies is defined as the traditional approach recognizing units that could be delimited on the basis of morphological characters, and ideally by discontinuities in several such, as distinct from biological, phylogenetic and ecological species. Morphospecies appears to be the robust species concept in studies with lower eukaryotes and some prokaryotes (Oliver and Beatty, 1996). Because of possible presence of numerous cryptic species among haemosporidian parasites (Bensch et al., 2004),

currently, the term morphospecies has been also accepted and used in malariology (see Hellgren et al., 2007a; Cosgrove et al., 2008; Garamszegi, 2009; Križanauskienė et al., 2009; Valkiūnas et al., 2008, 2009).

In this study, two common avian malaria morphospecies were linked to their DNA sequences of mitochondrial cyt *b* gene, which can be used for molecular diagnostics of malaria cause by these parasites. By doing these investigations, we continue recent research by Martinsen et al. (2006), Hellgren et al. (2007a) and Valkiūnas et al. (2007). As suggested by Valkiūnas et al. (2007), the experimental infections were used during this study. That enabled clear identification of morphospecies of *Plasmodium* parasites, which usually is difficult to do using light natural infections, and thus precise linkage lineage and morphological information.

1.2.2. Polymerase chain reaction (PCR)-based methods; a comparison with microscopy in the studies of avian malaria parasites

During the last 15 years, numerous PCR-based methods have been developed for diagnosing of haemosporidian infections (Feldman et al., 1995; Li et al., 1995; Bensch et al., 2000; Hellgren et al., 2004; Waldenström et al., 2004). These methods have been used increasingly to study diversity, distribution, specificity, ecology and different aspects of evolutionary biology of avian haemosporidian parasites (Bensch et al., 2000; Waldenström et al., 2002; Beadell et al., 2004; Fallon et al., 2005; Križanauskienė et al., 2006; Hellgren et al., 2004, 2007b). Numerous molecular biologists and ecologists addressed questions on the phylogenetic relations of avian *Plasmodium* and closely related haemosporidians (Ricklefs et al., 2004; Martinsen et al., 2008; Beadell et al., 2009). These studies were started by constructing phylogenetic trees of avian malaria and closely related parasites based on one mitochondrial cytochrome *b* gene (Bensch et al., 2000; Waldenström et al., 2002; Ricklefs et al., 2004; Martinsen et al., 2006) and continuing with studies using two or more different molecular markers (Bensch et al., 2004; Martinsen et al., 2008; Beadell at al., 2009).

In some comparative molecular studies, microscopy was shown to be significantly less sensitive than PCR-based methods in determining prevalence of haemosporidian infections in birds. Richards et al. (2002) compared several PCR assays and microscopy for detection of avian haemosporidians; they concluded that the PCR is faster, cheaper, and more reliable than microscopic blood smear examination for large-scale screening. According to Jarvi et al. (2002), PCR tests were 3- to 4-fold better than microscopy for detecting chronic blood parasite infections. Durrant et al. (2006) reported that specimens examined using both PCR-based techniques and blood smears showed an approximately 10-fold difference in prevalence of haematozoa, with PCR-based techniques detecting many more infections. These conclusions raised a question about the value of microscopy in field studies and the reliability of conclusions, which have been based on the microscopy data, in ecology of haemosporidian parasites.

However, in other hand, recent studies revealed that molecular methods being extremely sensitive also have shortcomings in detecting haemosporidian infections. A main problem is that the PCR techniques frequently are selective during simultaneous infections with species of *Plasmodium* and *Haemoproteus* or both (Pérez-Tris and Bensch, 2005; Valkiūnas et al., 2006a). So, alone they are insufficient when diagnosing mixed infections of haemosporidian parasites, which are common in wildlife. A combination of traditional microscopy and PCR-based methods has been recommended for this purpose and for better understanding of biology of these parasites (Valkiūnas et al., 2006a). Linkage of knowledge of traditional parasitology and molecular biology is important because the former remains an important source of information about basic life history strategies of these organisms and the latter provides new information about their phylogenetic relationships (Atkinson and van Riper, 1991; Bensch et al., 2000; Ricklefs and Fallon, 2002; Perkins and Schall, 2002; Valkiūnas, 2005). There is an urgent need to remedy this because few experts possess the knowledge to identify parasite species in many branches of parasitology, and

few people in the next generation of scientists are learning these taxonomic skills.

During this study we verified the sensitivity of microscopy in determining prevalence of haemosporidian infections in birds. The results of screening a large number of blood samples using 2 nested mitochondrial cyt *b* PCR assays and microscopy were compared in determining the prevalence of haemosporidian infections in naturally infected birds.

1.3. Necessity of development of new approaches and molecular markers for investigations of avian malaria parasites and closely related haematozoa

Most of the genetic analyses of avian *Plasmodium* and *Haemoproteus* parasites have employed sequencing of mitochondrial genes, in particular a part of the cytochrome *b* gene (Bensch et al., 2009). It was revealed, that diversity of parasite mitochondrial lineages is many-fold higher than expected from traditional morphological analyses. To understand levels of reproductive isolation between mitochondrial lineages, and to evaluate patterns of population structure of lineages occurring in different hosts, multiple nuclear markers are needed. The development of such markers has been effectively hindered. Bird *Plasmodium* parasites cluster together in separate clade and are more distantly related to mammalian *Plasmodium* parasites (Martinsen et al., 2008). Hence, although the whole genome from several mammalian *Plasmodium* species have been sequenced (Pain and Hertz-Fowler, 2009), it has been difficult to use these for constructing primers that amplify bird parasites. Markers for a few nuclear and apicoplast genes have nonetheless been constructed by using this approach (Feldman et al., 1995; Bensch et al., 2004; Martinsen et al., 2008), but these genes have a rather slow rate of evolution and therefore less useful for studies of speciation and population divergence. Microsatellite markers would be ideal for these questions as been demonstrated by studies of *Plasmodium falciparum* (Anderson et al., 2000), but directly isolating microsatellites from bird parasites is a much more challenging project than for mammalian *Plasmodium* species. In contrast to mammals, birds have nucleated erythrocytes and a sample of bird

blood contains much more host DNA than a sample of mammalian blood (Freed and Cann, 2006). Birds have an average genome size of approximately 1300 Mbp, much larger than the genome size of *Plasmodium* parasites (25-30 Mbp). Hence, even if only infected erythrocytes were harvested, the parasite DNA will consist of less than 3% of the total DNA yield, making random clone sequencing very inefficient.

The standard starting material for genetic analyses of avian malaria parasites and related haemosporidians is the total DNA extracted from whole blood collected from infected birds. Such material contains DNA not only from birds, but also from numerous other parasite species (Valkiūnas et al., 2006a). This can result in misfortunate linking of a DNA sequence data with parasite species observed in the blood smear from the same sample (Valkiūnas et al., 2008a).

Recent developments of laser microdissection microscopy make it now possible to excise single parasite cells from various tissue materials for genetic analyses of DNA or RNA (Jones et al., 2004). For example, this method has been employed to investigate gene expression profiles of liver-stages of several mammalian *Plasmodium* species (Sacci et al., 2002; Semblat et al., 2002). Although not yet tested, this novel technique has the potential to break new ground in studies of avian malaria parasites and related haemosporidians. First, isolation of pure parasite DNA for massive genome sequencing would enable the development of multiple and highly variable genetic nuclear markers. Second, by using single cell analyses of parasites in mixed infections one can confidently link morphology with DNA sequences (Dolnik et al., 2009). Third, precise genetic analyses of isolated ookinetes in hybridization experiments (Valkiūnas et al., 2008b) or oocysts in vectors (Razakandrainibe et al., 2005) can answer questions about basic reproductive biology, including species limits and levels of selfing.

An Olympus/MMI CellCut Plus microdissection system for isolating single cells of avian haemosporidian parasites had been used in the present

study. The new methods of harvesting cells, DNA extraction and single-cell PCR were described.

1.4. Specificity and virulence of *Plasmodium* species

The experimental research with avian malaria parasites of the genus *Plasmodium* has been developed increasingly from the end of the 19th century. The majority of experimental studies have been carried out with *Plasmodium gallinaceum, P. juxtanucleare*, *P. durae, P. lophurae* and *P. hermani,* which develop in poultry (Bennett et al., 1966; Garnham, 1966, 1980; Huchzermeyer, 1993; Permin and Juhl, 2002; Paulman and McAllister, 2005; Williams, 2005). Some experimental studies have also been done with *P. cathemerium, P. rouxi* in canaries and *P. relictum* in wild birds (Garnham, 1966; Corradetti et al., 1970; Riper et al., 1986; Atkinson et al., 1995). According to the studies with poultry, most of investigated parasites caused severe malarial infections. The most vulnerable infections and the greatest mortality rates have been recorded in juvenile birds of susceptible host species. However the virulence even of the same species varied markedly in different regions. For instance two strains of *P. juxtanucleare* were recorded in Asia and Neotropic region. The death was rarely recorded in domestic chickens in Asia but was common in the Neotropics where the mortality rate exceeded 90% (Bennett et al., 1966; Garnham, 1980). Furthermore, the Neotropical strains were lethal not only for juvenile chickens but also for adult birds. The effects of *P. relictum*, which has been recorded in over 300 bird species all over the world (Valkiūnas, 2005), were extensively studied in Hawaiian Islands. In 1826, the mosquito *Culex quinquefasciatus* was introduced to the Hawaii (Warner, 1968; Riper et al., 1986; Yorinks and Atkinson, 2000), allowing *P. relictum* (lineage GRW4) to spread all over the moist and warm lowlands of the islands (Atkinson et al., 1995; Beadell et al., 2006). The first studies with Hawaiian birds indicated that this parasite had reduced the populations or even caused extinction of some endemic bird species (Warner, 1968). Latter, experimental investigations, aiming to measure the effects of this parasite on different Hawaiian wild bird species, were initiated

(Riper et al., 1986; Atkinson et al., 2000; Yorinks and Atkinson, 2000; Atkinson et al., 2001a). It was shown that this lineage is highly virulent to many species of the endemic Drepanidae, but is less virulent to introduced passerines and Hawaiian thrushes (Atkinson et al., 1995). The experimentally confirmed negative effect of *P. relictum* on the drepanids, due to host-shift, raises concerns about effects of host-shifts that are likely to take place elsewhere, but are more difficult to record in non-island ecosystems. Migratory birds regularly carry malaria parasites from wintering areas with warm climates to their breeding grounds in the north (Waldenström et al., 2002; Valkiūnas, 2005; Hellgren et al., 2007b). It is possible that climate change could lead to change of distribution areas of vectors; that might increase probability for expansion of these parasites to new ecosystems in northern latitudes during global warming. Resident northern and arctic birds that have not been exposed to such parasites for substantial time might have lost or not developed their resistance to malarial infections, as is a case with many Hawaiian birds (Yorinks and Atkinston, 2000), and therefore might be at risk.

The information about vertebrate host specificity, pathogenicity and dynamics of parasitemia of *Plasmodium* spp. in different wild bird species is absent for the great majority of lineages of bird malaria parasites. From the end of the 20th century, the majority of studies on avian malaria parasites and other haemosporidians were developed mainly based on material collected from naturally infected birds. Prominent information about ecology, molecular biology, distribution, prevalences, diversity, and phylogeny has been accumulated (White et al., 1978; Hamilton and Zuk, 1982; Bensch et al., 2000; Hellgren et al., 2004, 2007b; Valkiūnas, 2005; Beadell et al., 2006). In such studies, data about infections (mainly prevalence and parasitemia) were collected from individual birds caught in mist nets or different types of traps. These catching techniques are biased towards catching relatively healthy individuals, i. e. parasite free or birds with low chronic infections (Valkiūnas, 2005). Such studies provide little information about true impact of malaria on different bird species, particularly during acute primary stage of infection.

During this study we measured the effects of the same lineages (SGS1) of *P. (Haemamoeba) relictum* to juveniles of different non-migrating Palearctic bird species under controlled experimental conditions. In respect that this parasite is a generalist (Valkiūnas, 2005), we expected that all species of small passerines should be susceptible and predicted that parasitemia and virulence should not differ between different close related host species.

1.5. Interaction between parasites and their hosts during simultaneous infections

The development of many species of avian malaria parasites in avian hosts has been insufficiently investigated. Moreover, even less research has been designed to clarify effects of simultaneous infections on avian hosts using experimental infections. However, simultaneous infections are common in wildlife. Precise field studies combining molecular and traditional methods revealed that *Plasmodium* spp. and other related haemosporidians are found in simultaneous infections in more than 43% of infected birds (Valkiūnas et al., 2006a), and as often as over 80% in some European bird populations (Valkiūnas et al., 2003). Effects of simultaneous infections on avian hosts are largely unknown.

Two main models of parasite interaction were demonstrated during simultaneous infections. First, synergetic, when one parasite species may cause a prolonged infection and/or increased establishment of another species in the host. Second, antagonistic effect, when one parasite species is capable in reducing the survival or fecundity of second species (Christensen et al., 1987). In Juhl and Permin's (2002) study with *Plasmodium gallinaceum* and *Ascaridia galli*, antagonistic effects between parasites were determined in infected birds. During this investigation *P. gallinaceum* caused a significant reduction in the establishment of the *A. galli*, compared to single infection with this nematode. At the same time, the double infection reduced the virulence of *P. gallinaceum* for avian hosts. Similar results were detected when *Plasmodium berghei* reduced establishment of the helminth *Hymenolepis diminuta* in mice (Fenwick,

1980). Synergetic interaction was detected in experimental study with simultaneous infection of two malaria parasites (Taylor et al., 1998). The virulence during simultaneous infection with two *Plasmodium chabaudi* genotypes was greater than that of single-clone infections. Zehtindjiev et al. (2008) reported death of great reed warblers *Acrocephalus arundinaceus* after experimental simultaneous infections of two generalist avian malaria parasites, *P. relictum* (lineage GRW4) and *P. ashfordi* (GRW2). However, because of small sample size in latter study, it is remain unclear if the high virulence and mortality were due to the mixed infection. According to Taylor et al. (1998), it is crucial to compare both virulence and within-host growth patterns in mixed and single infections for understanding of virulence strategies of parasites.

During this study we estimated the effects of simultaneous infection of two widespread species of malaria parasites on juveniles of three non-migrating Palaearctic bird species under controlled experimental conditions. That provides information for understanding patterns of development of simultaneous infections in different avian hosts. That is important for understanding pathological processes that take place in hosts during simultaneous infections.

1.6. Brief summary of drug development to treat avian malaria

Avian malaria parasites served as important models to develop antimalarial drugs, particularly during and after the Great World War II (Davey, 1951). In total more than 14000 compounds were tested using avian malaria models in the 20th century. Numerous studies were done on avian malaria treatment with pyrimethamine, quinine, chloroquine, proguanil and other 8-aminoquinolines in Europe (see reviews by Davey, 1951; Garnham, 1966; Valkiūnas, 2005). Researchers from the United States did precise investigation of effects of 8-aminoquinolines on bird *Plasmodium* spp., as well as pentaquine, isopentaquine and primaquine. Primaquine was recorded to be least toxic for human treatment of all 8-aminoquinolines using efficient doses (Davey, 1951). The main curative effect of this drug was recorded on late exoerythrocytic forms of malaria parasites. For that reason, the cure of heavy primary infections

required administration of a combination of this compound with other antimalarial drugs. A combination of primaquine with 8-aminoquinolines, pamaquin, quinine and other compounds were used to treat parasitemia. Resistance of avian malaria parasites to different drugs and their combinations was also recorded and investigated during this period of research (Davey, 1951).

The search for a more satisfactory drugs using avian malaria models continued until middle of 20th century, when rodent malaria were discovered (see Killick-Kendrick, 1974). Since then, avian malaria parasites lost their attraction in research of human malaria, including investigations for antimalarial drugs. For a long time experimental studies have not been performed with wild birds. As a result, the newest drugs for treatment of human malaria were not tested for the majority of avian *Plasmodium* species.

One of the newest and highly effective antimalarial drugs, Malarone™ (GlaxoSmithKline) is commercially available since the late 1990s (Wichmann et al., 2004). Malarone™ is a combination of Atovaquone and Proguanil (ATQ/PRO). Atovaquone was developed as a potential antimalarial drug, which causes parasite mitochondrial membrane potential collapse and inhibition of mitochondrial electron transport across the mitochondrial inner membrane via the cytochrome b-c_1 complex (Srivastava and Vaidya, 1999). Other compound, proguanil, has been found as antimalarial drug in early forties with tests against blood and exoerythrocytic forms of avian malaria parasites (Davey, 1951). During later studies with human malaria, it has been shown that proguanil acts as an activity enhancer to atovaquone (Srivastava and Vaidya, 1999). Many studies confirmed that ATQ/PRO is a useful agent for the treatment of uncomplicated *P. falciparum* malaria (Kuhn et al., 2005). Looareesuwan et al. (1999b) stated that usage of Malarone™ following by primaquine also is safe and effective for treatment of *P. vivax* malaria; Malarone™ acts against blood stages of malaria parasites and primaquine's curative effect is mostly against tissue stages. It worth mentioning that primaquine was used for treatment of related to *Plasmodium* spp. haemosporidian parasites (*Haemoproteus* spp.) in several recent studies (Merino et al., 2000; Marzal et al., 2005).

Because we were dealing with birds experimentally infected with malaria, we used this opportunity to carry out preliminary investigations with the aim to treat experimentally infected birds with Malarone™ and Malarone™ combining with primaquine. Effects of these drugs on avian malaria are unknown.

1.7. Genetic diversity of avian malaria parasites and a potential for their expansion to new regions

Species of *Plasmodium* and other haemosporidians are common dipteran-borne blood parasites, which have been reported in birds all over the world (Greiner et al., 1975; McClure et al., 1978; Atkinson and van Riper, 1991; Bishop and Bennett, 1992). It is confirmed by numerous studies that transmission of avian malaria parasites takes places in the Palearctic, including Scandinavian countries and countries of the Baltic States (Waldenström et al., 2002; Valkiūnas, 2005; Križanauskienė et al., 2006; Hellgren et al., 2007b). Low temperature is one of the obstacles for the transmission of malaria parasites. The temperature higher than 10°C is necessary not just for parasite development in the vector but also for mosquito activity (Valkiūnas, 2005). It seems probable that conditions for transmission of avian malaria and other haemosporidians developed in northern Europe, including the Baltic region, after the last glacial period about 10,000 years ago (Križanauskienė, 2007). Since then, during seasonal migrations from Afrotropic and Mediterranean regions, birds regularly have been transported parasites to northern areas and expose them to local blood sucking vectors. In the beginning, probably, the life cycle of parasites was not completed, but later some generalist parasites adapted to new environmental conditions (vectors and abiotic conditions). Migrating birds infected with haemosporidians are sources of infection for vectors and thus are possible sources for the future evolution of parasites, which can be transmitted in the north. The importance of avian migrations for blood parasite distribution and evolution is obvious (Hellgren et al., 2007b). It seems probable that seasonal bird migrations contributed to high prevalence and diversity of

avian malaria and other haemosporidian parasites, which already established transmission in the northern Palearctic (Valkiūnas, 2005).

Analyses of the mitochondrial cytochrome *b* gene of avian malaria parasites showed their remarkable genetic diversity (Bensch et al., 2009). Due to cosmopolitan distribution and phylogenetic relationships to agents of human malaria, these avian blood parasites are attractive model organisms to investigate such fundamental questions of biology and parasitology as mechanisms of maintenance of genetic diversity, host specificity, phylogeny, phylogeography, evolution of virulence and many other fundamental issues (e.g. Ricklefs et al., 2005; Hellgren et al., 2007b; Fallon et al., 2005; Pérez-Tris and Bensch, 2005). Determinant mechanisms of malaria parasites speciation, spreading and adaptation are still unclear; answers to these questions are important for better understanding of appearance new diseases.

Plasmodium relictum is the most widely distributed avian malaria species; it has been recorded in Eurasia, Africa, North and South America in more than 300 bird species belonging to different families (Valkiūnas, 2005). According to recent molecular studies, this species include broad range of mitochondrial cyt *b* gene lineages. One of the most prevalent lineages is SGS1, which is a widespread malaria parasite of birds in Europe and tropical Africa; it also has been recorded in Asia east to South Korea, so appears to be cosmopolitan in the Old World (Waldenström et al., 2002; Beadell et al., 2006; Hellgren et al., 2007b). Another widespread genetic lineage of *P. relictum* is GRW4. This lineage has been recorded in countries with warm climates like Nigeria, Papua New Guinea, USA (Hawaii) and others (Waldenström et al., 2002; Ricklefs and Fallon, 2002; Beadell et al., 2006). This lineage has not been recorded in resident birds in Europe so far. Other lineages of *P. relictum* have been recorded fragmentally too (Bonneaud et al., 2006; Križanauskienė et al., 2006; Pérez-Tris et al., 2007; Palinauskas et al., 2007). Huge genetic diversity of *P. relictum* and spotty distribution of its lineages provide opportunities to use this species in phylogeography studies.

Composition of *P. relictum* lineages in Europe is insufficiently investigated. This information is important for better understanding geographic distribution and phylogeography of this parasite; it also would provide data about patterns of spreading of *P. relictum* lineages to northern Europe. During our study we addressed this issue using the house sparrow as host species. This species is not migrating, so serves as indicator of the transmission at each study site (Valkiūnas et al., 2006b). A fragment of mitochondrial cyt *b* gene was amplified and sequenced for this purpose. The mitochondrial genes were approved being informative and suitable markers for studies in taxonomy of different organisms (Saccone, 1994; Avise and Walker, 1998). We predicted that South Europe, being refugia of birds and their haemosporidian parasites should contain more rich genetic diversity of lineages of *P. relictum*.

2. MATERIALS AND METHODS

2.1. Study site and material

The experimental work was carried out at the Biological Station "Rybachy" of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea (55° 05′N, 20° 44′E) between June and August, 2005 – 2008 (Fig. 2). The author collected this material (Table 1).

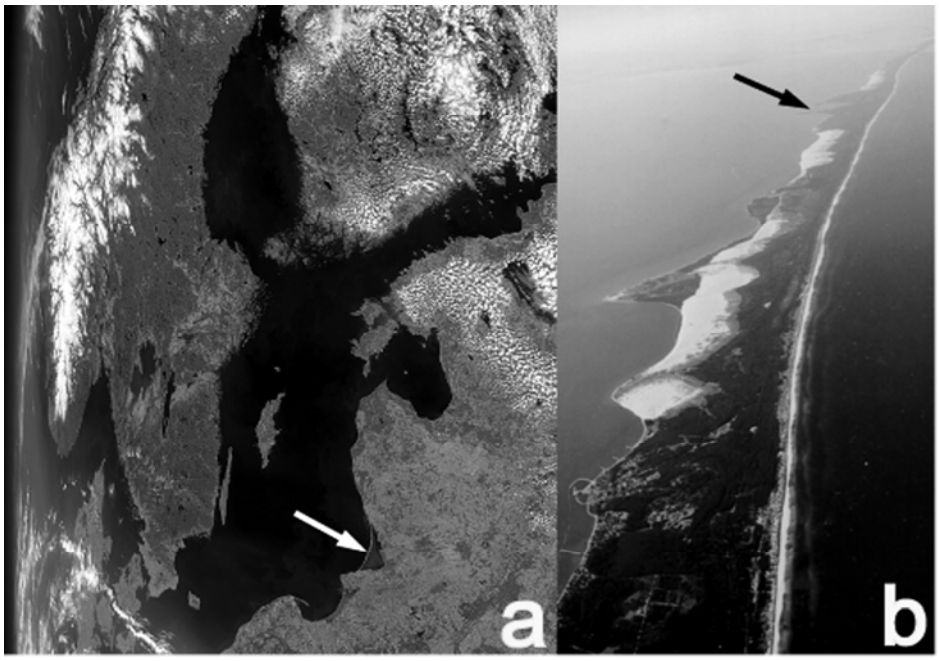

FIGURE 2. The main study site; Location of the Biological Station "Rybachy" (arrow) of the Zoological Institute, Russian Academy of Sciences on the Curonian Spit in the Baltic Sea: a - Baltic region, b - the Curonian Spit. (pictures from http://veimages.gsfc.nasa.gov; http://www.spauda.lt/nida).

Table 1. List of birds tested by microscopy and PCR-based techniques during studies on the Curonian Spit, 2005 – 2008.

Family	Species	No of birds tested/used in experiments
Emberizidae	*Emberiza schoeniclus*	2/0
Fringillidae	*Carpodacus erythrinus*	16/0
	Carduelis chloris	32/12
	Coccothraustes coccothraustes	16/2
	Fringilla coelebs	68/19
	Loxia curvirostra	39/34
	Spinus spinus	66/30
Laniidae	*Lanius collurio*	5/0
Muscicapidae	*Ficedula hypoleuca*	44/0
	Erithacus rubecula	7/0
	Luscinia luscinia	8/0
	Muscicapa striata	25/0
	Phoenicurus phoenicurus	2/0
	Saxicola rubetra	1/0
Passeridae	*Passer domesticus*	66/20
	P. montanus	8/0
Phylloscopidae	*Phylloscopus collybita*	4/0
	P. sibilatrix	8/0
	P. trochilus	76/0
Sylviidae	*Acrocephalus arundinaceus*	60/0
	A. palustris	103/0
	A. schoenoboenus	77/0
	A. scirpaceus	163/1
	Hippolais icterina	40/0
	Sylvia atricapilla	106/0
	S. borin	124/1
	S. communis	93/0
	S. curruca	51/0
	S. nisoria	2/0
Sturnidae	*Sturnus vulgaris*	35/24
Turdidae	*Turdus merula*	5/0
Total	31 species	1352/143

Additional material was also collected by the author's collaborators in Lithuania and other countries of Europe (Fig. 3), Africa and North America for comparative PCR-based, microscopic and phylogeographic analyses (see description below). The detailed description of these study sites are published by Valkiūnas et al. (2008c) and Marzal et al. (manuscript in preparation).

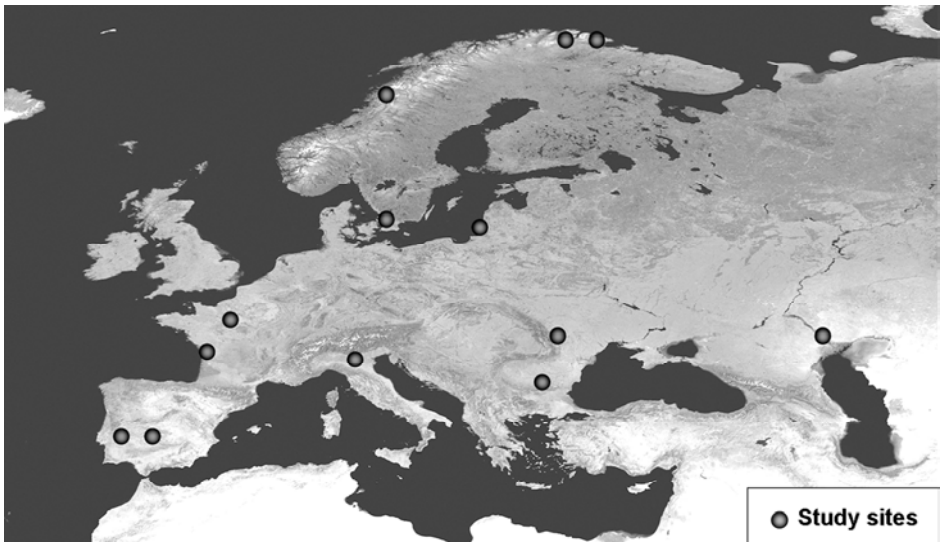

FIGURE 3. Locations (circles) where house sparrows were caught for investigation of geographic distribution of *Plasmodium relictum* mitochondrial cytochrome *b* gene lineages (picture from http://veimages.gsfc.nasa.gov)

2.2. Collection of material

2.2.1. Collection of birds

Birds were caught with mist nets (Fig. 4, a) and large 'Rybachy' type traps (Fig. 4, b). The birds were ringed, bled, and the majority of them were released after collection of blood samples and preliminary examination. Recipients and donors of infection were kept in captivity. Male birds were used as recipients during all experiments. Birds' age was determined according to Vinogradova et al. (1976).

FIGURE 4. Mist nets (a) and large 'Rybachy' type traps (b).

For molecular identification of two species of *Plasmodium*, 396 birds were caught and investigated by microscopy to determine naturally infected donors and uninfected birds, which were used as recipients of infection during experimental studies. This work was done in June – August 2005 (Table 2, exp. A).

Experiments with *Plasmodium relictum* (SGS1) were carried out in June – August 2006 (Table 2, exp. B). In all, 317 birds belonging to 23 species were caught and investigated for malaria parasites during this period of time. Sixty-five birds belonging to 6 species were kept in the cages for further experiments with this parasite.

The treatment experiments with Malarone™ were done in June – August 2007. In all, 446 birds belonging to 24 species were caught and examined by microscopy during this period of time. Twelve greenfinches and 4 chaffinches were kept for experiments.

Table 2. List of experimental infections carried out on the Curonian Spit, 2005 – 2008.

Expe-riment	Year	Donors of infection*	Parasite species and lineage	Infected recipient hosts
A	2005	*Coccothroustes coccothroustes* (1)	*Plasmodium relictum* (lineage SGS1)	*Passer domesticus* (2) *Loxia curvirostra* (2)
		C. coccothroustes (1)	*P. relictum* (SGS1)/ *P. circumflexum* (TURDUS1)**	*P. domesticus* (2) *L. curvirostra* (2)
B	2006 – 2007	*Acrocephalus scirpaceus* (1)	*P. relictum* (SGS1)	*Carduelis chloris* (6) *Fringilla coelebs* (6) *L. curvirostra* (6) *P. domesticus* (6) *Spinus spinus* (6) *Sturnus vulgaris* (6)
C	2008	*Sylvia borin* (1)	*P. relictum* (SGS1)/*P. ashfordi* (GRW2)**	*L. curvirostra* (6) *S. spinus* (6) *S. vulgaris* (6)

* Number of infected individuals is given in parenthesis.
** Simultaneous double infection.

For experiments with simultaneous infections of *P. relictum* (SGS1) and

P. ashfordi (GRW2) and for laser microdissection studies, 193 birds belonging to 21 species were caught and examined both by microscopy and PCR-based studies in May – August 2008 (Table 2, exp. C). During this period of time, 41 birds were kept in the laboratory for experimental studies.

In total, blood samples from 1352 passerine birds belonging to 31 species and 9 families were collected on the Curonian Spit for different studies (see Table 1). In total, 1273 additional birds belonging to 11 species and 9 families were collected for comparative PCR-based, microscopy and phylogeography analyses.

2.2.2. Obtaining of bird blood and preparation of the material for microscopic examination and DNA studies

Blood was taken by puncturing the brachial vein (Fig. 5, a). A drop of blood was taken from each bird to make 2 or 3 blood films on ready-to-use glass slides (Fig. 5, b). Blood films were air-dried within 5-15 sec after their preparation. In humid environments, a battery-operated fan to aid in the drying of the blood films. Smears were fixed in absolute methanol for 1 min on the day of their preparation. Fixed smears were air-dried and stained in a 10% working solution of a commercially purchased stock solution of Giemsa's stain, pH 7.0-7.2, at 20-25° C for 1 hr. Details of preparation and staining of blood films are described by Valkiūnas (2005). Stained smears were air-dried and packed into paper bands, so that they did not touch each other. All smears wrapped into paper and sealed in plastic packs were brought to Ecology Institute of Vilnius University for examination.

For laser microdissection microscopy, we also made two blood smears from birds with high parasitemia on special membrane slides (MMI-MembraneSlide, Molecular Machines & Industries). These films were fixed in absolute ethanol for 3 min. From each bird, one smear was stained with Giemsa, as described above and another one was saved unstained.

Approximately 50 µl of whole blood was drawn into heparinized microcapillaries from each bird (Fig. 5, c) and stored in SET-buffer (0.015 M

NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) for molecular analysis (Hellgren et al., 2004). The fixed samples were held at –20° C temperature in the field and later in the laboratory.

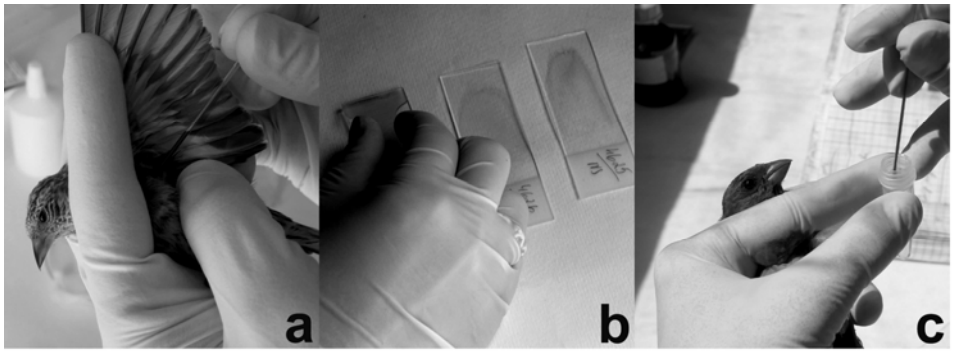

FIGURE 5. Obtaining of blood from birds and preparation of the material for microscopic examination and DNA analysis: a - puncturing brachial vein; b - making blood smears; c - storage of blood in micro tube with SET-buffer.

The samples from Africa and North America were fixed in lysis buffer, as described by Sehgal et al. (2001). Detail description of methods, which were used in this chapter of the Method section are presented by Valkiūnas et al. (2008c).

2.3. Blood smear examination; morphological and morphometric investigation of parasites

One blood film from each infected bird was examined. Approximately 100 – 150 fields were examined at low magnification (× 400), and then at least 100 fields were studied at high magnification (× 1,000). The microscopy of each sample took 20-25 min. In total, the approximate number of screened red blood cells was 5×10^5 in each blood film. Intensity of infection was estimated as a percentage by counting the number of parasites per 1,000 erythrocytes or per 10,000 erythrocytes if infections were very light, as recommended by Godfrey et al. (1987). Malaria parasites were identified according to Valkiūnas (2005). An Olympus BX51 light microscope equipped with digital camera and software DP-SOFT was used to prepare illustrations and to take measurements. For

comparative PCR and microscopy analysis Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used. For this study only good quality slides, i. e. without any features of lysis of cells and well-stained blood cells and parasites, were used for microscopic examination. To show differences in detectability of parasites in good-quality and bad-quality blood films, illustrations of the latter are provided from material which is deposited at the Institute of Ecology of Vilnius University, Vilnius, Lithuania.

The morphometric features used in the study of molecular identification of two avian malaria morphospecies were those defined by Valkiūnas (2005). Morphology of the parasites was compared with type material of *Plasmodium relictum* from its type vertebrate host, the Spanish sparrow, *Passer hispaniolensis* (Garnham Collection, the Natural History Museum, London; blood slide accession no. 225) and with type specimens of *P. circumflexum* from its type host, the Fieldfare, *Turdus pilaris* (a passage in a subinoculated canary, Garnham Collection, the Natural History Museum, London; the blood slide accession no. 270).

All positive blood slides, which are used in this study, are deposited in the Institute of Ecology of Vilnius University, Vilnius, Lithuania.

2.4. Extraction of DNA, PCR and sequencing

The standard phenol-chloroform protocol (Sambrook et al., 1989) was used for total DNA extraction from fixed blood, and a nested-PCR protocol (Bensch et al., 2000; Waldenström et al., 2004) was used for genetic analysis. We amplified a segment of the parasite mitochondrial cyt *b* gene using the initial primers HaemNF [5′ - CATATATTAAGAGAAITATGGAG - 3′] [I= a universal base, inosine] and HaemNR2 [5′ - ATAGAAAGATAAGAAATA CCATTC - 3′] which are general for species of *Haemoproteus*, and *Plasmodium* (Waldenstöm et al., 2004). For the second PCR, we used primers HaemF [5′-ATGGTGCTTTCGATATATGCATG -3′] and HaemR2 [5′ -GCATTATCTGG ATGTGATAATGGT - 3′] (Bensch et al., 2000).

The first PCR, with the primers HaemNF – NR2, was carried out in a 25 µl volume, and included 50 ng of total genomic DNA, 1.5 mM $MgCl_2$, 1X PCR buffer, 1.25 mM of each deoxynucleoside triphosphate, 0.6 mM of each primer, and 0.5 units *Taq* DNA polymerase. The cycling profile consisted of an initial denaturation for 3 min at 94° C, 30 sec at 94° C, 30 sec at 50° C, 45 sec at 72° C for 20 cycles, followed by final extension at 72° C for 10 min. For the second PCR we used 2 µl of the first PCR product, as template in a 25 µl volume with the primers HaemF – R2, including the same reagents and thermal conditions, as the first reaction, except with 35 cycles instead of 20 cycles.

One negative control was used per every 8 samples to control for false amplification due to the high sensitivity of the nested PCR. The amplification was evaluated by running 1.5 µl of the final PCR product on a 2 % agarose gel. For sequencing were used procedures as described by Bensch et al. (2000). Fragments were sequenced from the 5´ end with the primer HaemF, and new lineages were sequenced from 3´ end with the primer HaemR2. We used dye terminator cycling sequencing (big dye) and the samples where loaded on an ABI PRISM ^{TM} 310 sequencing robot (Applied Biosystems). Sequences were edited and aligned using the software BioEdit (Hall, 1999). We used the programmes MEGA, version 3.0 (Kumar et al., 2004), mrBayes version 3.1.1. (Ronquist and Heulsenbeck, 2003) and PAUP 4.0 (Swofford, 2001) to construct phylogenetic trees. To distinguish genetic differences between *Plasmodium* and *Haemoproteus* spp., the sequences were compared to their closest sequence matches in GenBank using the NCBI nucleotide blast search. The results were confirmed using microscopy. GTR+ G model of evolution (General Time Reversible model including rate variation among sites) was used defined by Modeltest 3.7 (Posada and Crandall, 1998). Two simultaneous runs were conducted with a sample frequency of every 100th generation over 1 million generations. Before constructing a majority consensus tree, 25% of the initial trees in each run were discarded as burn in periods. The phylogenies were visualized using Tree View 1.6.6. (software available from <http://evolution. genetics.washington.edu /phylip/software.html>). A Kimura 2-parameter

distance matrix was used to generate a neighbour-joining tree. The sequence divergence between the different lineages was calculated with the use of a Jukes-Cantor model of substitution, with all substitution weighted equally, implemented in the program MEGA 3.0 (Kumar et al., 2004).

The main molecular investigations were done in the Laboratory of Molecular and Evolutional Biology, Animal Ecology Department, Lund University in 2006 – 2009.

Samples from Africa and North America were screened for species of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* using different PCR based protocols. To obtain total DNA, the blood was extracted following a DNeasy kit protocol (Qiagen®, Valencia, California). To test *Leucocytozoon* spp. infections, the extracted DNA was used in a nested PCR reaction that amplifies region of mitochondrial cyt *b* gene. The first round of amplification the following primers developed by Perkins and Schall (2002) were used: DW2: 5'-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3', and DW4: 5'-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3'. The first PCR products were used as template for the second PCR reaction. The following primers for the second round were developed by Sehgal et al. (2006b): Leuco Cyt F: 5'-TCT TAC TGG TGT ATT ATT AGC AAC-3', and Leuco Cyt R: 5'-AGC ATA GAA TGT GCA AAT AAA CC- 3'. The reaction conditions for both PCR reactions are described in Valkiūnas et al. (2008c) study. *Plasmodium* and *Haemoproteus* spp. mitochondrial cyt *b* gene lineages were amplified using nested PCR with the same reaction conditions as Sehgal et al. (2006b) with the following primers: L15183: 5'- GTG CAA CYG TTA TTA CTA ATT TAT A-3' and H15730: 5'- CAT CCA ATC CAT AAT AAA GCA T- 3' (Fallon et al., 2003b; Szymanski and Lovette, 2005). The cycling profile is described by Valkiūnas et al. (2008c).

2.5. Dissection of single cells of parasites; extraction of DNA from single cells, PCR and sequencing

2.5.1. Dissection of single cells and extraction of DNA

Material for the laser microdissection microscopy was obtained from three individual birds. One siskin experimentally infected with *Plasmodium relictum* (intensity of parasitemia was 2%, mitochondrial cyt *b* gene lineage SGS1, GenBank accession no. AF495571), one siskin naturally infected with *Haemoproteus tartakovskyi* (parasitemia 8%, lineage SISKIN1, AY393806), and one chaffinch naturally infected with *H. majoris* (parasitemia 2%, lineage WW2, AF254972). Methods of experimental infection of siskin are described in section 2.6.1.

Laser microdissection microscopy of *P. relictum* (lineage SGS1) was carried out on films made immediately after withdrawal of the blood, and thus contained erythrocytic stages of parasites (gametocytes and meronts). For *H. tartakovskyi* and *H. majoris*, we treated the extracted blood before making blood films in a way to stimulate gametogenesis and fertilization of gametes to form extra-erythrocytic ookinetes. This was done by mixing the extracted blood containing mature gametocytes of these parasites with a 3.7 % solution of sodium acetate and exposure to the air, as described by Valkiūnas et al. (2008b). Mature ookinetes of both *Haemoproteus* species developed within 13 h *in vitro* (Fig. 6, a). Blood films on the membrane slides were prepared from this material, as described above. The intensities of ookinetes in both experiments were about 1%.

An Olympus IX 71 light microscope equipped with Olympus digital camera was used for microscopic examination of blood films and morphological analysis. The isolation of parasites was carried out in a room dedicated for this work to avoid contamination from PCR products. The Olympus/MMI Cellcut Plus® laser system (Molecular Machines & Industries) with PTP function software (Predefined Target Position) was used to cut single cells. By adjusting the contrast was possible to identify the parasites on the non-stained membrane slides and to capture parasite single cells on the silicon cap of micro-tubes (0.5

µl) (Fig. 6, a, d). The success of cell capture was confirmed by microscopic examination of the tube cap.

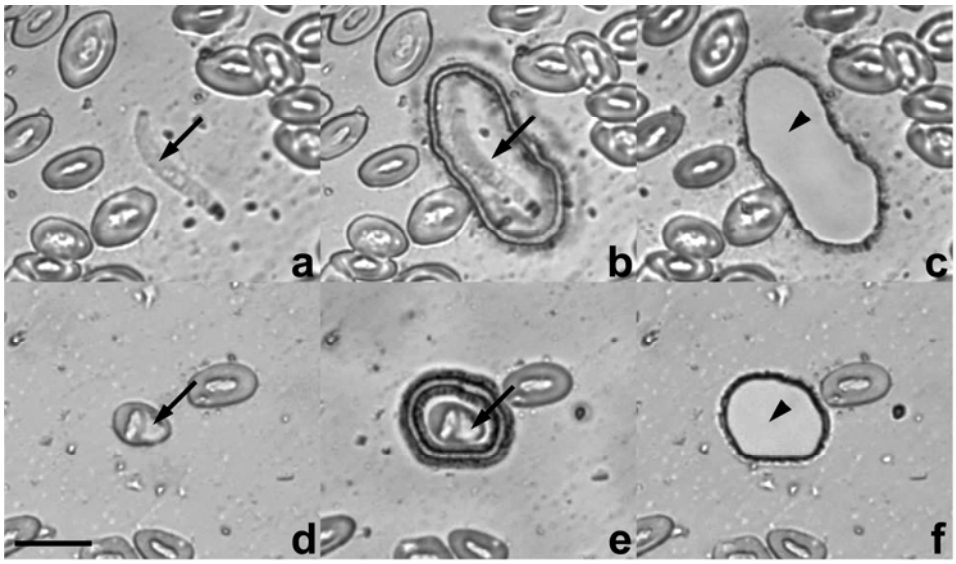

FIGURE 6. Laser microdissection of single cells of two haemosporidian species: a – c - ookinete of *Haemoproteus majoris* (lineage WW2,); d – f - gametocyte of *Plasmodium relictum* (SGS1) in its host cell; a, d - membrane slides with cells; b, e - membrane slides with dissected cells on the membrane, c, f - membrane slides after dissection of cells. *Arrows* - parasites; *arrow heads* - holes in the membranes after dissection of cells. Unstained thin blood films. Scale bar = 10 µm.

Table 3. Efficacy of one cell amplification of three different haemosporidian species. Each species was tested twice in batches of six independent isolates. The species identity of positive samples was confirmed by sequencing.

Parasite species and lineage	Number examined	Positive
Haemoproteus majoris (WW2)	6 / 6	6 / 5
Haemoproteus tartakovskyi (SISKIN1)	6 / 6	5 / 5
Plasmodium relictum (SGS1)	6 / 6	3 / 1

The multiple tubes with single ookinetes of *H. tartakovskyi* (SISKIN1), *H. majoris* (WW2), and gametocytes or meronts of *P. relictum* (SGS1) were

prepared (Table 3). The tubes with 100, 10 and 2 cells were also prepared for developing the extraction methods, primers and PCR protocols, and to investigate how the repeatability of the PCR depended on the number of isolated cells (Table 4).

Table 4. Efficacy of Chelex extraction and amplification of partial mitochondrial cytochrome *b* gene from different numbers of parasite cells. Four independent PCRs were performed from repeated samples from the same tube.

	Ookinetes of *Haemoproteus tartakovskyi* (lineage SISKIN1)		Erythrocytic stages of *Plasmodium relictum* (lineage SGS1)	
Number of cells used for PCR	10	2	10	2
Positive / Tested	4 / 4	3 / 4	3 / 4	1 / 4

A Chelex® (Bio-Rad) based extraction protocol was modified for a single cell extraction. Chelex® is a polyvalent chelating agent that is presumed to prevent the degradation of DNA by chelating metal ions that act as catalysts in the breakdown of DNA (Walsh et al., 1991). 0.2 g of Chelex suspended in 1,000 µl of ddH_2O was used and kept for 1 h in a 56° C water bath. The freshly prepared Chelex suspension was allowed to cool to room temperature. The tube was shaken before each aliquot of Chelex. The small drop of 25µl of Chelex suspension was attached to the wall of the tube with excised cells (excised cells were on the silicon cap of the tube). Then 0.7 µl of Proteinase K (10 mg/ml) was added to the Chelex drop, the tube was closed, turned upside-down and shaken down once so that the Chelex with Proteinase K was covering all the surface of the cap. The tube was kept in a water bath at 56° C for 1 h and mixed every 20 min followed by boiling in water for 12 min to inactivate the Proteinase K (Dolnik et al., 2009). The sample was centrifuged briefly to recover the extraction mix at the bottom of the tube. The silicon caps were replaced to standard caps and the tubes were centrifuged at 12000 RPM for 12 min. The

supernatant (about 10–15µl) was immediately transferred with a sterile micropipette into a new tube and was ready to use for PCR. Samples containing from 100 and 10 ookinetes were extracted also with standard phenol-chloroform method (Sambrook et al., 1989) and ammonium acetate method (Richardson et al., 2001).

2.5.2. Design of primers for small amount of DNA; PCR and sequencing

To increase the sensitivity of the PCR new primers for a nested PCR protocol to amplify shorter DNA fragments within the HaemF and HaemR2 region of the mitochondrial cyt *b* gene were designed (Bensch et al., 2000). For this purpose, extracted DNA's from two samples with 100 and 10 ookinetes were used and nested PCR's with different primer combinations were run. The primer combination that consistently and successfully amplified from samples containing 100 and 10 cells was chosen for the analyses. The outer primers in the nested PCR were HaemF [5´ - ATGGTGCTTTCGATATATGCATG -3´] and HaemR2 [5´ - GCATTATCTGGATGTGATAATGGT - 3´] (Bensch et al., 2000) and the products from this reaction (478 base pairs excluding primers) were re-amplified with the new primers F144 [5´ - AGTTACAGCTTTT ATGGGTTAT -3´] and R368 [5´ - GTATTTTTAAAGCTGTATCATACC - 3´] to generate a 224 bp fragment. Each reaction of 25 µl contained 14.4 µl of sterile deionized double distillated H_2O, 1.5 µl $MgCl_2$ (25 mM), 2.5 µl Roche® 10x PCR buffer, 2.5 µl dNTPs (1.25mM), 1.0 µl of each primer (10 µM), and 0.1 µl of *Taq* ® DNA polymerase (5 U/ µl). Two µl of template DNA (supernatant with single cell DNA after Chelex extraction) was added to the reagent mix of 23 µl.

The amplifications were evaluated by running 2.5 µl of the final PCR product on a 2 % agarose gel. Fragments were sequenced from the 5´ end with the inside forward primer F144. For sequencing, the procedures as described in section 2.4. were used.

2.6. Design of experiments for infection of birds

2.6.1. Experiments for development of molecular identification of morphospecies of avian malaria parasites

One hawfinch *Coccothraustes coccothraustes,* with a light natural infection of *Plasmodium (Haemamoeba) relictum* and one hawfinch with a light natural simultaneous infection of *P. (Haemamoeba) relictum* and *P. (Giovannolaia) circumflexum* were caught and kept in bird housing facilities in June 2005. They were used as donors of parasites to infect non-infected (recipient) birds (Table 2, exp. A). Eight juvenile common crossbills and eight juvenile house sparrows were caught at the study site. Four birds of each species were infected and four were used as negative controls. All birds were taken from the wild and kept indoors in a vector-free room under controlled conditions [20 ± 1 °C; 50 – 60% RH; the natural light-dark photoperiod (L:D), 17:7 h].

All recipient and control birds were shown to be uninfected with haemosporidian parasites both by microscopic examination of blood films and PCR before the inoculation experiments. On 19 and 30 of June, two house sparrows and two common crossbills were exposed to *P. relictum* by subinoculation of 250 µl of a freshly prepared mixture of infected blood, 3.7% solution of sodium citrate and 0.9% saline (4 parts blood to 1 part sodium citrate and 5 parts saline). This mixture was inoculated into the pectoral muscle of recipient birds as described by Iezhova et al. (2005) (Fig. 7).

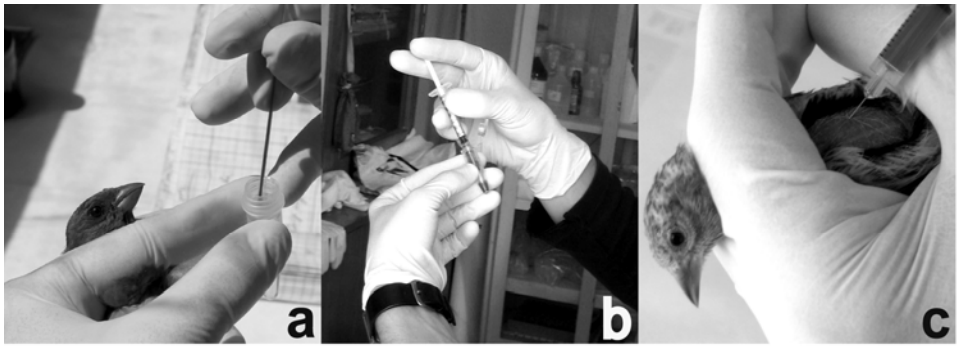

FIGURE 7. Subinoculation of a freshly prepared mixture of infected blood: a - infected blood mixing with sodium citrate and saline; b - taking infected blood mixture into syringe; c - inoculation of the infected blood into the pectoral muscle of recipient bird.

On 15 and 20 of June, two common crossbills and two house sparrows were infected with simultaneous infection as described above. Intensity of parasitemia both of *P. relictum* and *P. circumflexum* was 0.001% in the donor birds on each day of the exposure. The blood for microscopic examination and PCR was taken from all birds as described in section 2.2.2 (Fig. 5) once every three days for a period of two months. The isolated strains of malaria parasites were maintained in live birds for future experimental studies; they were also cryopreserved according to Garnham (1966).

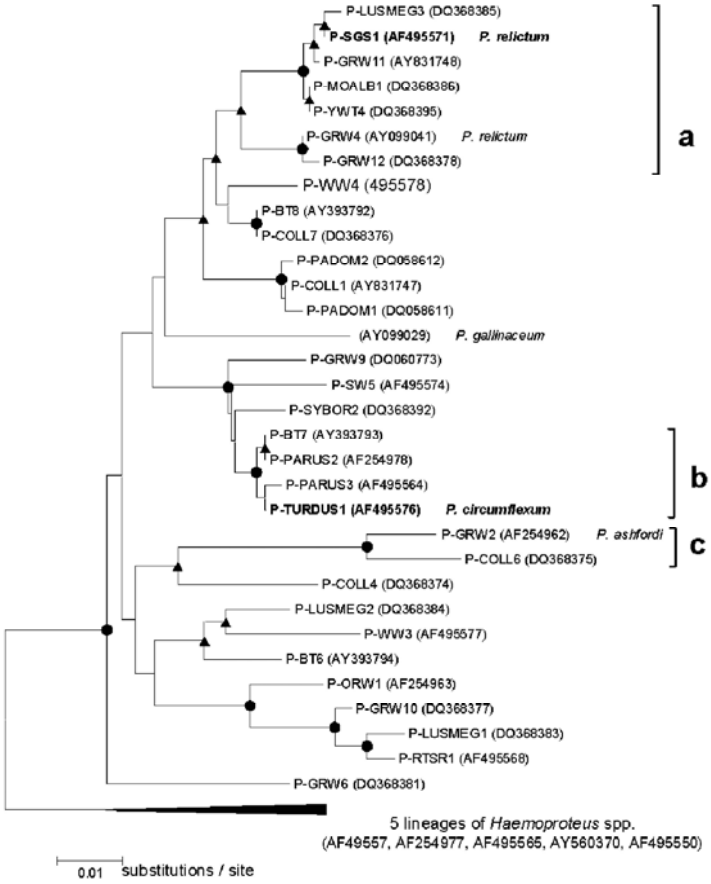

FIGURE 8. Neighbour – Joining tree of 32 lineages of *Plasmodium* spp. and five lineages of *Haemoproteus* spp. as the out group. The tree was constructed using the NJ method with Kimura 2-parameter distance matrix. Bootstrap values are represented by circles (>90%), squares (70 – 89%), and triangles (50 – 69%). Closely related (within a genetic distance ≤ 2.5%) to *Plasmodium relictum*, *Plasmodium circumflexum,* and *Plasmodium ashfordi* lineages of malaria parasites are marked by bars a, b and c, respectively. GenBank accession numbers of sequences are given after lineage names in parentheses.

In analyses, which was used for two morphospecies molecular identification (Fig. 8), we applied 32 lineages formerly deposited in GenBank, including the lineages SGS1 and TURDUS1 and lineages denoted as *Plasmodium gallinaceum*, *P. ashfordi* and *P. relictum*. Five *Haemoproteus* lineages (GenBank accession nos. AF49557, AF254977, AF495565, AY560370, AF495550) were used as the outgroup in the constructions of Neighbour-Joining (NJ) tree.

2.6.2. Experimental infection of birds with Plasmodium relictum (lineage SGS1)

One reed warbler with a light natural infection of *Plasmodium (Haemamoeba) relictum* as determined by microscopic examination of blood films, was caught and kept in bird housing facilities in June 2006. This bird was used as a donor to multiply parasite strain and then to infect uninfected (recipient) juvenile birds (Table 2, exp. B).

Infected blood from the naturally infected reed warbler was inoculated to intact juvenile chaffinches (2 birds) and juvenile common crossbills (2 birds), to multiply parasite strain. It was done as described in section 2.6.1. The birds with parasitemia between 0.01 and 5% were used in experimental inoculations. Mixture of infected blood from four donors was prepared and used for experimental infection.

Twelve juvenile common crossbills, chaffinches, house sparrows, siskins, and starlings were caught at the study site. All birds were taken from the wild and kept indoors as described in section 2.6.1. Six birds of each species were infected experimentally as described in section 2.6.1 (Fig. 7). Six birds of each species were used as negative controls; they were inoculated with the same amount of uninfected blood from the same host species, as experimental group. Different size birds were inoculated with different amount of infected blood (Garnham, 1966; Valkiūnas, 2005). The dose of mature erythrocytic meronts in each inoculum was the same for each bird species, but different in different bird species. It was calculated according to Garnham (1966). Approximately 150 μl

of infected blood solution was inoculated in siskins (intensity of mature erythrocytic meronts in the solution was 0.04 %, and the dose of the inoculated meronts was approximately 1×10^5). The same parameters were 200 µl, 0.06%, 2×10^5 in chaffinches; 250 µl, 0.05 %, 2×10^5 in house sparrows; 380 µl, 0.2%, 1×10^6 in crossbills; and 550 µl, 0.2%, 2×10^6 in starlings. Before the inoculation experiments, all recipient birds were proved to be uninfected with haemosporidian parasites both by microscopic examination of blood films and later by PCR.

On 1, 6, 8, 12, and 17 of July, six chaffinches, starlings, common crossbills, siskins and house sparrows were exposed to *P. relictum* (lineage SGS1), respectively. The observation time for all experimental and control birds was 30 days post inoculation (dpi), except for starlings and house sparrows, which was 27 and 24 dpi, respectively. Blood for microscopic examination and PCR was taken from all birds as described in section 2.2.2 (Fig. 5) once every three days for approximately one month. Body mass, body temperature and level of haematocrit were measured of all control and experimental birds between 08:00 h and 10:00 h every third day after experimental infection.

Birds were weighed using 'Sartorius portable' scale to the nearest 0.1 g. The temperature was measured to the nearest 0.1° C using 'Accurat microlife' thermometer, which was placed into cloaca of birds approximately for 40 s.

Level of haematocrit was measured by taking 50 µl of blood from the brachial vein into heparinised glass microcapilaries. Samples were immediately centrifuged for 5 min at 8000 RPM and were measured to the nearest 1% using microhaematocrit reader.

At the end of the study, experimental and control crossbills were decapitated and dissected. The brain, heart, liver, lungs and spleen were isolated and weighed using standard 'Chyo JL-180' analytical balances scale to the nearest 0.0001 g. These organs were fixed in 10% neutral formalin for future histological examination. After the experiment, control birds of all other species were released. The experimentally infected birds were either decapitated or maintained alive in order to maintain strains of malaria parasites. Forty blood

samples with *P. relictum* were cryopreserved according to Garnham (1966) for future experimental studies.

In 2007, the experimental work was repeated with greenfinches for more detailed investigation of avian malaria effect on phylogeneticaly closely related bird species. For experimental infection we used the same cryopreserved *P. relictum* (lineage SGS1) strain, which was used for experimental infections in 2006. To multiply the strain, blood from the cryopreserved samples was inoculated to 4 non-infected chaffinches, as described in section 2.6.1. Light parasitemia (<0.01%) developed in the infected chaffinches; these birds were used as donors for blood stages of the parasites to infect uninfected (recipient) greenfinches. Twelve juvenile male greenfinches (< 1 year old) were caught at the study site. Six birds were infected with malaria parasites and 6 individuals were used as negative controls. Laboratory conditions were the same as in 2006. On 21 July, 6 greenfinches were exposed to *P. relictum.* The birds were infected by inoculation of 200 µl of a freshly prepared mixture (intensity of mature erythrocytic meronts in the blood was 0.5% and the dose of the inoculated meronts was approximately 9×10^5). Infection was done as described in section 2.6.1 (Fig. 7).

The observation period for all experimental and control greenfinches was 30 (dpi), and it was 52 dpi for chaffinches. These birds were used also for treatment experiment, which is described below in section 2.7. During the pre-treatment time, blood for microscopic examination and PCR was taken as described in section 2.2.2 (Fig. 5). The body mass and value of haematocrit were measured as in 2006.

2.6.3. Experimental simultaneous infection of birds with Plasmodium relictum (lineage SGS1) and P. ashfordi (GRW2)

One garden warbler *Sylvia borin* with a light natural double infection of *Plasmodium (Haemamoeba) relictum* and *P. (Novyella) ashfordi*, as determined by microscopic examination of blood films, was caught and kept in bird housing facilities in June 2006. This bird was used as a donor of parasites to multiply the

strain and then to infect uninfected (recipient) juvenile birds in 2008 (Table 2, exp. C).

To multiply the strain, infected blood from the naturally infected garden warbler was inoculated to intact juvenile siskins (2 birds), as described in section 2.6.1 (Fig. 7). The third passage of originally isolated and then cryopreserved according to Garnham (1966) strain was used in this experiment. Mixture of infected blood from four donors was prepared and used for experimental infection.

Twelve juvenile starlings, siskins and common crossbills were caught at the study site and used as recipient hosts. The procedure for infection of the recipients and keeping them in the laboratory were the same, as described as in section 2.6.1. Approximately 150 µl of infected blood solution was inoculated to siskins (intensity of mature erythrocytic meronts in the solution was 0.3 %, and the dose of the inoculated meronts was approximately 2×10^6). The same parameters were 380 µl, 1%, 1×10^7 in crossbills; and 550 µl, 0.3%, 6×10^6 in starlings.

On 19, 28 of June and 1 of July 2008, six siskins, starlings and common crossbills were exposed to *P. relictum* (SGS1) and *P. ashfordi* (GRW2), respectively. The observation time for all experimental and control birds was 33 dpi. The blood for microscopic examination and PCR was taken the same as body mass and value of haematocrit measurements once every three days, as described in sections 2.2.2, and 2.6.2. At the end of the study, experimental birds were decapitated and dissected. The internal organs of all birds were stored in 10% neutral formalin for future examination, as described in section 2.6.2. Control birds of all species were released.

2.7. Treatment of experimental avian malaria with Malarone™ and a combination of Malarone™ and primaquine

Twelve greenfinches and 4 chaffinches from the experiment described in the section 2.6.2 were treated with the antimalarial drug Malarone™ in 2007. The total observation period was divided into three sub-periods: pre-treatment,

treatment and post-treatment (Fig. 9). After the pre-treatment period, which is described in section 2.6.2., all experimental and control birds were treated for 19 days (treatment period). The treatment was done with Malarone™; pills with fixed – dose combination of 250 mg of atovaquone and 100 mg of proguanil hydrochloride. During the treatment period a dose recommended for *P. falciparum* treatment in humans (7 mg/kg) (Looareesuwan et al., 1999a) was administered to each bird three times per day every third day; 21 dozes were given to each experimental bird in total. One doze contained 0.24 mg of Malarone™ dissolved in 50 µl of drinking water. This mixture was given to birds by oral intubation using a plastic micropipette. Greenfinches and chaffinches were bled every 6 days during the treatment period and once more 6 days after the last administration of the drug (Fig. 9). There was no possibility to keep greenfinches in captivity longer during this study. However, there was also an opportunity to test blood of infected chaffinches 43 days after the end of treatment (114 days after the experiment began) was presumable. Blood was taken for microscopy and PCR analyses from all birds, as described in sections 2.2.2 (see Fig. 5).

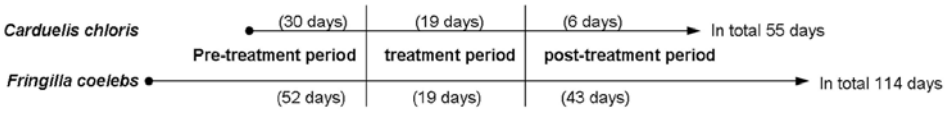

FIGURE 9. Diagrammatic representation of the time scale (periods) of experimental infection of greenfinches *Carduelis chloris* and chaffinches *Fringilla coelebs* with *Plasmodium relictum* (lineage SGS1), and treatment of the infected birds with Malarone™.

A treatment experiment using a combination of Malarone™ followed by primaquine was initiated in 2008. Such protocol of treatment was recommended for relapsing *P. vivax*-malaria by Looareesuwan et al. (1999b). For this experiment, siskins given a simultaneous experimental infection of *P. relictum* (SGS1) and *P. ashfordi* (GRW2) were used (Table 2, exp. C). The experimental infection during pre-treatment period is described in the section 2.6.3; other two

sub-periods were treatment and post-treatment (see Fig. 10). With reference to the Looareesuwan et al.'s (1999b) study, birds received sequential treatment with Malarone™ (once daily for three days), followed by treatment with primaquine (once daily for 14 days) (Fig. 10).

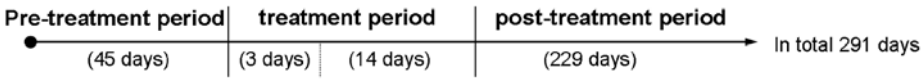

FIGURE 10. Diagrammatic representation of the time scale (periods) of experimental infection of siskins *Spinus spinus* with simultaneous infection of *Plasmodium relictum* (lineage SGS1) and *P. ashfordi* (GRW2), and treatment of the infected birds with Malarone™ and primaquine.

During this treatment experiment, birds received a dose of Malarone™ (21mg/kg). A dose recommended for treatment of *P. vivax* malaria in humans (30 mg of primaquine) was given to birds (Looareesuwan et al., 1999b). Both compounds were given by oral intubation. During the treatment period the birds were bled once after 3 days (the end of treatment with Malarone™) and once after 16 days (the end of treatment with primaquine) for microscopy and PCR-based diagnostics. All birds were also tested for parasitemia on days 31, 93, 115 and 229 during the post-treatment period. Blood was taken for microscopy and PCR analysis, as described in section 2.2.2.

2.8. Statistical analysis

The programme SPSS v14 was used for statistical analysis. Body mass, temperature and haematocrit values were compared between infected and control groups using repeated-measures ANOVA (Kirk, 1982). Throughout the experiment, mean differences in body mass, temperature and level of haematocrit within experimental and control groups were analyzed using the nonparametric Friedmann's test. Student's *t*-tests for independent samples were used to determine statistical significance between mean trait values. Prevalences were compared by Yates corrected chi-square test. A *P* value of 0.05 or less was considered significant.

3. RESULTS AND DISCUSSION

3.1. A comparative analysis of microscopy and PCR-based detection methods for avian malaria parasites and closely related haematozoa

To consider the sensitivity of microscopy and PCR-based detection methods broadly for species of haemosporidian parasites, not only *Plasmodium* spp. but also closely related parasites of the genera *Haemoproteus* and *Leucocytozoon* were used in this study (Fig. 11, a-d; Table 5). There was no significant difference in the prevalence of *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. infections in the same samples tested by microscopy and PCR (Table 5). The overall prevalence of infection, which was determined by combining results of both these methods, was higher than the overall prevalence, which was determined by each of these methods separately, but the differences were insignificant. Importantly, both microscopy and PCR diagnostics showed the same trends of prevalence of *Plasmodium, Haemoproteus* and *Leucocytozoon* spp. infections in the same samples at each study site and in the overall sample (Table 5). Compared to microscopic examination, the sensitivities of the nested PCR protocols for the detection of haemosporidian infections were similar.

Table 5. Birds tested and outcome of the microscopic examination of blood films and the PCR screening of the same samples.

Study site and bird species	No. tested	No. positive											
		Microscopic examination				PCR screening				Both methods combined			
		H*	P†	L‡	Total	H	P	L	Total	H	P	L	Total
Cameroon													
Cyanomitra olivacea	193	39	84	71	137	31	64	92	135	46	102	104	160
Gallus gallus	71	0	0	5	5	0	0	5	5	0	0	5	5
Total	264	39 (14.8)§	84 (31.8)	76 (28.8)	142 (53.8)	31 (11.7)	64 (24.2)	97 (36.7)	140 (53.0)	46 (17.4)	102 (38.6)	109 (41.3)	165 (62.5)
Uganda													
Gallus gallus	77	0	0	22 (28.6)	22 (28.6)	0	0	22 (28.6)	22 (28.6)	0	0	22 (28.6)	22 (28.6)

Study site and bird species	No. tested	No. positive											
		Microscopic examination				PCR screening				Both methods combined			
		H*	P†	L‡	Total	H	P	L	Total	H	P	L	Total
Lithuania													
Acrocephalus palustris	12	0	0	-‖	0	0	1	-	1	0	1	-	1
Sylvia communis	17	8	2	-	10	10	5	-	15	10	5	-	15
Phylloscopus trochilus	28	6	0	-	6	6	0	-	6	6	0	-	6
Total	57	14 (24.6)	2 (3.5)	-	16 (28.1)	16 (28.1)	6 (10.5)	-	22 (38.6)	16 (28.1)	6 (10.5)	-	22 (38.6)
Russia													
Ficedula hypoleuca	27	23	5	-	27	22	4	-	26	23	5	-	27
Parus major	21	20	3	-	21	20	1	-	21	20	3	-	21
Total	48	43 (89.6)	8 (16.7)	- -	48 (100)	42 (87.5)	5 (10.4)	- -	47 (97.9)	43 (89.6)	8 (16.7)	- -	48 (100)
California													
Bubo virginianus	5	0	1	5	5	0	1	5	5	0	1	5	5
Buteo jamaicensis	8	7	5	5	8	8	0	5	8	8	5	6	8
Megascops kennicottii	6	0	2	6	6	0	4	6	6	0	4	6	6
Tyto alba	7	0	1	5	6	0	1	6	6	0	1	6	7
Total	26	7 (26.9)	9 (34.6)	21 (80.8)	25 (96.2)	8 (30.8)	6 (23.1)	22 (84.6)	25 (96.2)	8 (30.8)	11 (42.3)	23 (88.5)	26 (100)
Grand total	472	103 (21.8)	103 (21.8)	119 (25.2)	253 (53.6)	97 (20.6)	81 (17.2)	141 (29.9)	256 (54.2)	113 (23.9)	127 (26.9)	154 (32.6)	283 (60.0)

* *Haemoproteus* sp.
† *Leucocytozoon* sp.
‡ *Plasmodium* sp.
§ Percentage of birds positive.
‖ The data are absent.

The results of this comparative analysis do not support the conclusions of Richards et al. (2002), Jarvi et al. (2002), Fallon et al. (2003b) or Durrant et al. (2006) regarding a much lower sensitivity of microscopy in comparison to PCR-based methods for determining the prevalence of haemosporidian infections. These discrepancies are likely due to the following main shortcomings of microscopy methods, which were used in these studies.

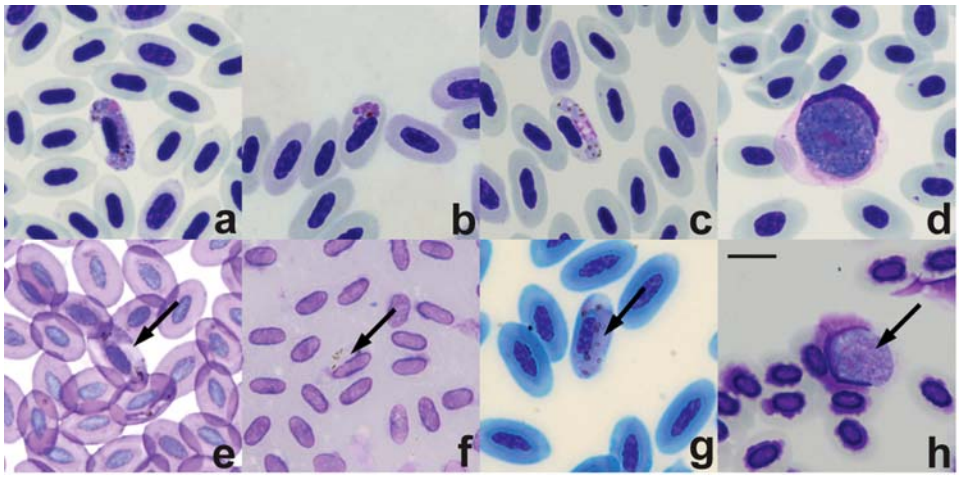

FIGURE 11. Blood stages of haemosporidian parasites as they are seen in good-quality (a – d) and bad-quality (e – h) blood films: a, e - gametocytes of *Haemoproteus* spp.; b, f - erythrocytic meronts and c, g - gametocytes of *Plasmodium* spp.; d, h - gametocytes of *Leucocytozoon* spp.; e, g - blood films with good fixation and bad staining; parasites' and their nuclei are hardly distinguishable; f, h - bad fixation and staining of blood films; blood cells are destroyed because of haemolysis and their nuclei are hardly seen. Arrows - parasites in bad-quality preparations. Giemsa-stained thin blood films. Bar = 10 μm.

Richards et al. (2002) used poor quality slides for microscopic examination in their study. The slides were stained several years after their preparation (R. N. M. Sehgal, personal communication). It is difficult to obtain a good quality of cell staining by Giemsa in such old material using traditional techniques (Garnham, 1966). The staining of blood films was pale, and corpuscles stained too blue, so the nuclei of parasites are poorly seen or even invisible. Additionally, numerous slides from this study contain haemolysed blood cells, which testify to the insufficient desiccation and fixation of blood films, or both in the field. The conclusion of this paper, that PCR is more reliable than blood smear analysis for large-scale screening reflects not the insensitivity of microscopy as such, but shows that microscopy of poor quality blood films is an unreliable method, and should be discouraged. It is much easier to see parasites in good quality slides (compare Fig. 11, a – d and e – h).

To determine prevalence of infection, Jarvi et al. (2002) screened approximately 50,000 red blood cells in each blood film with chronic parasitemia. This is enough to determine the intensity of infection (Godfrey et al., 1987), but is too small a number of the cells to determine the prevalence of infection, especially in light chronic infections (Garnham, 1966; Valkiūnas, 2005). Their conclusion that the PCR tests are 3- to 4-fold better than microscopy for detecting chronic infections can be explained by this shortcoming of their microscopy protocol.

The protocol for preparation, fixation and staining of blood films was not described by Durrant et al. (2006). The quality of the slides was not good (R. C. Fleischer, personal communication). Furthermore, the authors examined only 20,000 red blood cells during this study, which was certainly not enough to determine prevalence of infections, especially light chronic ones. In that study, PCR-based techniques showed an approximately 10-fold difference in prevalence of *Haemoproteus* and *Plasmodium* spp. infections, with PCR-based techniques being much better. That is in part due to shortcomings of their microscopy protocol.

We agree with Jarvi et al. (2002) that both microscopy and PCR screening slightly underestimate prevalence of infection of blood parasites in naturally infected birds. Combined results of both these methods usually show higher prevalence data (Table 5). However, the difference in prevalence of infection between these 2 methods is insignificant. The overall prevalence of haemosporidian blood parasites, as determined by microscopy (53.6%) and PCR diagnostics (54.2%), was marginal. In some of samples, microscopy was slightly more sensitive than PCR, and in some samples, the opposite (Table 5). However, in all samples both these methods showed the same trends of prevalence; this is essential information for the investigation of patterns of the distribution of blood parasites in wildlife.

According to the present study, both microscopy and PCR-based detection methods underestimate approximately the same number of the patent infections of haemosporidian parasites (Table 5). The main shortcoming of

microscopic examination of blood films is the low sensitivity in determining exceptionally light infections (< 0.001%) when just a few parasites are present in blood films. Such light parasitemias might be overlooked even with increased observation time. The main shortcoming of the current nested PCR-based protocols is a limited sensitivity in reading simultaneous infections of haemosporidian parasites, especially parasites belonging to the same genus or relative genera, i.e. *Haemoproteus* spp. and *Plasmodium* spp. This is mainly because amplification is often highly selective during simultaneous infections; it is sometimes not related to the intensity of parasitemia with a particular parasite (Valkiūnas et al., 2006a). To determine the true species composition of haemosporidian parasites in each individual host, PCR diagnostics will need to be improved. Specific primers for targeting *Haemoproteus* spp. and *Plasmodium* spp. should be used. A combination of approaches of microscopy and PCR-based methods is recommended for development of such protocols. A better understanding of this group of parasites currently requires a comparison and synthesis of microscopy and molecular data.

PCR-based methods are particularly attractive because they provide sequence information for phylogenetic and epidemiological studies of parasites and for the diagnosis of parasitic disease (Santiago-Alarcon et al., 2008; Perkins and Schall, 2002; Jarvi et al., 2002; Bensch et al., 2004; Ricklefs et al., 2004; Kimura et al., 2006; Križanauskienė et al., 2006; Martinsen et al., 2006; Valkiūnas et al., 2006a; Palinauskas et al., 2007; Perkins et al., 2007). However, microscopy also remains an important tool in the investigation of blood parasites. It has the following advantages in comparison to the current PCR-based techniques in detecting infections of blood parasites in naturally infected birds. First, microscopy is an inexpensive investigation method, which is available in each laboratory; technical staff can be easily trained for the routine screening of blood films. Second, microscopy provides an opportunity to determine or verify the identity and intensity of infections, and with PCR this is especially difficult to do with simultaneous infections of closely related species of parasites. Currently this is particularly important with the rapid accumulation

of sequence data that should be linked with data of traditional taxonomy (Hellgren et al., 2007a; Palinauskas et al., 2007). It is important to note that simultaneous infections of haemosporidians belonging to the same and different genera and subgenera are common and, in some bird species, have been recorded in over 80% of all samples that were positive by microscopic examination of blood films (Valkiūnas et al., 2003). Third, in spite of relatively long duration of microscopy of each sample (20-25 min during this study), such examination provides opportunities for simultaneous determination and verification of taxonomically different parasites, i. e. numerous species of *Haemoproteus, Plasmodium, Leucocytozoon, Trypanosoma, Atoxoplasma, Hepatozoon, Babesia,* microfilariae and some other (Bishop and Bennett, 1992). Presently, different PCR protocols must be used for the detection of parasites belonging to different genera, and even some different species of the same genus (Valkiūnas et al., 2006a); this is expensive and also time-consuming.

It is important to note that blood films, which are used for microscopic examination, should be of good quality (see Fig. 11, a-d), i. e. they should be thin, clean from artifacts, properly fixed and stained. Unfortunately, the quality of blood films, which are used for microscopy, has never been addressed in recent molecular studies of avian blood parasites, but it is essential for reliable prevalence results obtained using this method (Cooper and Anwar, 2001).

Preparation of good quality blood films is cheap and easy. However, even small errors in standard techniques can render films entirely unsuitable for examination. The following oversights in the preparation of blood films are common (Hewitt, 1940; Shute and Maryon, 1960; Garnham, 1966; Cooper and Anwar, 2001; Valkiūnas, 2005). First, contamination by dust or flies, exposure to strong sunlight, excessive heat, or humidity frequently spoil blood films. Second, slow drying of blood films (> 30 sec), which usually is the case in humid climates, leads to rapid changes of morphology of mature gametocytes of haemosporidians due to the onset of gametogenesis. Identification of haemosporidian species usually is difficult or even impossible in such material. Battery-operated fans can be used to aid in the drying of the blood films during

field studies. Third, the use of non-absolute methanol for fixation leads to haemolysis of blood cells, usually during the subsequent staining of blood films. Methanol should be kept in hermetic bottles, and not reused if frequently exposed to air in humid environments. Fourth, the late staining of blood films after their preparation. Ideally, blood films should be stained soon after their fixation. However, this is often difficult to do during field studies. It is possible to achieve the satisfactory staining if the slides are stained 1-2 months after their fixation. After that time, the blood films become alkaline due to exposure of air and humidity, and as a result, the cells stain too blue with Giemsa. Our experience shows that good staining quality cannot be achieved if staining of fixed slides is carried out later than 3 months after their preparation (Valkiūnas, 2005); it is usually unsatisfactory after 6 months (G. Valkiūnas, unpublished data). If there is no opportunity to stain blood films soon after fixation, they can be stored by placing them in plastic hermetic boxes with a desiccating material, and keeping them in a refrigerator. Such blood films stained well even after approximately a year or more.

It is important to note that the length of time for scanning blood films for scientific research hardly can be standardized because different examiners have different experience, skills and conscientiousness. Microscopic examination of blood films for approximately 20-25 min, as described above, gives good results in detecting the prevalence of avian haemosporidians in comparison to the PCR-based methods (Table 5).

Jarvi et al. (2002) recommended the use of a combination of approaches for the reliable diagnostics of blood parasites in wildlife. These would ideally include examination of blood smears, PCR tests and suitable serological methods. It is important to note that both microscopy and PCR diagnostics are insensitive in detecting latent infections, when blood parasites are absent from the peripheral circulation (Jarvi et al., 2002). These tools provide better results in the detection of blood parasites when the majority of infections are patent. That takes place in different seasons of the year at different latitudes, but usually coincides with the maximum breeding period of birds (Valkiūnas, 2005) when

parasitemias are relatively high and the transmission of infection to juvenile birds takes place. For better understanding of the distribution of haemosporidians in wildlife using microscopy and PCR tools, collecting of blood samples during the period of the active transmission of blood parasites is recommended.

In conclusion, both microscopic examination of blood films and nested PCR-based diagnostics show the same trends in prevalence of infection of haemosporidain parasites in naturally infected birds. Thus, microscopy is a valuable method in field studies if blood films are of good quality and they are examined properly by skilled investigators. We recommended continued use of optical microscopy in the research of haemosporidian parasites of vertebrates. Importantly, this tool provides important information how molecular methods can be further improved and most effectively applied, especially in the field studies of parasites.

3.2. Molecular identification of *Plasmodium relictum* and *Plasmodium circumflexum*; linkage of PCR-based and morphology data and some taxonomic consideration in identification of *Plasmodium* spp.

According to PCR analysis and microscopic examination of blood films, haemosporidian parasites were not observed in recipient and control birds prior to infection and all negative controls remained uninfected throughout the course of the experiment.

Two lineages SGS1 and TURDUS1of malaria parasites were identified using PCR. Infections of the lineage SGS1 became patent between 8 and 14 (on average 11) dpi and infections of the lineage TURDUS1 became patent between 20 and 24 (on average 22) dpi. The highest parasitemia of lineage SGS1 (20%) developed in one of the common crossbills at 21 dpi, and the highest parasitemia of the lineage TURDUS1 (0.1%) developed in one of the house sparrows at 26 dpi, respectively. Blood smears with high parasitemia from experimental infections were used to describe morphology of these parasites.

Plasmodium (Haemamoeba) relictum (Grassi and Feletti, 1891) (Fig. 12, Table 6).

DNA sequences: Mitochondrial cyt *b* lineage SGS1 (478 bp, GenBank accession no. AF495571).

Avian hosts: The lineage SGS1 has been recorded in 29 species of birds belonging to eight families of Passeriformes (Waldenström et al., 2002; Beadell et al., 2006; Reullier et al., 2006; Hellgren et al., 2007b).

Site of infection: Mature erythrocytes; no other data.

Representative blood films: Voucher specimens (accession nos. 20230 NS, 20232 NS, USNPC 99591, G464933, *Loxia curvirostra*, 3 August 2005, collected by V. Palinauskas) were deposited in the Institute of Ecology (Vilnius University, Vilnius, Lithuania), in the U. S. National Parasite Collection (Beltsville, USA), the Queensland Museum (Queensland, Australia), respectively.

The main morphological characters of lineage SGS1 blood stages were similar to those of *Plasmodium (Haemamoeba) relictum* in its type vertebrate host, the Spanish sparrow at the type locality (Sicily, Italy). This lineage certainly belongs to this morphospecies.

Table 6. Morphometry of mature erythrocytic meronts and gametocytes of *Plasmodium relictum* (lineage SGS1) and *Plasmodium circumflexum* (lineage TURDUS1) (n=25)

Feature	Measurements (μm)*	
	P. relictum	*P. circumflexum*
Meront		
Length	4.1-7.1 (5.6 ±0.8)	8.1-22.4 (18.1 ± 3.2)
Width	3.2-6.1 (4.4 ± 0.7)	1.4-2.7 (1.8 ± 0.4)
Area	13.1-27.7 (20.1 ± 4.2)	20.5-40.6 (29.0 ± 5.6)
No. of merozoites	11-22 (15.5 ± 2.7)	13-28 (20.4 ± 4.1)
Macrogametocyte		
Length	6.0-10.5 (7.7 ± 0.8)	9.5-15.3 (11.9 ± 1.8)
Width	3.1-5.9 (4.6 ± 0.7)	1.6-3.1 (2.3 ± 0.4)
Area	19.7-29.6 (22.5 ± 1.9)	18.7-34.0 (26.3 ± 3.7)
Microgametocyte		
Length	4.5-9.1 (7.2 ± 1.1)	11.1-20.5 (15.9 ± 3.0)
Width	4.0-6.7 (5.2 ± 0.7)	1.7-2.9 (2.2 ± 0.3)
Area	18.8-29.8 (23.6 ± 2.3)	24.6-45.0 (33.6 ±5.0)

* Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

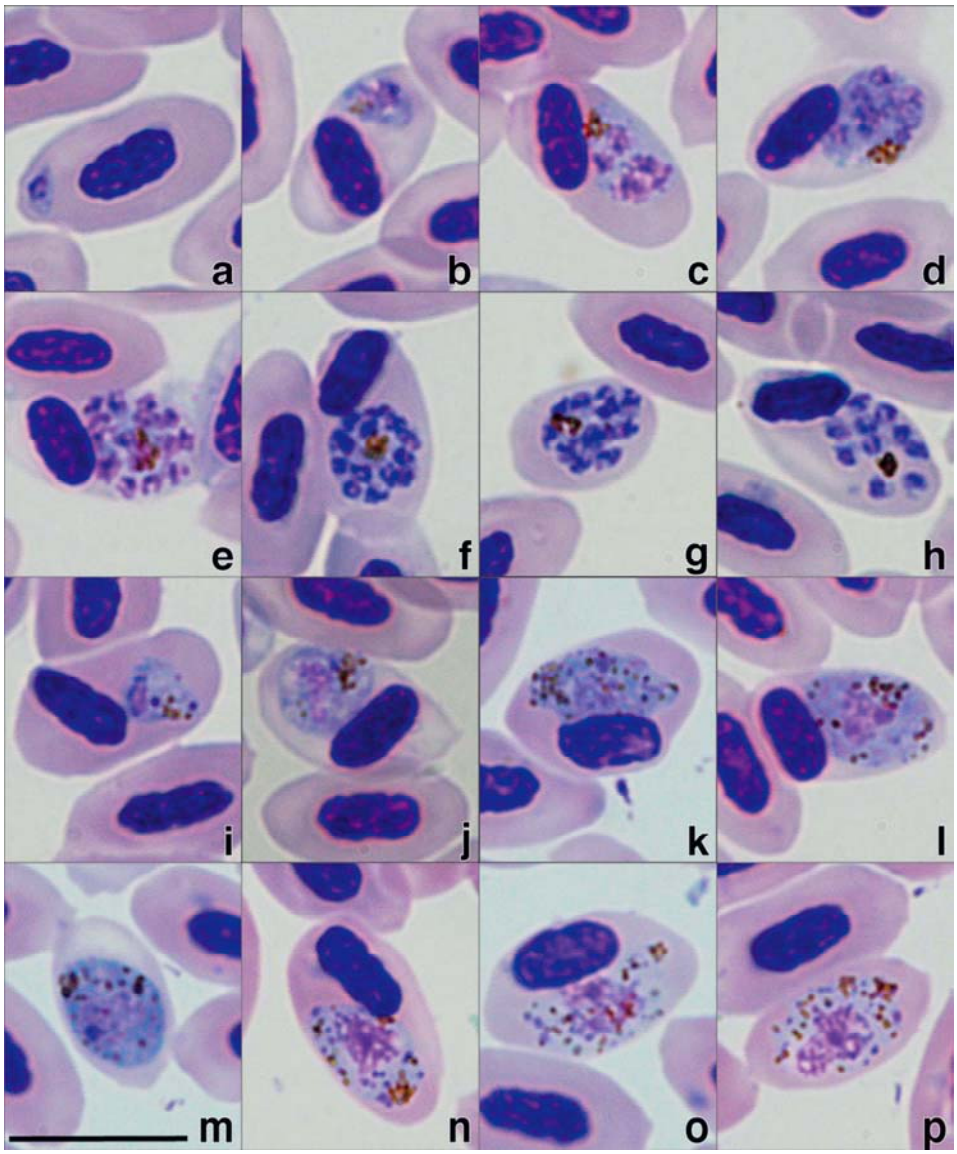

FIGURE 12. *Plasmodium relictum* (lineage SGS1) from the blood of common crossbill *Loxia curvirostra*: a, b - trophozoites; c – h - erythrocytic meronts; i – m - macrogametocytes; n – p - microgametocytes. Scale bar = 10 μm.

Trophozoites (Fig. 12, a, b) are roundish or oval. Pigment granules and small vacuoles appear in some early trophozoites. The nuclei of infected erythrocytes are markedly displaced by intermediate sized trophozoites.

Erythrocytic meronts (Fig. 12, c-h). Growing parasites are variable in form, sometimes irregular in shape (Fig. 12, c), but the majority of full-grown

meronts are roundish or oval. The meronts markedly displace the nuclei of infected erythrocytes; they can occupy more then half of the cytoplasmic space in the erythrocytes and sometimes can enucleate the host cells (Fig. 12, g). Pigment granules are aggregated in prominent loose clumps. The number of merozoites in mature meronts most frequently ranges between 10 and 18.

Macrogametocytes (Fig. 12, i-m) are often oval or roundish (Fig. 12, j, l, m), but irregularly shaped parasites also are common (Fig. 12, k). The parasites markedly displace the nuclei of infected erythrocytes toward the pole and can enucleate the host cells (Fig. 12, m). Pigment granules usually are roundish or oval, black or dark-brown, randomly scattered throughout the cytoplasm, they sometimes are seen in loose clumps (Fig. 12, j).

Microgametocytes (Fig. 12, n-p). The general configuration and other features are as for macrogametocytes with the usual sexual dimorphic characters. Microgametocytes with irregular and ameboid outline (Fig. 12, o, p) are more common then macrogametocytes.

Plasmodium (Giovannolaia) circumflexum (Kikuth, 1931) (Fig. 13, Table 6).

DNA sequences: Mitochondrial cyt *b* lineage P-TURDUS1 (472 bp, GenBank accession no. AF495576).

Avian hosts: The lineage TURDUS1 has been recorded in 13 species of birds belonging to seven families of Passeriformes and one family of Falconiformes (Hellgren, 2005, Hellgren et al., 2007b; A. Križanauskienė, unpublished).

Site of infection: Mature erythrocytes; no other data.

Representative blood films: Voucher specimens (accession nos. 19290 NS, 19291 NS, *Loxia curvirostra* and USNPC 99590, G464934, *Passer domesticus*, 4 and 15 July, 2005, collected by V. Palinauskas) were deposited in the Institute of Ecology (Vilnius University, Vilnius, Lithuania), in the U. S. National Parasite Collection (Beltsville, USA), in the Queensland Museum (Queensland, Australia), respectively.

The main morphological characters of lineage TURDUS1 blood stages were similar to those of *P. (Giovannolaia) circumflexum* from its type vertebrate host, the fieldfare during development of this parasite in the canary *Serinus canaria* Linnaeus.

Trophozoites (Fig. 13, a-c) are variable in form, frequently elongated with apparent pigment granules and vacuoles. The nuclei of infected erythrocytes are not displaced (Fig. 13, a, b) or only slightly displaced (Fig. 13, c).

Erythrocytic meronts (Fig. 13, d-j) grow around the nuclei of erythrocytes from earliest stages of their development and finally can completely encircle the nuclei (Fig. 13, h, i). The meronts do not displace or only slightly displace the nuclei of infected erythrocytes. Position of young meronts usually lateral in relation to the host cell nucleus and filling of the cytoplasm is variable (Fig. 13, d-g). Pigment granules are aggregated into loose clump, which usually are located near the end of growing parasites (Fig. 13, d, e), but also present in sub-terminal position in nearly mature parasites (Fig. 13, g). The number of merozoites in mature meronts most frequently ranges between 16 and 25.

Macrogametocytes (Fig. 13, k-n) grow around the nuclei of infected erythrocytes as halteridia (Fig. 13, l) and finally can completely encircle the nuclei (Fig. 13, n). The parasites do not or only slightly displace the host cell nucleus laterally (Fig. 13, n). Pigment granules are usually roundish or oval, randomly scattered throughout the cytoplasm or present as loose clumps (Fig. 13, k).

Microgametocytes (Fig. 13, o, p). The general configuration and other features are as for macrogametocytes with the usual sexual dimorphic characters.

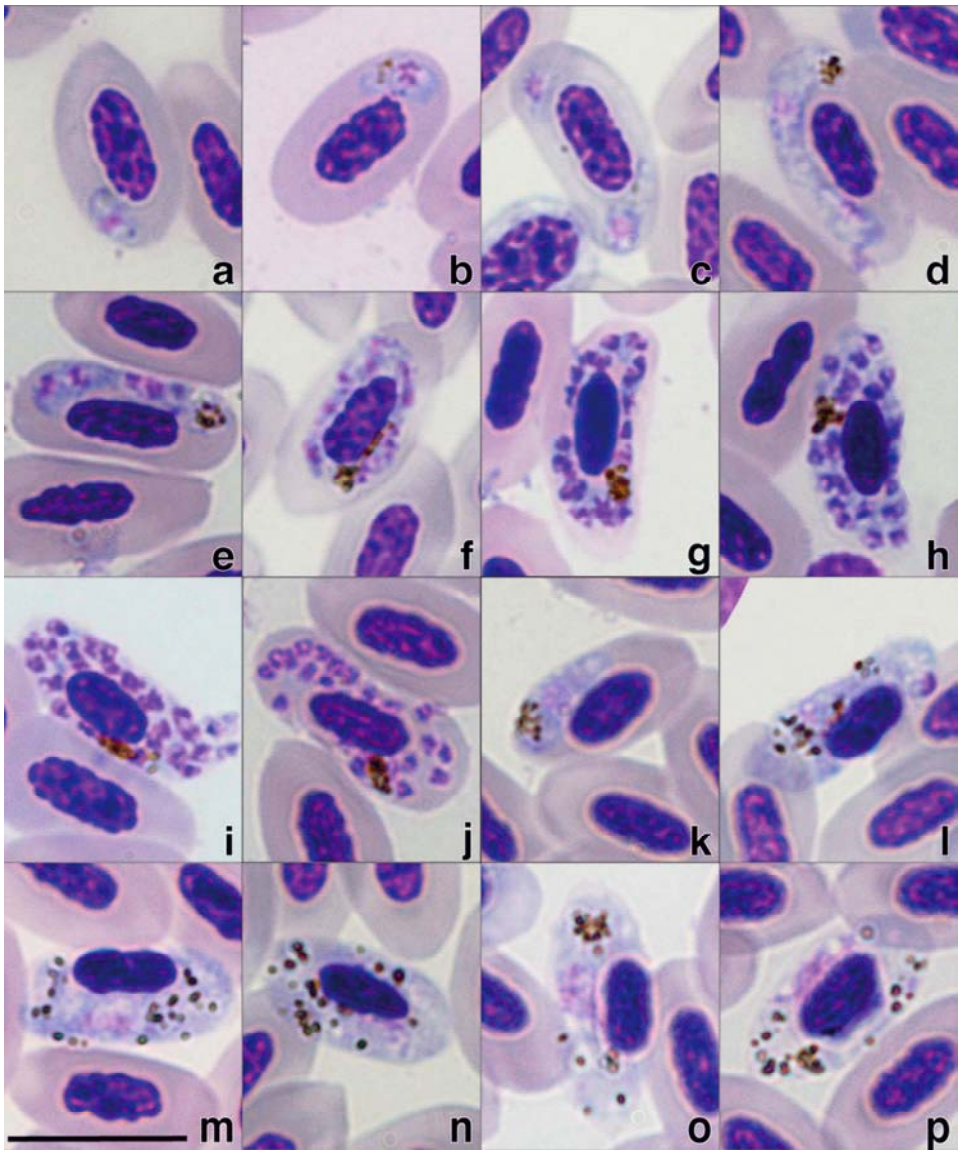

FIGURE 13. *Plasmodium circumflexum* (lineage TURDUS1) from the blood of house sparrow *Passer domesticus*: a – c - trophozoites; d – j - erythrocytic meronts; k – n - macrogametocytes; o, p - microgametocytes. Scale bar = 10 μm.

The lineages SGS1 and TURDUS1 belong to clearly different clades (Fig. 8, clades a and b), which are in accord with their clear morphological differences (Figs. 12, 13) in traditional taxonomy. The sequence divergence between these two lineages is 4.1%. The lineage SGS1 belongs to a diverse

group of lineages (Fig. 8, a) containing six other lineages within genetic distances up to 2.5% between them. The lineage TURDUS1 has three closely related lineages with a genetic distance of < 0.5 % between them. Both clades (a and b) are clearly separated from *Plasmodium ashfordi* (the lineage GRW2), which belongs to the subgenus *Novyella* and has only one sister lineage in the phylogenetic tree with genetic divergence of 2.5% (Fig. 8, c). Inside morphospecies, the sequence divergence between lineages of *P. relictum* (clade a) and *P. circumflexum* (clade b) is < 2.5% (in average 1.7% and 0.3%, respectively).

Recent molecular studies (Perkins and Schall, 2002; Ricklefs and Fallon, 2002; Waldenström et al., 2002; Beadell et al., 2004, 2006; Pérez-Tris and Bensch, 2005; Szymanski and Lovette, 2005; Santiago-Alarcon et al., 2008), which are based mainly on genes of the mitochondrial genome, have shown that the genetic diversity of avian malaria parasites is much greater than their morphological diversity (Valkiūnas, 2005). Over 500 cyt *b* gene lineages of avian malaria parasites have been deposited in GenBank. However, the great majority of these lineages have been identified only to the generic level. It is still unclear what the genetic bounds of morphospecies are, and how the sensitive PCR-based techniques can be used in identification of avian *Plasmodium* spp. and other haemosporidian parasites. Additionally, it is important to develop molecular identification of *Plasmodium* spp. because the great majority of natural malaria infections are light and frequently cannot be identified to species level using microscope even by experts.

Wiersch et al. (2005), Martinsen et al. (2006, 2007), Hellgren et al. (2007a) and Valkiūnas et al. (2007) have made the first steps on linking traditional taxonomy and genetic data. In the present study, two common mitochondrial cyt *b* gene lineages of two morphospecies of avian malaria parasites were identified using highly reliable experimental data. That was done after detailed investigation of blood films with experimental parasitemia. All blood stages of the parasites, important for morphospecies identification, were available during this study; that usually is not the case during light natural

infections. So, clear identification of specific identity of the lineages SGS1 and TURDUS1 was possible.

It seems probable that a diverse group of lineages of clade a (Fig. 8) belongs to the morphospecies *P. relictum*, which is the type species of the subgenus *Haemamoeba*. This is probably an example of morphospecies variation within avian *Plasmodium* spp. The remarkable genetic diversity of *P. relictum* (see Bensch et al., 2000; Beadell et al., 2006; Hellgren et al., 2007b) probably reflects (i) a local adaptation of different lineages of this parasite to develop in a huge range of avian hosts, belonging to different families and even orders of birds, (ii) an adaptation to develop in numerous species of vectors (mosquitoes of the family Culicidae), which are involved in its transmission in different parts of the world, and (iii) an adaptation to development in different climatic zones (see Valkiūnas, 2005).

It is worth noting that morphological features and measurements of two morphologically well-investigated lineages of *P. relictum* (SGS1 and GRW4) markedly overlap (Table 6, also see Table 1 in Valkiūnas et al., 2007). There were no significant differences between main diagnostic features of these two lineages of *P. relictum*, except one feature, which was more frequently recorded in parasites of the SGS1 lineage. Mainly, macro- and especially microgametocytes of SGS1 more frequently possess irregular or ameboid margins (Fig. 12, k, o, p), than GRW4. This character can be used for morphological identification of these two lineages, which are different in their ability to develop in northern latitudes of the Holarctic (see below).

Morphological features and measurements of TURDUS1 lineage match well with *P. circumflexum* from its type vertebrate host (Table 6, Valkiūnas, 2005). Circumnuclear erythrocytic meronts (Fig. 13, g-i) and gametocytes (Fig. 13, n, p), which clearly distinguish *P. circumflexum* from other species of the subgenus *Giovannolaia*, are common in the TURDUS1 lineage.

The results obtained from molecular and morphological analyses during this study are similar and complement each other. Differences between *P. relictum* (SGS1) and *P. circumflexum* (TURDUS1) are clearly seen in the

neighbour-joining tree (Fig. 8). There was shown by Valkiūnas et al. (2008d) that no differences between the topology of phylogenetic trees constructed using either the Bayesian or neighbour-joining approaches were recorded, and that phylogenetic relationships are reflected. Molecular differences are well-supported by microphotographs (Figs. 12, 13) and morphometry (Table 6). This study is in accord with conclusion by Martinsen et al. (2007), Hellgren et al. (2007a) and Valkiūnas et al. (2007) that phylogenetic trees constructed based on the mitochondrial cyt *b* gene analyses are matching well with data of traditional taxonomy, which is important for future development of systematics and investigation of evolutionary biology of the parasites.

The lineage SGS1 is transmitted in northern, western and southern European countries (Hellgren, 2005; Hellgren et al., 2007b; A. Marzal et al., manuscript in preparation). It is also transmitted in Eastern Europe, including Ukraine and the European slip of the Ural Mountains in Russia (Hellgren et al., 2007b; Beadell et al., 2006; V. Palinauskas, unpublished data). According to Waldenström et al. (2002), transmission of the SGS1 lineage takes place in Nigeria. Moreover, the same lineage was determined in birds in South Korea (Beadell et al., 2006). That testifies to the wide range of transmission of the lineage SGS1, which probably is cosmopolitan in distribution.

The lineage GRW4 of *P. relictum* has been recorded all over the world in countries with warm climates, such as USA (in Hawaii), Nigeria and Papua New Guinea and others (Waldenström et al., 2002; Ricklefs and Fallon, 2002; Beadell et al., 2006). This lineage was not recorded in resident birds in northern Europe. It is probable that the SGS1 lineage of *P. relictum* is more adapted for transmission in northern latitudes and GRW4 has not developed such ability so far. The sequence divergence between these 2 lineages is 2.2%. Both SGS1 and GRW4 lineages have a wide range of avian hosts, which belong to different families and orders of birds, mainly species of Passeriformes (Waldenström et al., 2002; Hellgren, 2005; Beadell et al., 2006; Reullier et al., 2006; Hellgren et al., 2007b).

Transmission of the lineage TURDUS1 of *P. circumflexum* takes place all over Europe (Hellgren, 2005; Hellgren et al., 2007b; Wood, personal communication). Waldenström et al. (2002) did not find this lineage in Africa and thus attributed it to a group of Europe-transmitted lineages. According to personal communication of A. Križanauskienė, TURDUS1 lineage was recorded in Yekaterinburg district on the European slip of the Ural Mountains and at one site in Irkutsk district near the Lake Baikal, Russia. It is probable that this lineage is common in Eurasia. There are no data about transmission of this lineage in tropical areas so far. Closely related lineage BT7, with the sequence divergence of only 0.02% (Fig. 8, b) has been found in Europe and Siberia (Hellgren, 2005; Hellgren et al., 2007b; V. Palinauskas, unpublished data). Beadell et al. (2006) have found this lineage in birds from South Korea and USA. It is probable that the BT7 lineage belongs to the same morphospecies, *P. circumflexum*. According to microscopy data (Valkiūnas, 2005), this species has been recorded in all zoogeographical regions except the Antarctic. It is possible that this morphospecies unites numerous lineages, which differ from each other in ability for transmission in southern and northern latitudes as is the case with lineages SGS1 and GRW4 of *P. relictum*; that warrants further investigation.

Using sequences of mitochondrial cytochrome *b* and cytochrome oxidase I genes of *Plasmodium* spp., Martinsen et al. (2007) raised a novel question that subgenera *Novyella* and *Giovannolaia* do not represent monophyletic groups of avian malaria parasites. However, based on material obtained from experimental *Plasmodium* infections by Wiersch et al. (2005), Valkiūnas et al. (2007) and the present study (Fig. 8), the lineages of *P. (Haemamoeba) relictum*, *P. (Giovannolaia) circumflexum*, and *P. (Novyella) ashfordi* formed clearly different clades in the phylogenetic tree; that supports the validity of these subgenera. It is important to note that in Martinsen et al's (2007) molecular study, malarial infections were selected from naturally infected birds using microscopy methods. In this case, it is difficult to rule out completely, a possibility that low-grade mixed infections can be present in a sample, but missed even during a long-time microscopy. In the latter case, a sequence might

be obtained and assumed to be that of the parasite seen under the microscope, but there might be a low-grade infection of another species, that is the one amplified, as was documented by Valkiūnas et al. (2006a). Current PCR-based diagnoses of haemosporidian infections assume that the construction of primers from highly conserved regions of the parasites' DNA should amplify all species belonging to *Haemoproteus,* and *Plasmodium* equally (Ricklefs and Fallon, 2002; Waldenström et al., 2004). The study by Valkiūnas et al. (2006a), however, indicates that this is not necessarily true, because the amplification is often highly selective during simultaneous infections. Because nested PCR based methods underestimate simultaneous infections, one should be careful interpreting data obtained from naturally infected bird. That should be taken into consideration in molecular systematic and phylogenetic studies.

It is important to note that the subgeneric identity of many species of avian malaria parasites is unclear in traditional taxonomy; morphological features of some species are on the bound of definitions of the subgenera *Giovannolaia* and *Novyella* (Garnham, 1966; Valkiūnas et al., 2006a). It is possible that subgeneric identity of some *Plasmodium* species might be changed in the future; definitions of old subgenera might be corrected and new subgenera of avian malaria parasites might be established. However, further field and experimental investigations combined with DNA studies of parasites are needed to clarify and develop traditional taxonomy of avian malaria parasites. Further work to increase the number of linkages between *Plasmodium* DNA sequences and their corresponding morphospecies is an urgent necessity.

3.3. Laser microdissection of single cells for PCR-based studies of *Plasmodium* spp. and closely related blood parasites

The samples extracted with phenol-chloroform and ammonium acetate methods failed to amplify with the new primers (see chapter 2.4 of the method section). Similarly, repeated examination of Giemsa stained MMI-MembraneSlide (Molecular Machines & Industries) slides never yielded any positive PCR amplification irrespective of the extraction method (phenol-

chloroform or Chelex), so all the following experiments were carried out on isolates from unstained MMI slides.

The standard primers HaemFN/HaemRN and HaemF/R2 for nested PCR of *Haemoproteus* and *Plasmodium* failed to amplify parasite DNA when tested on samples containing extracts from 100 and 10 ookinetes. A combination of primers targeting shorter fragment of the cyt *b* gene (224 bp, HaemF/HaemR2 and F144/R368) amplified successfully from both samples (100 and 10 ookinetes).

In each of the PCR reactions only a fraction (2 µl) of the total volume of the supernatant (10 - 15 µl) from the extracted samples was used. DNA extracted from 10 and 2 cells yielded higher PCR success for *Haemoproteus tartakovskyi* (SISKIN1) than for *Plasmodium relictum* (SGS1) (Table 4). The success was 100% from the sample extracted from 10 cells of *Haemoproteus* spp. ookinetes and high (75%) also from samples with 2 cells of these parasites. From extracts of *P. relictum* erythrocytic stages, the positive amplifications were 75% from 10 cells and 25% from 2 cells.

Twelve samples containing extracts of single ookinetes from either *H. tartakovskyi* (SISKIN1) or *H. majoris* (WW2) (Fig. 6, a – c), showed positive amplifications in 11 (92%) and 10 (83%) PCR runs respectively (Table 3). The resulting sequences were clear and matched perfectly the parasite lineages found in these birds. From 12 samples of *P. relictum* (SGS1) (Fig. 6, d – f) four (33%) successful PCRs were obtained and the sequences also perfectly matched the parasite sequence from the bird.

Massive genome sequencing of parasite DNA, either by random clone sequencing using the traditional Sanger protocol or the new methodology of pyrosequencing (Ellegren, 2008) requires substantial amounts of pure parasite DNA, safely isolated from the host DNA. We here demonstrate that laser microdissection microscopy is a powerful method for isolating pure parasite DNA. From slides with high parasitemia (1% or more) one day of work is sufficient for harvesting 1,000 cells into one single tube by using the Olympus /MMI CellCut Plus microdissection system. With a genome size of 25 Mbp for

malaria parasites, 1,000 cells should approximately contain 25 pg of DNA (Doležel et al., 2003). Protocols for setting up a pyrosequencing project recommend DNA quantities in the range of a few µg. Hence, it seems not tractable to isolate such quantities (>40,000 cells) using a CellCut Plus microdissection system. This problem could be solved if the isolated parasites DNA can be multiplied using whole genome amplification approach. Commercial kits for whole genome amplification of single cells of human DNA are available, e.g. GenomePlex® Single Cell Whole Genome Amplification Kit (Sigma), however these are not expected to be useful for these parasites as they are targeting on repeated elements in the human genome for initiating the amplification (Barker et al., 2004). Hence, without knowledge of the bird malaria genome such an approach must rely on arbitrary primers. A variety of such protocols are available (Zhang et al., 1992), but arbitrary primer approaches may result in biased amplifications of the genome. Multiple sequencing from a biased genome library might however still be fruitful as long as it will yield plenty of unique nuclear sequences.

The few alternative approaches to obtain high amount of pure DNA from avian malaria parasites and other haemosporidians have so far proved difficult. Gentle lyses of erythrocytes with EDTA-20 and repeated steps of Percoll gradient centrifugation (Graczyk et al., 1994) can provide large quantities of DNA, but purity maybe compromised due to presence of host DNA. Hence, this is a good method for enriching the sample of parasite DNA but may not give the purity for efficient large scale sequencing. Dissection of oocysts from vector midguts (each mature oocyst of *Plasmodium* spp. contains several hundreds of sporozoites or parasite genomes) (Valkiūnas, 2005) is likely to yield relatively pure parasite DNA, but this approach faces the same problem in terms of quantity as here described approach based on laser microdissection microscopy.

Our experiments suggest that Giemsa staining interferes with the PCR amplification of the parasite cytochrome *b* gene. This was a surprising result since Giemsa stained material has been successfully used as template in other PCR experiments on bird haemosporidians (Scopel et al., 2004; Beadell and

Fleischer, 2005). According to Freed and Cann (2006) there are many critical stages where parasite DNA can be negatively affected by inappropriate storage solution. Moreover the PCR reaction can be directly inhibited by different reagents. If Giemsa is a PCR inhibitor, it might be that the small extraction volumes are not sufficient for rinsing the sample from Giemsa. A study using Giemsa stained bone marrow demonstrated increased PCR success when more sophisticated extraction methods were used (Poljak et al., 2000).

Although the issue of species limits of avian haemosporidians is under debate, most bird species are hosts of multiple species sometimes as many as 10 (Bensch et al., 2007; Pérez-Tris et al., 2007). Individuals with simultaneous infections are therefore common and even predominat in some populations (Valkiūnas et al., 2003). The primers used for screening these parasites may not be equally efficient for all parasites as can be revealed by careful microscopic examinations (Valkiūnas et al., 2006a). Isolation of single cells from samples where morphological analyses suggest mixed infections but standard PCR typing only recover a single parasite lineage will be a useful approach to recover parasites presently missed by the standard molecular techniques. Here presented results from experiments with single cell analyses of *Plasmodium* and *Haemoproteus* parasites collected with the Olympus/MMI CellCut Plus microdissection system suggest that it should be a straightforward approach.

There are several examples of closely related parasite lineages that appear to be reproductively isolated despite co-occurring in the same avian host individual (Pérez-Tris and Bensch, 2005; Pérez-Tris et al., 2007) and therefore also likely to be engorged into the same vector were the sexual process is taken place. Genetic analyses of isolated ookinetes in hybridization experiments (Valkiūnas et al., 2008b) or oocysts in vectors (Razakandrainibe et al., 2005) can be greatly assisted by laser microdissection microscopy and improve our understanding of the underlying mating patterns and mechanisms behind the presumed reproductive isolation between parasite lineages. Understanding how reproductive isolation is maintained is a key to understand patterns of diversification and speciation in the diverse group of haemosporidian parasites.

In conclusion, laser microdissection microscopy is a promising new technology for solving long standing questions in the research of avian malaria and other haemosporidian parasites. That also should be true for blood parasites of fish, amphibians and reptiles, which red blood cells possess nuclei. For better use of the isolated parasites, protocols for whole genome amplification need to be developed along with multiple nuclear markers for precise identification of parasite lineages. Such a bioassay will allow for detailed analyses of the reproductive biology of these parasites and deepen our understanding of the evolution of this remarkably diverse and species rich group of parasites.

3.4. Development of *Plasmodium relictum* (lineage SGS1) infection in experimentally infected birds

It is important to note that 4 of 6 bird species, which were used during this study, were hatched during winter time (common crossbill) or early spring (house sparrow, siskin and greenfinch) when transmission of *Plasmodium* spp. is absent in the northern Palearctic and at our study site (Valkiūnas, 2005). Thus, they have had slim chances to get natural infection in the nests at our study site; that was supported during this study. Because of that, juvenile siskins, crossbills, greenfinches and house sparrows are particularly convenient model bird species for experimental research in northern Europe.

According to our studies the lineage SGS1 belongs to this morphospecies *Plasmodium relictum* (see section 3.2 and Fig. 12). *P. relictum* is characterised by remarkable genetic diversity; it contains many different ecological strains, which might be good biological species or subspecies (Bensch et al., 2004). For instance, the lineages SGS1 and GRW4 of *P. relictum* morphologically markedly overlap (see section 3.2 and Table 5), but they differ in their geographic distribution. The main transmission area of GRW4 is tropical Africa, North America and several Oceanic islands including the Hawaii (Beadell et al., 2006). The lineage SGS1, on the other hand, infects birds in tropical Africa, most of Europe up to north, and in Asia east to South Korea (Waldenström et al., 2002; Ricklefs and Fallon, 2002; Beadell et al., 2006). Different lineages

could also be different in their ability to infect different host species as well as having different potential to affect birds. Remarkably little is known about virulence of the same malaria parasites for different avian hosts (Atkinson et al., 2001a).

3.4.1. Specificity of Plasmodium relictum (lineage SGS1) for birds in regard to their phylogenetic relationships

According both to PCR analysis and microscopic examination of blood films, recipient and control birds were malaria-free prior to experimental infection and all negative controls remained uninfected throughout the course of the experiment.

Based on microscopic examination, the isolated parasite was attributed to the morphospecies *Plasmodium relictum*. One mitochondrial cyt *b* gene lineage SGS1 (478 bp, GenBank accession no. AF495571) of this morphospecies was present in our samples (Fig. 12). All experiments were done with this lineage.

The susceptibility of 6 passerine bird species to *P. relictum* was different. Infection developed in all experimental siskins, crossbills, chaffinches and greenfinches. The susceptibility of house sparrows to this parasite varied between individuals; the parasitemia developed in 3 of 6 infected birds. Parasites were not recorded in any experimental starlings neither by microscopy nor PCR.

Mortality was not recorded in any of the experimental or control birds during this study.

A key finding of the present study is that susceptibility of different bird species to the same lineage of *P. relictum* (SGS1) is markedly different. This parasite developed in all four species of birds belonging to the Fringillidae. The house sparrows were also susceptible. However, there was marked variation in susceptibility to the experimental infection in this bird on individual level. Additionally, light transient parasitemia developed in the susceptible individuals of the house sparrow. Starlings were resistant to the infection. It seems that common starlings have strong suppression abilities for *Plasmodium* parasites

because in the nature, species of *Plasmodium* have been rarely recorded in this bird species using both microscopy and PCR-based diagnostic methods (Bennett et al., 1982; Bishop and Bennett, 1992; Valkiūnas, 2005; S. Bensch, unpublished).

It is worth noting, that house sparrows and starlings are species, which markedly expand the range of their distribution during last century (Sakai et al., 2001). It is possible that resistance to avian malaria is one of the factors contributing to the global expansion of these bird species. According this study, the starlings seems to be innately resistant to the lineage SGS1 of *P. relictum*, so this bird species can be used as a model species for investigation of mechanism of such kind of resistance to avian malaria.

3.4.2. Prepatent period and dynamic of parasitemia of Plasmodium relictum (lineage SGS1) in experimentally infected birds

Prepatent period varied between 3 and 21 (mean 11.5) dpi in different bird species. The shortest prepatent periods were observed in common crossbills 3-12 dpi (mean 7), greenfinches 3-15 dpi (mean 8) and siskins 6-15 dpi (mean 9). The longest prepatent periods, which ranged between 9 and 19 (mean 14.3) and 12-21 (mean 16.5) dpi, were recorded in house sparrows and chaffinches, respectively.

Mean intensity of parasitemia varied markedly between bird species (Figs. 14, 23). Intensity of parasitemia was light in chaffinches, house sparrows and greenfinches (Figs. 14, a, c and 23, a). No obvious peaks of parasitemia were seen in house sparrows and chaffinches and low intensity maintained during the experimental time in these bird species. In these two species, the highest intensity of parasitemia (0.1%) was recorded in two chaffinches at 18 and 24 dpi and in one house sparrow (0.04%) at 24 dpi. In greenfinches two minor peaks of mean parasitemia were recorded on 12 dpi (0.8%) and 24 dpi (0.02%) (Fig. 23, a). The highest individual parasitemia (5%) was seen in one greenfinch at 12 dpi. In other greenfinches, parasitemia was between 0.02% and 0.5% (mean 0.1%) during both peaks of parasitemia.

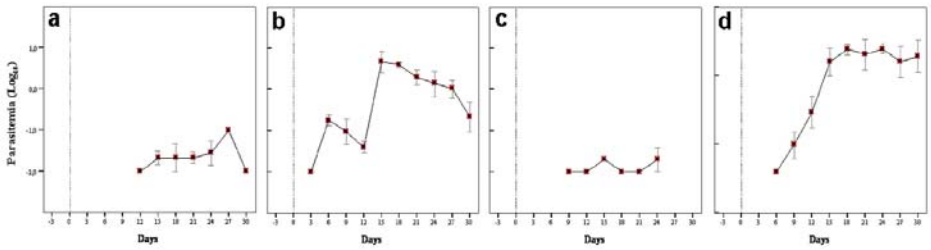

FIGURE 14. Dynamics of mean parasitemia (expressed in logarithmic scale) of *Plasmodium relictum* (lineage SGS1) in experimentally infected passerine birds: a - *Fringilla coelebs*; b - *Loxia curvirostra*; c - *Passer domesticus*; d - *Spinus spinus*. Vertical dotted line indicates the day of inoculation. Error bars show mean +/- 1.0 SE.

Mean parasitemia in crossbills was much higher then in before mentioned bird species with small individual variations (Fig. 14, b). The highest parasitemia (50%) developed in one common crossbill at 15 dpi. After that the parasitemia dropped in this individual bird. A second peak of parasitemia (12%) in this bird was recorded at 24 dpi. Intensity of parasitemia was lower (8-13%) in other common crossbills. Two peaks of parasitemia were seen in four experimental crossbills. The first one with mean intensity of 8.3% (range between 5 and 13%) was seen at 15-18 dpi, and the second one reaching 3.1% (range 1.3-6%) was recorded at 21-27 dpi. In two crossbills only one peak of parasitemia was recorded with maximum intensity 8% and 5% at 15 and 18 dpi, respectively.

Parasitemia in experimental siskins developed as high as in crossbills (Fig. 14, d, b). In these birds high parasitemia maintained till the end of experiment. Two peaks of parasitemia were seen in four siskins, with maximum intensity of both peaks approximately 18% (range 12-26%) at 15-21 dpi and at 24-30 dpi, respectively. In two siskins only one peak of parasitemia was recorded, with maximum parasitemia reaching 30% and 12% at 30 and 21 dpi.

Plasmodium relictum (SGS1) certainly is pathogenic, but reasons why it did not cause mortality during this study are unclear. Parasitemia was extremely

high in some bird species (Fig. 14, b, d). Using *P. gallinaceum* model, Paulman and McAllister (2005) have concluded that experimental infection of birds with malaria parasites by inoculation of blood lead to high mortality. This is certainly not the case for the lineage SGS1. In other experimental studies with avian malaria parasites, mortality varied depending both on parasite and host species. Zehtindjiev et al. (2008) reported several cases of death in great reed warblers infected with *P. relictum* (GRW4). However, because of simultaneous infection with *P. ashfordi*, it is difficult to rule out that the mortality was due to the mixed infection during their study. Heavy mortality occurred in experimental studies with different species of honeycreepers (Drepanidinae) on Hawaii islands. After sporozoite-induced infection of the lineage GRW4, mortality rate ranged between 63 and 65% in apapine *Himatione sanguinea* and Hawaii amakihi *Hemignathus virens*; it was up to 90% of iiwi *Vestiaria coccinea*, (Yorinks and Atkinson, 2000; Atkinson et al., 2000; Atkinson et al., 1995). Such high mortality could be explained by lack of co-adaptation to malaria parasites of Hawaiian endemic honeycreepers. Nevertheless, Hawaiian thrushes (*Myadestes obscurus*) appear to be tolerant to this lineage of *P. relictum* (Atkinson et al., 2001b).

Controversial results on survival of chickens were obtained during experiments with *P. (Haemamoeba) gallinaceum*. In some studies this parasite caused mortality of chickens (Permin and Juhl, 2002; Juhl and Permin, 2002; Williams, 2005), in others studies, birds survived but decrease of body mass was recorded (Paulman and McAllister, 2005). The different results on mortality obtained in the infected chickens could be explained by differences in bird age, the way of blood inoculation (Garnham, 1966; Williams, 2005), and probably different genetic lineages of *P. gallinaceum* (the latter issue remains unresolved). Moreover, factors that determine whether a bird will survive an infection or not might be related to individual genetics of the host and to its overall physiological condition during infection (Gustafsson et al., 1994; Sorci et al., 1997; Yorinks and Atkinson, 2000). It worth noting, that according to Paulman and McAllister (2005), route of inoculation appears to affect the ability

to recover from *P. gallinaceum*. On the other hand, the intensity and dynamics of parasitemia are not affected by the size of inoculum if chickens are infected by blood passage (Permin and Juhl, 2002). Mechanism of that remains unknown; that warrants further investigation.

The development of parasitemia in the majority of the infected birds was similar to a scheme, which is general for avian malaria parasites, i. e. prepatent period, first peak of parasitemia, crisis, second peak of parasitemia and chronic infection (Valkiūnas, 2005).

During the present study we obtained experimental information about the development of *P. relictum* (SGS1) in 2 groups of phylogenetically relatively distant fringillids. These were chaffinch, on one hand, and siskin, crossbill and greenfinch on the other hand. The obtained information provides an opportunity to compare the development of this parasite in different species of fringillid birds in relation to their phylogenies. In this study, the phylogenetic information of different fringillids was based on the Treplin et al.'s (2008) and Nguembock et al.'s (2009) analyses. According to our study, the development of the parasite is markedly different in different fringillids. Mean prepatent period of the infection in greenfinches (mean 8 dpi) overlapped with the prepatent period in the phylogenetically close crossbills and siskins (7 and 9 dpi, respectively) and it was significantly shorter than in the phylogenetically more distant chaffinch (16.5 dpi) and house sparrow (14.3 dpi) ($P < 0.05$). In Zehtindjiev et al.'s (2008) study with mixed infection with two species of *Plasmodium*, prepatent period of *P. relictum* (GRW4) (12-15 dpi) overlapped with our data in chaffinches and house sparrows. The mean of prepatent period in crossbills greenfinches and siskins (7 and 9 dpi) was similar to the results from the other studies, which were performed with *P. relictum* (GRW4) using sporozoite-induced infections in Hawaii islands (4-8 dpi) (Atkinson et al., 2000; Yorinks and Atkinson, 2000; Atkinson et al., 2001b). According to Permin and Juhl (2002), the prepatent period can vary depending on the size of inoculum. Mechanism of that remains largely unresolved in avian malaria parasites. It is also unclear if that is true in all species of avian *Plasmodium,* which markedly differ in their live history

strategies (Valkiūnas, 2005). Hence, mode of experimental infection should be standardized in future experiments so that different studies are more comparable.

It is worth noting that ability to infect birds does not depend on dose of inoculated parasites. Starlings were inoculated by the largest dose of erythrocytic meronts, but they were resistant. Meanwhile in siskins, which were infected with the lowest dose of mature meronts, parasitemia developed in all experimental birds, and it was one of the highest between infected species. These results are in accord to Permin and Juhl (2002), who concluded that dynamics of parasitemia does not depend on dose of erythrocytic meronts in the inoculated blood.

There were marked individual variations in dynamics of parasitemia during this study (Fig. 14). Nevertheless, within species, the experimental birds had similar dynamics of parasitemia. In some house sparrows greenfinches and chaffinches, parasitemia declined to undetectable levels after appearance of parasites in the blood. It seems that some birds of these species have greater immunological ability to control *P. relictum* (SGS1) infections. The appearance of secondary parasitemia in some chaffinches and greenfinches testifies for the weakening of immunity. Two peaks of parasitemia were seen in most of the crossbills and siskins. The parasitemia was more synchronized in crossbills and peaks appeared mostly in the same time, whereas siskins had broad range of peak appearance time with holding high mean parasitemia till the end of experiment (Fig. 14, b, d). It might be that two individuals of both species that had one peak of parasitemia, probably have stronger immune system and suppressed parasitemia. The second peak could appear later in these birds, like in Atkinson et al.'s (2000) study, where second peaks were recorded in 48 dpi. Interestingly, the intensity of parasitemia in greenfinches was similar to what was observed in the phylogenetically more distant chaffinch (Figs. 14, a and 23, a).

3.4.3. The effects of Plasmodium relictum (lineage SGS1) on body mass, temperature and haematocrit value of birds

There were no significant differences in body mass between experimental and control birds for any of host species (Fig. 15). Some differences in mean body mass between sampling days were recorded both within experimental and control groups. In chaffinches and starlings, mean body mass varied statistically significant between sampling days within both control and experimental groups without any relation to infection (Fig. 15, a, e). Body mass of crossbills and siskins did not differ from control birds at the peaks of parasitemia (Figs. 14, b, d and 15, b, d). There was significant decrease of body mass in experimental group of house sparrows in the beginning of experiment, but the same decrease was also recorded in control birds, so it hardly can be attributed to effect of the infection (Fig. 15, c).

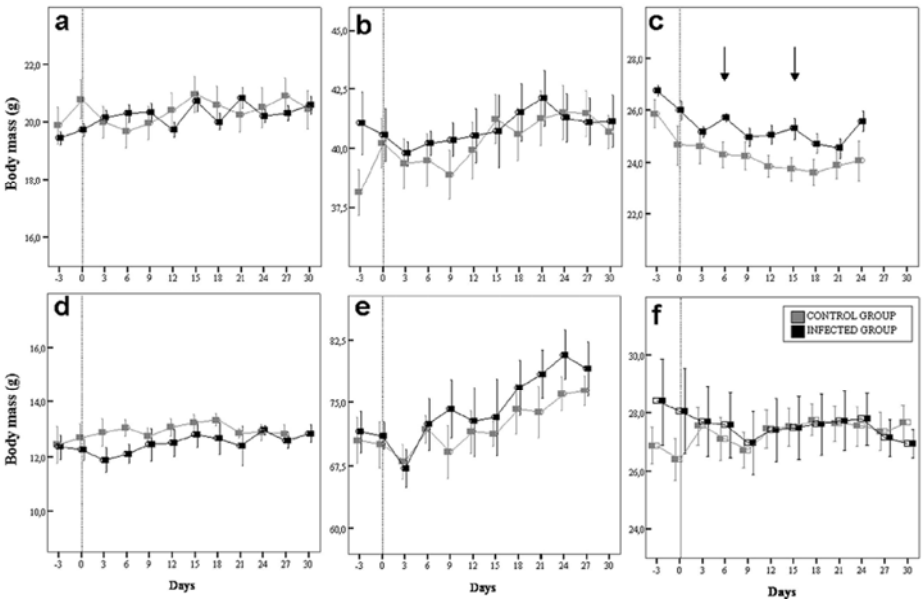

FIGURE 15. Mean body mass of experimentally infected with *Plasmodium relictum* (lineage SGS1) and control passerine birds: a - *Fringilla coelebs*; b - *Loxia curvirostra*; c - *Passer domesticus*; d - *Spinus spinus*, e - *Sturnus vulgaris*, f - *Carduelis chloris.* Vertical dotted line indicates the day of inoculation. The arrows indicate the significant differences ($P < 0.05$). Error bars show mean +/- 1.0 SE.

There were no significant differences in body temperature between the experimental and control birds for any of host species (Fig. 16); the temperature

was not measured during the experiments for greenfinches. Marked individual variation in the body temperature was seen both in experimental and control birds without significant changes of temperature in experimental birds during peaks of parasitemia (Figs. 14 and 16). In one crossbill with 50% parasitemia, we recorded a temperature of 43.0 °C at 15 dpi. In two other individuals with 12% and 13% parasitemias, temperatures of 42.4 and 43.8°C were recorded at 24 dpi. The mean temperature in the control group of crossbills during peak parasitemia of experimental birds were 42.7 (± 0.4) and 42.8°C (± 0.9), respectively. In three individual siskins with the highest parasitemias of 15%, 20% and 25%, temperatures of 41.3, 41.8, and 42.6°C were recorded at 15, 18 and 21 dpi, respectively. Mean temperature in control siskins were 42.3 (± 0.5), 42.1 (± 0.4), and 42.4 (± 0.5) at the same time.

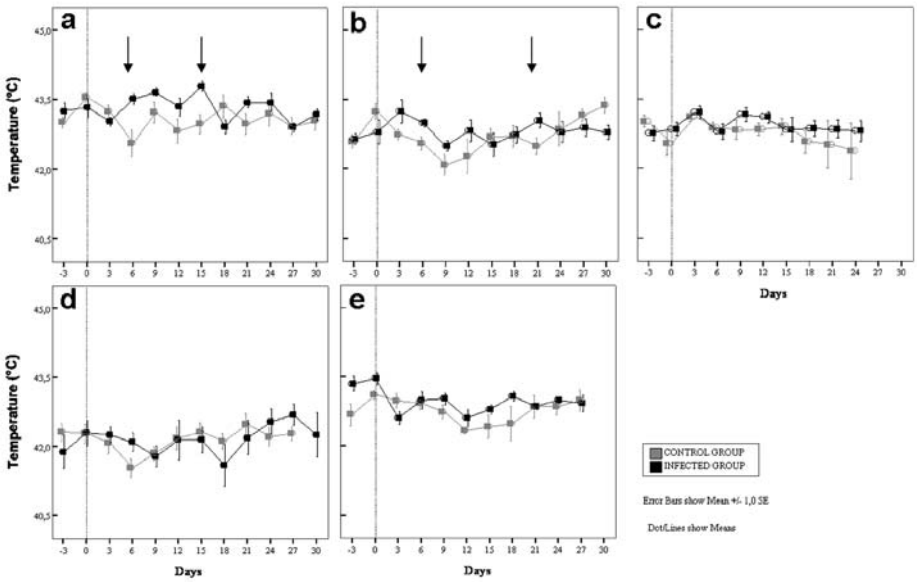

FIGURE 16. Mean body temperature of experimentally infected with *Plasmodium relictum* (lineage SGS1) and control passerine birds. Other symbols are as in Fig. 15.

Changes of temperature were recorded in all bird species within both experimental and control groups between sampling days. In chaffinches and

starlings, these differences were significant both in experimental and control groups ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 16, a, e). In house sparrows and siskins, temperature differed significantly between days of observation within experimental groups ($P < 0.001$ and $P < 0.05$, respectively) (Fig. 16, c, d). In crossbills, temperature changes were significant only in control group ($P < 0.01$) (Fig. 16, b).

Experimental infection significantly affected the haematocrit values in crossbills and siskins. The intensity of parasitemia was particularly high in these bird species (Fig. 17, b, d), and the value of haematocrit was significantly lower in experimental groups compared to controls ($P < 0.01$ for both bird species) (Fig. 17, b, d). According to pair wise comparisons, the first significant differences were seen at 15 dpi in both species. In these two bird species, significant differences were recorded within each experimental group between all sampling days after 15 dpi ($P < 0.001$ for both species).

There were no differences discernible in haematocrit value between experimental and control groups in house sparrows, starlings and greenfinches (Fig. 17, c, e, f) in statistical repeated measures analysis ($P > 0.05$). Significant differences were recorded in the haematocrit value within each experimental and control group of chaffinches during course of the experiment. There was slight decrease of haematocrit level in both these groups (Fig. 17, a). In pair-wise comparisons, the mean haematocrit value of infected greenfinches was significantly lower than in the control birds on 24 dpi ($P = 0.03$), which coincides with the time for the second parasitemia peak (Figs. 17, f and 23, a). Post infection, the mean haematocrit values were however lower in the infected birds than in the control birds at each of the ten times the groups were compared (Fig. 17, f). Using each day of comparison as an independent observation, this outcome is highly significant (sign test, $P < 0.001$) hence supporting the conclusion that malaria infections are negatively affecting the haematocrit values.

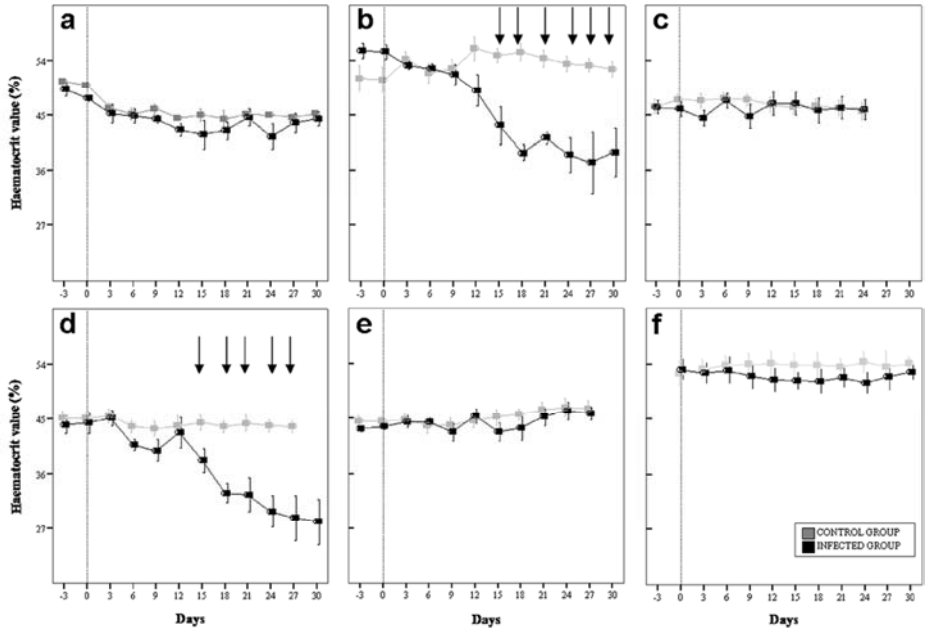

FIGURE 17. Mean haematocrit value in experimentally infected with *Plasmodium relictum* (lineage SGS1) and control passerine birds. Other symbols are as in Fig. 15.

No effect on body mass of malaria parasites were detected, even for the bird species with high parasitemia (Figs. 14, b, d and 15, b, d). Insensitivity of body mass change of birds to experimental infection can be explained by the beneficial experimental conditions with food offered *ad libitum*. It is probable that experiments in nature would demonstrate different results, mainly because of food shortage, impact of predators and other unfavourable conditions. We did not measure food consumption during this study, but infected siskins appeared to spend more time feeding in comparison to control ones. It is worth noting that when an experimenter entered the room with birds, experimental siskins kept on feeding, whereas control siskins and other bird species always took off on the roosts. In heavily infected crossbills, such behaviour as in experimental siskins was not observed. It seems that heavy parasitemia influences behaviour of the siskins, which warrants further investigation.

No differences were recorded in body mass of juveniles of infected and not infected great reed warbler by Zehtindjiev et al. (2008) either. In Atkinson et al.'s (2000) study amakihi birds infected with *P. relictum* (GRW4) were emaciated and had atrophy of pectoral muscles. These authors also recorded significantly decreased body mass of the infected birds in a later study with omao thrushes (Atkinson et al., 2001b). They speculated in the discussion that loss of body mass could have been due to stress associated with captivity (Atkinson et al., 2001b). This elucidation might be used to explain the body mass loss in experimental and control house sparrows (Fig. 15, c) during our study.

Hayworth et al. (1987) reported that *P. relictum* infection does not act as a pyrogenic agent. This study is in accord to our observations (Fig. 16). The large variation in body temperature of the birds makes it difficult to detect temperature changes using traditional methods. Seed and Manwell (1977) also stated that malaria infection does not influence body temperature of birds, except in pigeons. However, Williams (2005) revealed abnormal temperatures (the highest or the lowest) in some chickens infected with *P. gallinaceum* during a peak of parasitemia and before bird death. According to that study, a febrile response to malaria was weak and transient. Because of marked variation and effects of stress, it seems probable that changes of body temperature, unlike for human malaria, is not a good parameter to measure virulence of avian *Plasmodium* spp.

Development of *Plasmodium* spp. leads to marked loss of red blood cells and resulting anaemia. During heavy parasitemia, abnormal breakdown of red blood cells due to haemolysis have been well documented (Garnham, 1966; Springer, 1996; Permin and Juhl, 2002). One of the general causes of anaemia during bird haemosporidioses is the active removal of infected red blood cells from the blood circulation by the cells of the reticulo-endothelial system in the spleen, liver, bone marrow, and some other organs. Destruction of erythrocytes during the development of *Plasmodium* spp. is also associated with the development of numerous erythrocytic meronts and direct destruction of red

blood cells (Valkiūnas, 2005). The changes in the chemical composition of the blood plasma are observed during malaria, which enhances the effect of erythrocytes destruction (Seed and Manwell, 1977). Decrease of the plasma pH and increase of proteins concentration in the blood is observed during the increase of parasitemia. This leads to the decrease of the oxygen-binding capacity of haemoglobin and to the lessening of the effective circulation in the capillaries.

In recent studies on *P. relictum* in small passerine birds, haematocrit value of hosts has not been measured mainly because the amount of blood, which can be withdrawn from small passerines, is small for such measurements (Atkinson et al., 2000). During our experiment, negative controls remained healthy and showed relatively stable haematocrit levels (Fig. 17); it seems that multiple blood sampling had no effect on that. There were no differences between experimental and control groups in haematocrit level in experimental birds with low intensity of parasitemia and in not infected starlings (Figs. 14, a, c, and 17, a, c). However, the haematocrit value of experimental greenfinches decreased and maintained at a slightly lower level than in control birds during this study (Fig. 17, b). In contrast, the haematocrit values decreased markedly in the closely related siskins and crossbills when mean parasitemia increased to more than 10% (Figs. 14 b, d and 17, b, d). It seems that a decrease of haematocrit value during *P. relictum* (SGS1) malaria infections takes place mainly during heavy parasitemia and is associated with destruction of red blood cells and active removal of infected erythrocytes from circulation (Permin and Juhl, 2002; Williams, 2005).

The results on haematocrit value changes in crossbills and siskins are in accordance with *P. gallinaceum* studies in chickens (Permin and Juhl, 2002; Paulman and McAllister, 2005; Williams, 2005). During peak of parasitemia when haematocrit level decreases markedly birds should be most vulnerable, and juveniles with weak immune system should be less competitive and may die, particularly in wildlife.

3.4.4. Plasmodium relictum (lineage SGS1) effects on internal organs of birds

After a month of infection, the spleen and liver of experimentally infected crossbills were hypertrophied (Figs. 18 and 19).

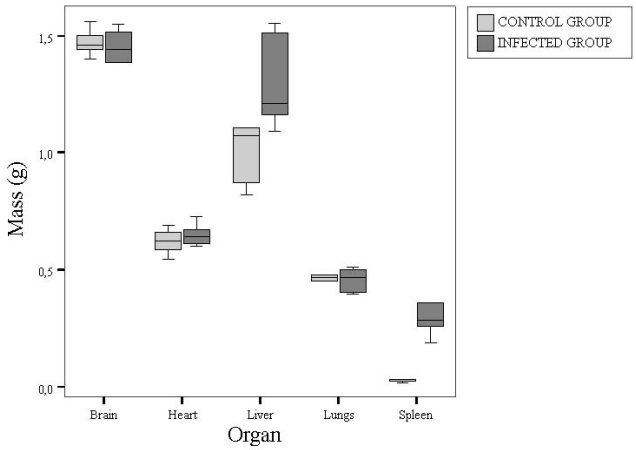

FIGURE 18. Effect of *Plasmodium relictum* (lineage SGS1) on mass of internal organs of experimentally infected and control common crossbills *Loxia curvirostra*. Measurements were taken from control and experimental birds 36 days post infection. In box plot graphic, boxes are represented with median lines and SE bars.

The mean mass of spleen and liver for infected birds was 11 and 1.2 times greater than of control birds. These organs were dark-brown or even black in colour in the experimental birds. There were no differences discernible in mass of other organs between experimental and control crossbills.

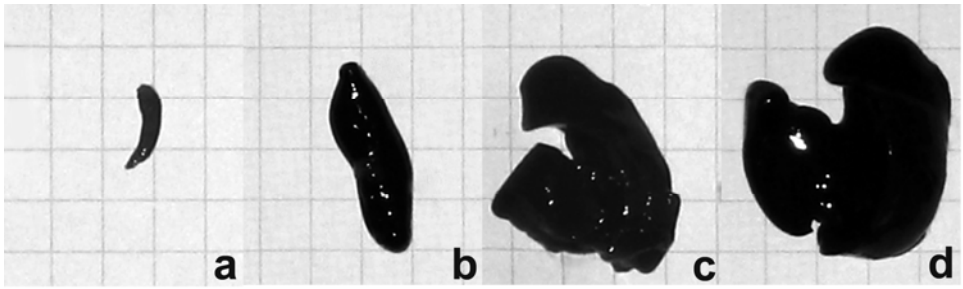

FIGURE 19. Spleen (a, b) and liver (c, d) of control (a, c) and experimentally infected (b, d) common crossbills. Scale square = 25 mm^2.

Damage of internal organs by exoerythrocytic meronts is an important factor leading to death of malaria-infected birds (Garnham, 1966; Valkiūnas, 2005). We did not examine internal organs of birds for tissue stages of malaria parasites during this study. According to earlier histological examinations, the majority of secondary exoerythrocytic meronts, which were induced by inoculation of erythrocytic merozoites of *P. relictum* into canaries, develop in brain; a few of them have been observed in liver, but not in other organs. However, there is no information about effects of particular lineages of *P. relictum* to internal organs of birds.

Hypertrophy of spleen and liver was observed during this study (Figs. 18 and 19). That is in line with results from other investigations on *P. relictum, P. gallinaceum* and other avian malaria parasites (Garnham, 1966; Atkinson et al., 2000; Yorinks and Atkinson, 2000; Permin and Juhl, 2002; Paulman and McAllister, 2005; Williams, 2005). The hypertrophy of these organs is associated with the hyperplasia of lymphoid-macrophage cells (Garnham, 1966; Seed and Manwell, 1977; Permin and Juhl, 2002). During heavy *Plasmodium* spp. infections a great amount of insoluble pigment is accumulated in the macrophages of the spleen and liver, and these organs obtain black hue. Consequences of these pathological changes for wild free-living birds remain unknown. Because susceptibility of different avian host to the same lineage of parasites is different, the pathology in their organs should be also different; that warrants further investigation.

Significant differences in virulence of the same malaria lineage for two bird species belonging to the genus *Carduelis* (siskin and greenfinch) and similarity of parasitemia development in siskins and crossbills (genus *Loxia*) were unexpected. It worth noting that our results about the virulence of *P. relictum* (SGS1) to different fringillid birds are in accord to phylogenetic study by Nguembock et al. (2009), according to which siskin is phylogenetically more closely related to crossbill than to greenfinch. The virulence of this parasite is high in the phylogenetically closely related siskin and crossbill and is lower in the more distantly related greenfinch and chaffinch. Mechanism of different

virulence of the same lineage in phylogenetically closely related hosts remains unclear. To answer this question, further experimental investigations are needed.

Providing beneficial living conditions for the birds in captivity, *P. relictum* (SGS1) did not cause mortality, but the anaemia and marked hypertrophy of spleen an liver hardly is neutral for birds in nature, particularly during energy consuming periods of time such as seasonal migration, breeding and unfavourable environmental conditions. For better understanding of malaria parasite – wild bird interactions, additional laboratory studies combined with field observations should be designed.

3.5. Development of simultaneous infection with *Plasmodium relictum* (lineage SGS1) and *P. ashfordi* (GRW2) in experimentally infected birds

Plasmodium relictum (lineage SGS1) and *P. ashfordi* (GRW2) are generalists and are cosmopolitan in distribution. The lineage SGS1 has been recorded in 29 bird species belonging to eight families of the Passeriformes in Eurasia and Africa (Waldenström et al., 2002; Beadell et al., 2006; Hellgren et al., 2007b; Palinauskas et al., 2007). *Plasmodium ashfordi* (GRW2) belongs to the subgenus *Novyella*, which unites the avian malaria parasites with small erythrocytic meronts and elongated gametocytes (Valkiūnas, 2005); this parasite has been recorded in 5 species of African long-distance migrants in Europe (great reed warbler *Acrocephalus arundinaceus*, melodious warbler *Hippolais polyglotta*, willow warbler *Phylloscopus trochilus*, garden warbler *Sylvia borin*) and in African resident bird species (lesser swamp-warbler *Acrocephalus gracilirostris*) (Bensch et al., 2000; Waldenström et al., 2002; Westerdahl et al., 2005; Hellgren, 2006; Reullier et al., 2006). The lineage GRW2 of *P. ashfordi* can be attributed to the group of lineages, which are transmitted only in Africa because this parasite has never been recorded in European resident birds or in juveniles of African migrants.

The effect of single infection of the *P. relictum* (SGS1) on 6 passeriform Palearctic bird species was investigated by Palinauskas et al. (2008, 2009). The

effect of this parasite on different hosts differed markedly. The severity of infection varied within birds of the Fringillidae even in phylogenetically closely related species. *Plasmodium ashfordi* (GRW2) have been reported in simultaneous infections with *P. relictum* (GRW4) by Zehtindjiev et al. (2007), but it remains unclear how they interacts and influence avian hosts during such infections.

3.5.1. Specificity of Plasmodium ashfordi (lineage GRW2) for experimentally infected birds

According both to PCR analysis and microscopic examination of blood films, haemosporidian parasites were absent in recipient and control birds prior to infection and all negative controls remained uninfected throughout the course of the experiment.

After microscopic examination, the isolated parasites were attributed to the morphospecies *Plasmodium relictum* and *P. ashfordi*. The mitochondrial cyt *b* gene lineages SGS1 (478 bp, GenBank accession no. AF495571) and GRW2 (478bp, GenBank accession no. AF254962) of mentioned morphospecies were present in our samples.

The susceptibility of birds belonging to three passeriform species to simultaneous infection with *P. relictum* (SGS1) and *P. ashfordi* (GRW2) was different. Both infections developed in all experimental siskins and crossbills. The morphological features of *P. ashfordi* were in accord to original description of this species by Valkiūnas et al. (2007). Moreover, in erythrocytes was recorded development of mature micro and macrogametocytes. These stages of parasites later develop in vectors. Starlings were resistant both to *P. relictum* and *P. ashfordi* parasites.

The control birds of all three species and experimentally infected siskins and starlings remained alive during this study. The mortality was recorded in experimental crossbills. Of 6 infected crossbills, 2 died after 30 and 33 dpi. The death occurred approximately 10 days after marked increase (> 50%) of

parasitemia. Last samples taken before death showed high intensities of parasitemia (> 90%) in both crossbills.

During this study, all infected siskins and crossbills were susceptible to *P. relictum* (SGS1); that is in accord to Palinauskas et al. (2008). As it was expected, *P. ashfordi* (GRW2) developed in all infected siskins and crossbills too. The development of *P. ashfordi* (GRW2) was recorded in the Northern Palearctic birds for the first time. This information is of epidemiological significance because mature gametocytes, which are stages infective for vectors, develop in infected birds. During cause of evolution, that could lead to establishment of complete the life cycle in mosquito vectors and then establishment of transmission. It is remains unclear at what stage development of this parasite is blocked in European species of mosquitoes; that needs further investigation.

As in previous study by Palinauskas et al. (2008), starlings were resistant to *P. relictum* (SGS1). Moreover, development of *P. ashfordi* (GRW2) was not recorded in these birds either. It seems that this successful invasive bird species has innate resistance for these malaria parasites; that might contribute to the relatively resent expansion of this bird species in the New World (Sakai et al., 2001). Resistance to malaria is an evolutionary advantage. It worth mentioning that starlings are naturally infected by numerous species of helminths and coccidians, but these parasites are less virulent than species of *Plasmodium* (Hair and Forrester, 1970; Mazgajski and Kędra, 1998; Sakai et al., 2001).

3.5.2. Prepatent period and the dynamics of parasitemia of Plasmodium relictum (lineage SGS1) and P. ashfordi (GRW2) during simultaneous infection in experimentally infected birds

According to microscopic examination, prepatent period of *P. relictum* (SGS1) varied between 3 and 6 dpi both in siskins and crossbills with mean prepatent period of 4.5 and 4, respectively. *Plasmodium ashfordi* (GRW2) appeared in the blood stream significantly later than *P. relictum*, 9 to 27 dpi

(mean 14) in siskins and 18 to 30 dpi (mean 24) in crossbills ($P < 0.01$ for both bird species in comparison to *P. relictum*).

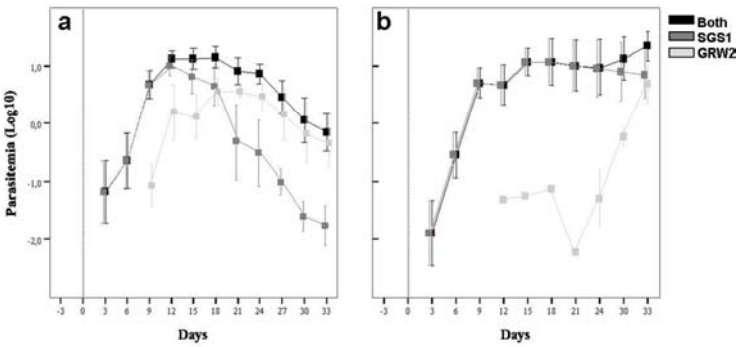

FIGURE 20. Dynamics of total mean parasitemia (expressed in logarithmic scale) of simultaneous infection *Plasmodium relictum* (lineage SGS1) and *P. ashfordi* (GRW2) in experimentally infected birds: a - *Spinus spinus*; b - *Loxia curvirostra*. Vertical dotted line indicates the day of inoculation. Error bars show mean +/- 1.0 SE.

Parasitemia was recorded in all infected siskins and crossbills throughout entire observation time (Fig. 20). The parasitemia of two species appeared to develop in parallel. The mean intensity of parasitemia of two *Plasmodium* species varied between bird species and individuals of the same species. The development of these malaria parasites differed significantly in infected siskins. Occurrence of *P. relictum* in blood of all infected siskins was followed by occurrence of *P. ashfordi* (Fig. 20, a). The peak of parasitemia of *P. relictum* was recorded in all siskins between 6 and 21 dpi; in mean, parasitemia was 23.6% during the peak. The highest parasitemia (58%) developed in one siskin at 18 dpi; after that the parasitemia of *P. relictum* dropped in five infected siskins and maintained at low level throughout the experimental time.

The time of decrease of parasitemia of *P. relictum* overlapped with increase of parasitemia of *P. ashfordi.* Once the lineage GRW2 of *P. ashfordi* reached a peak of parasitemia, it was present consistently at higher level than *P. relictum* (SGS1) (Fig. 20, a) in 5 siskins until the end of experiments. In one siskin, unlike other 5 birds, slight increase of *P. ashfordi* (GRW2) parasitemia

was followed by increase of *P. relictum* (SGS1) parasitemia. In this bird, parasitemia of *P. ashfordi* was seen at low level until the end of observation time. The highest intensity of *P. ashfordi* (GRW2) parasitemia (up to 20%) was recorded in two siskins at 18 and 27 dpi, when the parasitemia peak (mean 10.3%) occurred in all birds between 18 and 33 dpi. There were significant differences in the time of appearance of peaks of parasitemia of these two parasites both in siskins and crossbills (both $P < 0.01$).

Parasitemia in crossbills was more variable between individuals. Intensity of parasitemia of *P. ashfordi* reached the highest peaks (5, 18 and 23%) in three crossbills. Later, after slight decrease of parasitemia, *P. ashfordi* (GRW2) was present in higher intensity comparing to *P. relictum* (SGS1) until the end of experiments; that also was the case with most of siskins (Fig. 20, a). In other three crossbills, *P. ashfordi* occurred between 24 and 30 dpi and reached intensity of parasitemia of only < 0.001%, while parasitemia of *P. relictum* increased up to 76% and even more than 90% in two birds. These two crossbills died during acute parasitemia (> 90%) and one bird (with intensity 76% of parasitemia at its peak) survived with following decrease of parasitemia. In the majority of siskins and crossbills, only one peak of parasitemia was recorded, with an exception in one crossbill, with two peaks of *P. relictum* (maximum intensity was of 30% and 90%) at 9 and 33dpi, respectively, and in one siskin, with *P. ashfordi* (maximum intensity was 19% and 20%) at 18 and 33 dpi, respectively.

In comparison to single infection of *P. relictum* (SGS1) (Palinauskas et al., 2008), the prepatent period of the same parasite during simultaneous infection was significantly shorter both in siskins ($P = 0.02$) and crossbills ($P = 0.05$) (Fig. 20). That probably can be explained by stronger suppression of the immune system of host during simultaneous infection; however mechanisms of this phenomenon remain unresolved. Mean prepatent period of *P. ashfordi* (GRW2) was 14 and 24 dpi in siskins and crossbills, respectively. That was in accord to Zehtindjiev et al.'s (2008) study, in which mean prepatent period of *P.*

ashfordi (GRW2) during simultaneous infection was 25 dpi in the experimentally infected juvenile great reed warblers. Such long prepatent period is rather characteristic for many so far investigated malaria parasites of the subgenus *Novyella* (Garnham, 1966; Valkiūnas, 2005).

Mean parasitemia of simultaneous infection tends to be higher than single infection (see Figs. 14 and 20). In both siskins and crossbills, maximum mean parasitemias were also higher during simultaneous infections, 29 % and 53%, than single infections, 18% and 15%, respectively. It seems that synergetic effect due to simultaneous development of two avian malaria species is present. The dynamics of parasitemia of *P. relictum* (SGS1) during simultaneous infection with *P. ashfordi* (GRW2) was different from the development of the same parasite lineage during single infection (Valkiūnas, 2005; Palinauskas et al., 2008). Only one peak of parasitemia of *P. relictum* (SGS1) was recorded in infected siskins during this study; that was not the case during single infections of the same lineage in the same host when clearly expressed first peak of parasitemia, crisis and second peak of parasitemia have been recorded in the majority of infected siskins (Palinauskas et al., 2008). During this study, the decrease of parasitemia of *P. relictum* (SGS1) overlapped with increase of parasitemia of *P. ashfordi* (GRW2) (Fig. 20, a). In siskins, intensity of parasitemia of *P. relictum* (SGS1) remained low (mean 0.1%) after the crisis to the end of the experiment (Fig. 20, a). Parasitemia of *P. ashfordi* (GRW2) after peak of parasitemia also slightly decreased, but mean parasitemia of this parasite maintained; it was 3.7% on day 33 dpi (Fig. 20, a).

There were marked individual variations in dynamics of *P. relictum* (SGS1) parasitemia in infected crossbills. Of 3 individuals with extremely high intensity of *P. relictum* (SGS1) parasitemia, 2 birds died. Interestingly that parasitemia of SGS1 in both birds appeared early (3 and 6 dpi); it was increasing until it reached > 90% at 30 and 33 dpi. *Plasmodium ashfordi* (GRW2) parasitemia was recorded between 24 and 30 dpi in these heavily infected birds. It was at low level (< 0.01%) when two of these birds died. Mortality due to malaria parasites were observed in experimental studies with *P.*

relictum (GRW4) in endemic Hawaiian birds (Yorinks and Atkinson, 2000; Atkinson et al., 2000), and also in some studies with *P. gallinaceum* in chickens (Permin and Juhl, 2002; Williams, 2005). All these experimental studies were designed using single infections. It is probable that simultaneous infection was the main reason of mortality in our study. According to Palinauskas et al. (2008) *P. relictum* (SGS1) does not cause death of experimentally infected crossbills. In this case, probably due to synergetic relations between two parasites, high SGS1 parasitemia developed, which likely was the main reason of death of 2 infected birds. The mortality from double infection with *P. ashfordi* (GRW2) and *P. relictum* another lineage GRW4 was recorded in Zehtindjiev et al.'s (2008) study. Unlike to our study, parasitemia of *P. ashfordi* (GRW2) reached up to 85% which in the Zehtindjiev et al.'s (2008) study, so probably caused mortality. In our study, intensity of *P. ashfordi* (GRW2) was relatively low, so the recorded mortality hardly can be attributed to the direct effect of this infection.

Factors that determine predominant parasitemia of certain species during simultaneous infections in different hosts are unknown. Survival of a host after infection might be determined by individual genetics and physiological conditions during experiment (Sorci et al., 1997; Yorinks and Atkinson, 2000). During this study, the dynamic of parasitemia of *P. relictum* (SGS1) and *P. ashfordi* (GRW2) appeared to be negatively correlated in siskins, when increase of intensity of one malaria parasite overlapped with decrease of another (Fig. 20, a). It remains unclear if 1) strategies of development of two parasites are determined by their genetics and 2) they could be changed during simultaneous infection of another lineage (Read et al., 2002). Based on comparison with single *P. relictum* (SGS1) infection (see Palinauskas et al., 2008), it seems probable that SGS1 strategy did change during simultaneous infection; that manifests in longer prepatent period and changes of dynamics of parasitemia (two peaks of parasitemia instead of one peak); mechanisms of this phenomenon warrant further investigation.

3.5.3. The effects of Plasmodium relictum (lineage SGS1) and P. ashfordi (GRW2) simultaneous infection on body mass and haematocrit value of birds

There were no overall significant difference in body mass between experimental and control birds in any of investigated host species (Fig. 21). Individual body mass variation was seen both in experimental and control birds without significant changes between these groups. In pair wise comparison, significant difference in body mass between control and experimental siskins was recorded 3 and 9 dpi. Interestingly, infected siskins gained more weight ($P < 0.05$) in the beginning of infection in comparison to controls (Fig. 21, b). In two crossbills with 90% parasitemias, we recorded significant decrees of body mass, which was 46 g and 45 g before experimental infection and dropped down to 34 g and 33 g on 30 and 33 dpi, respectively. In heavily infected siskins (parasitemia 45 and 58%) decree of body mass was recorded from 14.3 g and 14.1 g to 12.8 g and 13.7 g, respectively. Some significant differences ($P < 0.05$) in mean body mass between sampling days were recorded both within experimental and control groups, so likely without relation to infection (Figs. 20 and 21).

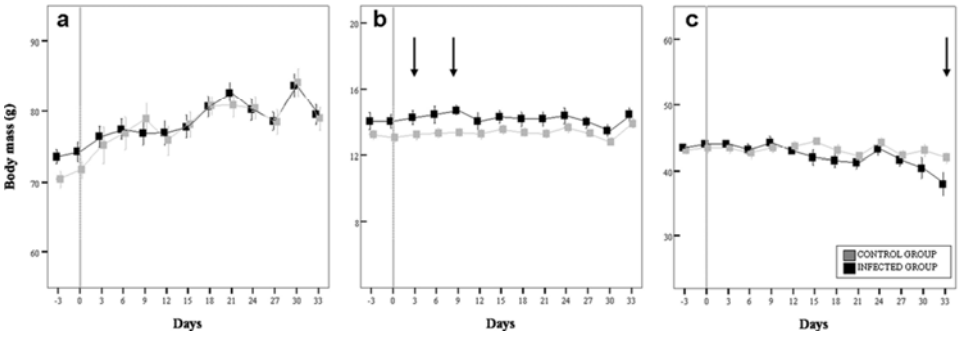

FIGURE 21. Mean body mass of experimentally infected and control passerine birds: a - *Sturnus vulgaris;* b - *Spinus spinus* ; c - *Loxia curvirostra*. Vertical dotted line indicates the day of inoculation. The arrows indicate the significant differences ($P < 0.05$). Error bars show mean +/- 1.0 SE.

Experimental infections significantly affected the haematocrit value in crossbills and siskins. The intensity of parasitemia was particularly high in

infected siskins and crossbills (Fig. 20, a, b), and the value of haematocrit was significantly lower in experimental groups compared to controls ($P < 0.01$ for both bird species) (Fig. 22, a, b). According to pair wise comparisons, the first significant differences in the haematocrit values between experimental and control groups were seen at 12 dpi in siskins and at 3 dpi in crossbills, respectively. Change of haematocrit value was recorded in siskins and crossbills also within each experimental group between sampling days ($P < 0.001$ for both species) (Fig. 22, a, b). That was not the case within control groups of these two bird species ($P > 0.05$) (Fig. 22, a, b). There were no differences discernible in haematocrit value between experimental and control groups in starlings (Fig. 22, c). In pair wise comparison of haematocrit value, the differences between control and experimental groups of starlings were statistically insignificant during this study ($P > 0.1$).

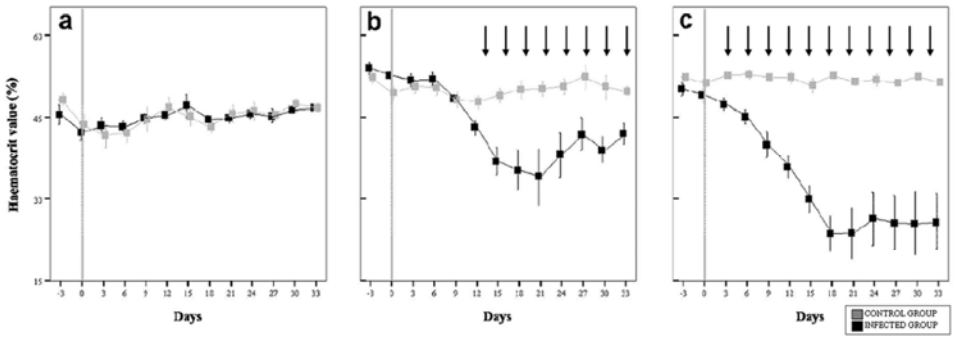

FIGURE 22. Mean haematocrit value in experimentally infected and control passerine birds. Other symbols are as in Fig. 21.

Obtained results about no visible influence of simultaneous malaria infections on body mass of birds (Fig. 21) are in accord to Palinauskas et al.'s (2008) study with single infection of *P. relictum* (SGS1); in that study the changes in body mass were not recorded in experimental groups even during heavy parasitemia. That can be explained by beneficial experimental conditions, absence of predators, and offering food *ad libitum*. It should be taken in consideration that the cost of infections in captivity, when birds are kept in

cages and food is easily available, is milder than in the wild (Valkiūnas, 2005; Møller and Nielsen, 2007). However, the significant decrease of body mass of two highly infected crossbills illustrates effect on body mass (Fig. 21, c). That is in accord to Atkinson et al's (2000) study, where the effect on body mass can be observed in extreme situations just before birds die.

Value of haematocrit markedly depends on the intensity of parasitemia (see Figs. 20 and 22). In siskins low value of haematocrit both in single infection of *P. relictum* (SGS1) and in simultaneous infection with *P. ashfordi* (GRW2) was always recorded during high parasitemias (see Figs. 14, 20). The same patterns, when increase of parasitemia was followed by decrease of haematocrit value, are recorded in the study by Palinauskas et al. (2008) and in present experiment. In both studies, the value of haematocrit decreased significantly when parasitemia increased more than 10%. Two infected crossbills died after intensity of parasitemia reached > 90%. At that time, values of haematocrit were 9.4% and 11.1% in these individuals; the same parameters in control birds varied between 49.5% and 53% (mean 51.4%). Five times lower haematocrit value, anaemia and anoxia can be defined as main reasons of death. In these two crossbills, peripheral blood colour changed from normal dark red to pink; that can be easily seen by visual observation. As in Palinauskas et al.'s (2008) study with single SGS1 infection, the birds with high simultaneous parasitemia (> 10%) were with ruffled feathers and showed apathy. Such behaviour together with food shortage, influence of predators and other unfavourable conditions could be determinant factor of death and natural selection in wildlife. It seems probable that heavily infected birds would not survive even with lower parasitemias in the wildlife; that corresponds to available field observations (Valkiūnas, 2005).

Poor body condition of experimental siskins and crossbills as well as mortality among crossbills indicate that simultaneous infections of avian malaria parasites are virulent; that also points out importance of primary infections of malaria parasites to their avian host. Our results show that simultaneous infections act synergistically on hosts, but at the same time such

infections are correlated negatively (Fig. 20). It is known that investigated avian malaria parasites of the subgenera *Haemamoeba* and *Novyella* are characterised by different prepatent periods and dynamics of parasitemia (Valkiūnas, 2005); although detailed development in avian hosts are still unknown for many species of these parasites. Interestingly, prepatent period and dynamic of parasitemia of *P. relictum* (SGS1) during simultaneous infection was shifted in siskins in comparison to single infection (see Palinauskas et al., 2008). It remains unknown how much such shifting is related to host immune system and how much determined and stable are these patterns of development of avian malaria parasites. To understand some of these patterns of development during simultaneous infection, additional experimental information on the parasites' life-history traits, genetics and immunology should be provided.

In conclusion, patterns of development of the same lineages of avian malaria parasites are markedly different in different avian hosts, even phylogeneticlly closely related (Palinauskas et al., 2008, 2009), so it is hardly possible to generalise the effect of all avian malaria parasites to their hosts, as frequently is a case in the current evolutionary studies, particularly studies, which are based solely of material collected from wildlife. We thus encourage experimental investigation of markedly diverse avian malaria parasites on level of host populations and genetic lineages of the parasites. For better understanding evolutionary biology and patterns of virulence of avian malaria, additional experimental studies combined with field observations are needed.

3.6. Efficacy of the antimalarial drug Malarone™ and a combination of Malarone™ and primaquine for treatment of avian malaria

Light parasitemia of *Plasmodium relictum* (lineage SGS1) was present in all experimental greenfinches (mean intensity 0.005%) and chaffinches (0.04%) before treatment with Malarone™ (Fig. 23). On day 6 following the treatment, parasitemia was absent from all infected greenfinches and chaffinches, as determined both by microscopic and PCR-based methods. Parasites were not recorded in any of the birds during the remaining treatment period (Fig. 23).

However, parasites were seen in all infected chaffinches 43 days after last administration of the drug, with mean intensity of parasitemia of 0.26% (Fig. 23).

No negative effects of Malarone™ on birds were recorded, as determined by visual observation of infected and control groups.

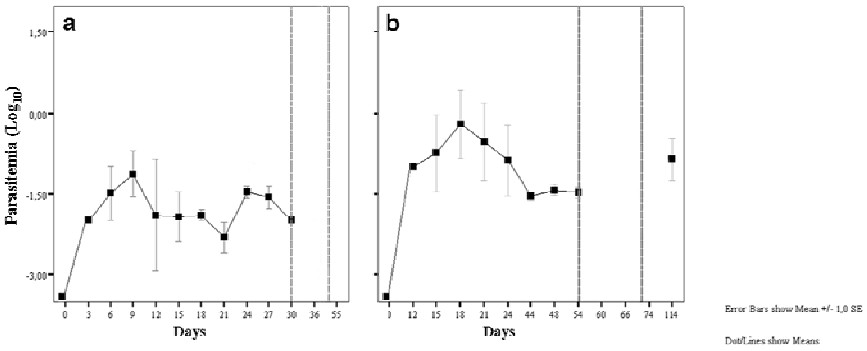

FIGURE 23. Dynamics of mean parasitemia (expressed in logarithmic scale) of *Plasmodium relictum* (lineage SGS1) in experimentally infected greenfinches *Carduelis chloris* (a) and chaffinches *Fringilla coelebs* (b). Vertical dotted lines indicate a period between the beginning and the end of treatment with Malarone™.

The combination of Malarone™ and primaquine was used to treat experimental malaria infection in 6 siskins. Light parasitemia (mean intensity 2%) of both *P. relictum* (SGS1) and *P. ashfordi* (GRW2) was present in all experimentally infected birds before the treatment (Fig. 24). According to microscopy and PCR-based results, parasitemia was absent in 4 of 6 infected birds 3 days after treatment with Malarone™. Thus, this drug is effective against this simultaneous infection, but 3-day treatment is not enough to cure parasitemia of these parasites in all birds. During the following treatment with primaquine (this drug was given for 14 days after the final administration of Malarone™), parasitemia appeared in 2 more infected birds (mean intensity < 0.01%). Thirty-one days after the post-treatment period, parasitemia (mean 2.9%) was recorded in all treated birds. In other words, simultaneous infection

relapsed in all experimental birds, so the treatment with Malarone™ and primaquine, at least in used doses, is not effective against simultaneous infection of *P. relictum* (SGS1) and *P. ashfordi* (GRW2). Visible parasitemia (mean 0.5%) in siskins remained till the end of post treatment observation, i. e. 291dpi (Fig. 24).

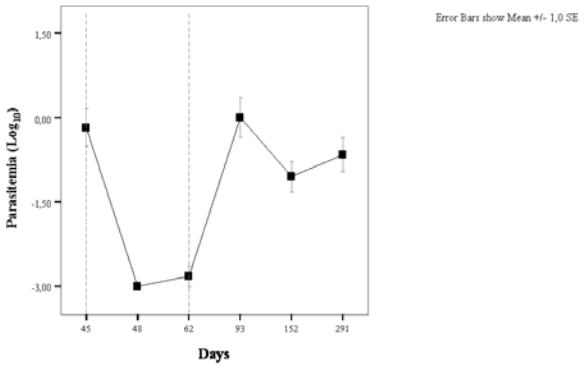

Figure 24. Dynamics of mean parasitemia (expressed in logarithmic scale) of *Plasmodium relictum* (lineage SGS1) and *P. ashfordi* (GRW2) simultaneous infection in experimentally infected siskins *Spinus spinus*. Vertical dotted lines indicate a period between the beginning and the end of treatment with Malarone™ and primaquine.

These experimental studies show that Malarone™ is efficient for clearing *P. relictum* (SGS1) and *P. ashfordi* (GRW2) blood stages. First experimental treatment indicates that, using this drug, the parasitemia clearance time is within 6 days. That is in accord to studies on *P. falciparum* and *Plasmodium vivax* infections in humans where this time was 3-6 days and 7 days, respectively (Looareesuwan et al., 1999a). The relapses of *P. relictum* and *P. ashfordi* infections in four chaffinches and 6 siskins during these two experimental studies show that tissue (exoerythrocytic) stages of these malaria parasites were not affected by Malarone™. The secondary exoerythrocytic meronts (phanerozoites), which are responsible for relapses of *P. relictum* infection, persist in endothelial cells of capillaries in different organs of birds (Garnham, 1966; Valkiūnas, 2005). According to present studies, they are not affected by the Malarone™ treatment; that leads to relapse when Malarone™ concentration

decreases. Actually, this is not unexpected because the Malarone™ treatment does not affect tissue stages (hypnozoites) of *P. vivax* in humans (Looareesuwan et al., 1999a).

During experimental treatment of siskins, two birds were not cured and malaria parasites were observed in the end of treatment time (after 3 days) with Malarone™. It seems that protocol that was used for malaria treatment in chaffinches and greenfinches (19 days of treatment) is more effective. Although less effect of Malarone™ in siskins can be due to more severe simultaneous infection; that needs further investigation. However, it is clear that primaquine, unlike in Looareesuwan et al's (1999b) study with *P. vivax*, did not affect exoerythrocytic stages of *P. relictum* (SGS1) and *P. ashfordi* (GRW2). Malaria parasites appeared in two infected siskins within 14 days during treatment with primaquine (Fig. 24). Low effect of primaquine on avian malaria infection also could be explained by primaquine doses, which were used during this study; that needs further investigation.

It should be noted that treatment of avian malaria is poorly developed, and effects of the majority of antimalarial compounds on avian *Plasmodium* spp. erythrocytic and exoerythrocytic stages have been insufficiently investigated. Frevert et al. (2007) reported that treatment by quinine and chloroquine prevented development both of the blood and tissue stages of *Plasmodium gallinaceum* in White Leghorn chickens. Other 8-aminoquinolines look promising for further investigations of avian malaria treatment because drugs of this group have an action on early tissue stages of human malaria parasites (Davey, 1951; Looareesuwan et al., 1987). It is possible that combination of Malarone™ and 8-aminoquinolines could be effective for treatment against both avian malaria blood and tissue stages; that warrants further investigation in controlled experiments. Moreover, new antimalarial drugs, such as cyclosporin and artemisin, should be tested for bird malaria treatment.

In conclusion, Malarone™ can be used for treatment of primary acute parasitemia of avian malaria, which is known to be the most dangerous and even

lethal stage of malaria infection in some bird species (Garnham, 1966; Valkiūnas, 2005). Treatment against tissue stages of avian malaria needs to be developed.

3.7. Geographic distribution of *Plasmodium relictum* lineages in the house sparrow *Passer domesticus* in Europe

House sparrow and its most prevalent avian malaria parasite *P. relictum* were used in analysis of haemosporidian parasites distribution. House sparrow is a sociable bird belonging to the Passeridae (Cramp and Perrins, 1994). Being sedentary, this bird spread, with human assistance, on almost all continents and ocean islands during last two centuries (Summers-Smith, 1988). Investigation of prevalence and occurrence of infection in this sedentary bird is useful because reflects transmission of haemosporidian parasites at each study site (Valkiūnas et al., 2006b).

In all, 801 house sparrows were sampled in Bulgaria (36 birds), France (231), Italy (48), Norway (123), Romania (104), Russia (Curonian Spit and Saratov, 48), Spain (191) and Sweden (20) (Fig. 3) during this study. After molecular investigation of blood samples, 17 mitochondrial cytochrome *b* gene lineages were recorded (Fig. 25). Twelve of these were parasites of the genus *Plasmodium* and 5 belonged to the genus *Haemoproteus*. Five lineages of malaria parasites (SGS1, GRW11, PADOM08, PADOM20 and PADOM21) belonged to the morphospecies *Plasmodium relictum* (Fig. 25). Three lineages (COLL1, PADOM01 and PADOM02) clustered together; their genetic distances differ less than 4% from lineages clustered with *P. relictum* (Fig. 25). To ascertain whether these three lineages certainly belong to the morphospecies *P. relictum*, morphological identification of their blood stages has to be done.

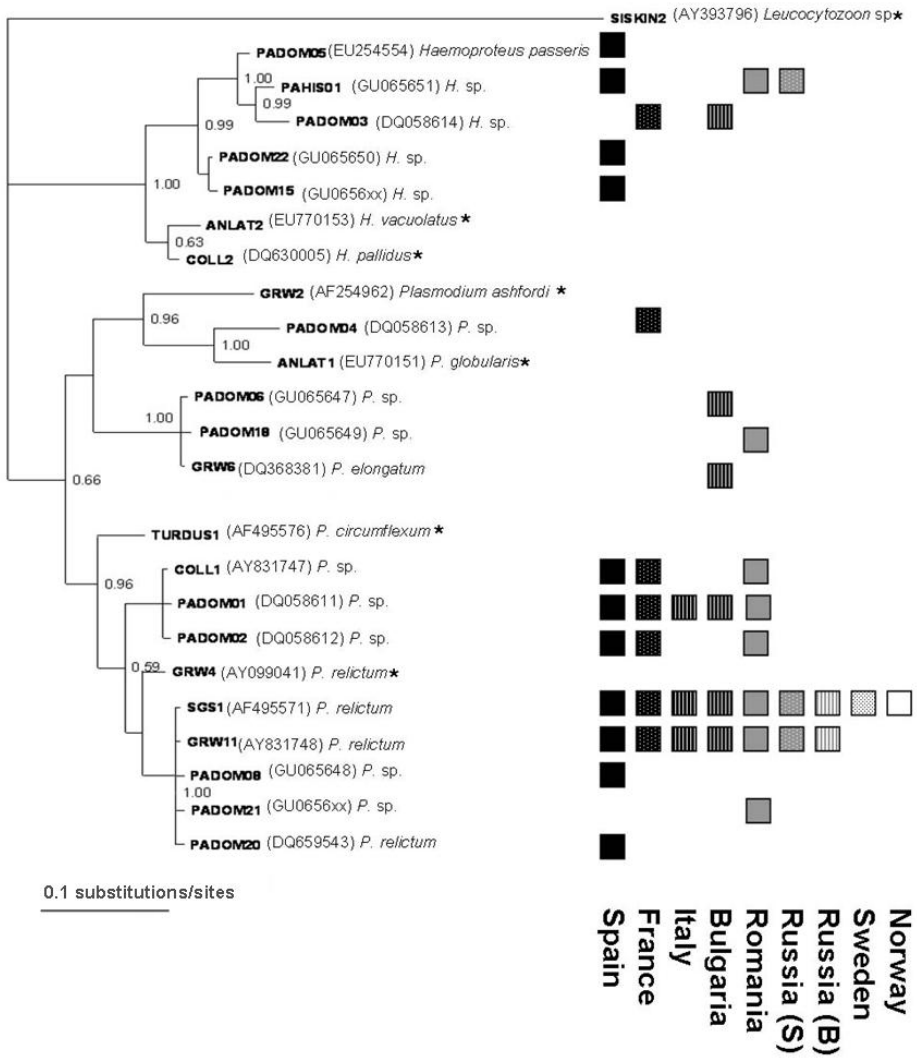

FIGURE 25. Occurrence of different haemosporidian mitochondrial cytochrome *b* gene lineages in house sparrows *Passer domesticus* in Europe. Bayesian phylogeny of 16 *Plasmodium* spp. and 7 *Haemoproteus* spp. lineages. The Bayesian consensus tree was constructed by sampling every 100th generation over 1 million generations and visualised in Tree View 1.6.6. after discarding 25% of the trees as burn in period. *Leucocytozoon* spp. (SISKIN2) was used as out group. Nodal support values indicate posterior clade probabilities. GenBank accession numbers are provided in the brackets.
*- Gene lineages used from MalAvi data base (Bensch et al., 2009).

Malaria parasites require relatively high temperature for development in mosquito vectors, so it seems probable that the faunae of avian haemosporidians

including *Plasmodium* spp. parasites in the northern Europe developed after last glacial period (Valkiūnas, 2005). However, the origins of certain lineages of avian malaria parasites and mechanisms that determine expansion of some lineages of the parasites to northern latitudes and the Baltic region have been insufficiently investigated. This problem is not only of theoretical interest, but also has practical significance. Establishment of transmission of new diseases in new regions and hosts can lead to shift of virulence, changes of epidemiological situation and new epidemics leading to disappearance of some bird species (Atkinson et al., 2000, 2001a; Kilpatrick, 2006), so warrants precise investigation.

After molecular investigation of blood samples from the house sparrow from the Curonian Spit (Russia) and other districts of Europe, it was recorded that diversity of malaria parasites differs between regions of the southern (6-10 lineages recorded) and northern Europe (1-2 lineages) (Fig. 25). It seems probable that evolution of *P. relictum* lineages is related to the southern Europe. Interestingly, only the SGS1 lineage of *P. relictum* is transmitted in all investigated districts up to the North Polar Circle (69° 45′N, 30° 00′E). Transmission of this malaria parasite was recorded for the first time so far to the north during this study. Based on these data we can assume that *P. relictum* (SGS1) have expanded to the northern Europe together with migrant birds from southern refuges after the last glacial period. That should be a time-consuming event because, before transmission is established, parasites had to adapt to local mosquitoes and to develop ability to complete sporogony at unfavourable temperature conditions in northern regions. Our study shows that mitochondrial cytochrome *b* gene markers are insufficient to answer the question about exact refuges (eastern or western), from which *P. relictum* (SGS1) spread from to the northern Europe. Precise phylogeographic studies require knowledge of genetic variation within studied species (Avise, 2004) using additional markedly variable markers.

Another lineage of *P. relictum*, which is common in the house sparrow, is GRW11 (Fig. 25). The transmission of this lineage was recorded in 5 districts

from South Europe up to the Curonian Spit in the Baltic Sea. This parasite was not recorded in house sparrows or juveniles of other bird species in Sweden so far (Waldenström et al., 2002; Hellgren et al., 2007b). According to available data, it is possible that a northern area of transmission of this lineage is eastern cost of the Baltic Sea on the Curonian Spit. Because this lineage have not been recorded in Romania and Russia (Saratov), it is possible that this lineage arrived to the Baltic region with birds from western refuges of Europe; that needs more detailed investigation. Three lineages of *P. relictum* were recorded either in western (PADOM08 and PADOM20 in Spain) or eastern (PADOM21 in Romania) parts of South Europe.

In conclusion, it seems probable that active expansion of avian malaria parasites takes place from South Europe towards North Europe after last glacial period. The certain time of establishment of the successful transmission of avian malaria in the northern Europe remains unclear. *Plasmodium relictum* (SGS1) is particularly successful during this expansion, so is a convenient model organism to study mechanisms of adaptation for such expansion. It is possible that already adapted in the Baltic region parasites (lineage GRW11) continue to spread further to the north. At the same time, new southern origin malaria parasites are arriving with migrant birds both from the western and eastern South Europe to the Baltic region. Further expansion of new avian malaria parasites can be important for appearance of new diseases in resident birds and for changing epidemiological situation in the future. The mechanisms contributing to spreading of malaria parasites lineage to new European ecosystems need clarification. For phylogeographic studies, more precise investigations are needed using vector studies and more informative microsatellite markers and nuclear markers. In the Baltic region, *P. relictum* lineages SGS1 and GRW11 are convenient model organisms for such phylogeography studies.

4. GENERALIZATION

Both microscopic examination of blood films and nested PCR-based diagnostics show similar and consistent estimates of prevalence for malaria and related blood parasites among naturally infected birds. In microscopic analyses it is essential that blood films are of good quality and that they are examined by skilled investigators. This tool is especially important during simultaneous infections, when more than one species of the same genus or different haemosporidian genera are present in the same bird. The strength of molecular approaches is the detection of exceptionally light infections, which are common in naturally infected wild birds, and that morphologically cryptic parasites can be identified. This study shows that a combination of microscopic and PCR analyses recover a higher proportion of individuals infected by avian malaria and closely related haemosporidian parasites than if either of the methods are used alone. When only microscopy or current protocols of PCR are used separately, slight underestimation of the overall prevalence data has been reported. According to results of this study, approximately the same number of the patent infections is overlooked by microscopy or unread by PCR in the same samples. We recommend using both microscopic and PCR-based methods during field and laboratory investigations. Importantly, microscopy provides information of how molecular methods can be improved and applied most effectively in the future.

By using microscopy, a huge body of information has accumulated about the biology of avian malaria and related blood parasites. This information is necessary for development of new ecological and evolution theories based on genetic information. Thus, precise comparison between microscopy data and genetic information is crucial. After experimental infections of birds with *Plasmodium* spp., heavy parasitemias develop; that provides opportunities for morphological identification of genetic lineages of avian malaria parasites. Importantly, identification of morphospecies is highly reliable using this method because natural infections are usually light and often not all life stages important

for morphological identification are present. Due to successful experimental infections, we identified two mitochondrial cytochrome *b* gene lineages (SGS1 and TURDUS1) which can be used for molecular identification of two widespread avian malaria parasites, *Plasmodium relictum* and *P. circumflexum,* respectively. Clear morphological differences between blood stages of these parasites are consistent with genetic differences between their lineages. After genetic analysis, these two parasites appeared in different clades in the phylogenetic tree. *P. relictum* (SGS1) clustered together with another *P. relictum* lineage GRW4; that was confirmed after detailed morphological examination using morphometry (no significant differences were found between blood stages of these two lineages). Meanwhile *P. circumflexum* clustered on a separate branch of the tree, representing more distant species of *Giovannolaia* subgenus. Results of this study, together with results by Martinsen et al. (2007), Hellgren et al. (2007a) and Valkiūnas et al. (2007), are the first important steps in developing molecular diagnostics of avian malaria and other haemosporidian parasites and clarifying the traditional taxonomy using DNA analysis of the haematozoa.

Due to common simultaneous infections in wildlife and great amount of host DNA in each sample, ideally, malaria and other intracellular blood parasites should be investigated on the cell level when the same cell is seen under the microscope and then is used for DNA analysis. However the development of methods providing sequence data from one cell is a challenging work. During this study we developed new protocols for one cell dissection, DNA extraction and PCR. That provides an opportunity to obtain mitochondrial cytochrome *b* gene sequences from one parasite cell. This approach was developed using the Olympus/MMI CellCut Plus. This method provides an opportunity to collect large amount of clean parasite DNA, which can be used for development of multiple nuclear markers; that is essential for precise genetic analysis of haemosporidians. Using microsatellites and other nuclear markers, genetic analyses of parasites in hybridization experiments, evaluation of phylogeography of blood parasites, and investigation of many other issues

would be possible. Our study shows that laser microdissection microscopy is a convenient tool for this new direction of investigations.

According to our results *P. relictum* (lineage SGS1) infects birds from the families Fringillidae and Passeridae. Starlings are resistant to this *P. relictum* lineage. The level of tolerance to malaria parasites is different between bird species of the same family; it is not strictly related to phylogenetic relations of hosts. Dynamics of parasitemia was similar in phylogenetically closely related crossbills and siskins, but in greenfinches, it was more similar to distantly related chaffinches instead of closely related siskins. The infection of *P. relictum* (SGS1) did not cause mortality in experimentally infected birds, but negative effects on bird's health were evident in the heavily infected siskins and crossbills. The body mass of infected birds was not affected by *P. relictum* (SGS1) parasitemia. The loss of energy spent to cope with disease probably is compensated by plenty of food, which was given *ad libitum* in captivity. It should be taken in consideration that food shortage, impact of predators and other unfavourable conditions are present in nature; that probably increase negative effect of malaria parasites on their avian hosts. That warrants further investigation. According to this study, high severity of infection in experimentally infected birds is due to marked loss of red blood cells and pathology caused in the haemopoetic organs (spleen, liver). The direct destruction of erythrocytes and active removal of infected cells lead to low haematocrit values and resulting anaemia in heavily infected birds.

It was shown for the first time that *P. ashfordi* (lineage GRW2) infects birds of the Fringillidae (siskins and crossbills), common Palearctic passeriform birds. As is the case with *P. relictum* (SGS1), starlings are resistant to the *P. ashfordi* (GRW2) infection. Mechanisms of this resistance remain unknown.

During simultaneous infection with *P. ashfordi* (GRW2), the prepatent period of *P. relictum* (SGS1) becomes shorter in simultaneous infection comparing to single in experimentally infected siskins and crossbills. The dynamic of parasitemia during the simultaneous infection differs also in comparison to single infection of the same lineages of *P. relictum*. During the

simultaneous infection, the mean parasitemia has been recorded to be significantly higher than during single infection of *P. relictum* (SGS1). Moreover, high parasitemia (more than 90%) leads to death of some crossbills during simultaneous infection; it was not recorded during single *P. relictum* (SGS1) infection. Thus, the development of *P. relictum* (SGS1) infection is shifted and synergistic effect of two avian malaria parasites is present due to simultaneous infection. Extremely low haematocrit values and significant differences in body mass between control and heavily infected (parasitemia about 90%) crossbills indicate severe effect of simultaneous infection on infected birds during primary infections. To understand mechanisms of relationships of parasites during simultaneous infections and their influence on immune system and physiological condition of hosts, further experimental investigations combining single and simultaneous infections with the same lineages of parasites are needed.

The efficient cure of malaria infected birds is an important issue in experimental studies, zoos and some conservation projects. It was shown for the first time that the treatment with Malarone™ of experimentally infected birds is highly efficient for *P. relictum* (lineage SGS1) and *P. ashfordi* (GRW2) blood stages. The acute (most dangerous for birds) primary infections can be reduced and cleared using Malarone™ in doses, which are recommended for humans. However this drug does not affect exoerythrocytic stages of both *P. relictum* (SGS1) and *P. ashfordi* (GRW2), so the relapses of both species have been recorded. Using adjusted doses of primaquine, which are recommended for treatment of relapsing human malaria, also do not affect exoerythrocytic stages of *P. relictum* (SGS1) and *P. ashfordi* (GRW2). Unlike hypnozoites of *P. vivax,* which are located exclusively in liver cells, phanerozoites of avian malaria are located in endothelial cells of capillaries in many organs and tissues, and they are not affected by this drug. Other 8-aminoquinolines or different doses of primaquine should be tested for complete treatment of malaria caused by these parasites.

According to Marzal et al. (manuscript in preparation), transmission of *P. relictum* (lineage SGS1) can take place as far north as in northern Norway (69° 45´N, 30° 00´E) close to the North Polar Circle. This is the first study reporting avian malaria transmission at such high northern latitudes. The closely related *P. relictum* lineage GRW11 does not complete its life cycle so far in northern Europe as SGS1, but is transmitted in the Baltic region (the Curonian Spit, Russia). Both these lineages are widespread all over South Europe and may have originated from numerous southern refuges. It seems probable that these lineages of *P. relictum* have expanded in the Baltic region and northern Europe relatively recently after the last glacial period. Five cyt *b* gene lineages of *P. relictum* are transmitted in South Europe (SGS1, GRW11, PADOM08, PADOM20 and PADOM21). It is still unclear why unlike GRW11 and SGS1 lineages, the other 3 genetic lineages have not expanded to the northern Europe so far. Due to already established transmission of the lineages GRW11 of *P. relictum* in the Baltics, this parasite might be the agent of emerging malaria in more northern regions of Europe in the future. The mechanisms contributing to the spread of certain malaria parasites lineages to new northern European ecosystems need clarification.

CONCLUSIONS

1. Microscopy is a reliable method for determining patterns of distribution of avian malaria parasites and other haemosporidians in naturally infected birds. Importantly, blood films should be of good quality and should be examined properly by skilled investigators. We encourage using optical microscopy in studies of blood parasites in parallel to the now widely employed molecular tools.

2. Mitochondrial cytochrome *b* gene lineages SGS1 and TURDUS1 belong to the morphospecies *Plasmodium relictum* and *P. circumflexum* respectively, which are widespread malaria parasites of birds; these two lineages can be used for molecular identification of malaria infections caused by *P. relictum* and *P. circumflexum*.

3. A new method for malaria parasite single cells dissection, DNA extraction and PCR was developed; it is a promising tool for DNA studies of avian malaria and other haemosporidian parasites.

4. *Plasmodium relictum* (lineage SGS1) infects birds of the Fringillidae and Passeridae families. This parasite causes disease of varying severity in experimentally infected birds, particularly due to massive destruction of red blood cells and pathology caused in haemopoetic organs. No effects of this infection on birds' body mass and temperature were recorded. Starlings are resistant to this lineage of *P. relictum*.

5. *Plasmodium ashfordi* (lineage GRW2), the malaria parasite of tropical origin which is common in some African migrants, infects European birds belonging to the Fringillidae. This parasite therefore has broad host specificity and might be potentially dangerous as a possible agent of emerging avian malaria in the future. Starlings are resistant to *P. ashfordi* (GRW2).

6. *Plasmodium relictum* (lineage SGS1) and *P. ashfordi* (GRW2) act synergistically during simultaneous infection in birds of the Fringillidae; these parasites cause death of some infected birds, particularly due to massive destruction of red blood cells. No effects of simultaneous infection on body mass or temperature of infected birds were recorded.

7. Treatment of infected birds with Malarone™ is efficient for clearing blood stages of *Plasmodium relictum* (lineage SGS1) and *P. ashfordi* (GRW2), but not exoerythrocytic stages of these malaria parasites. Primaquine does not affect exoerythrocytic stages of avian malaria using doses recommended for humans. Malarone™ is recommended to cure parasitemia of avian malaria.

8. Two mitochondrial cytochrome *b* gene lineages (SGS1 and GRW11) of *Plasmodium relictum* are transmitted in the Baltic region. The lineage SGS1 is transmitted also in far North Europe, whereas transmission of the lineage GRW11 has not been recorded in Scandinavian countries so far. Both these lineages are transmitted and widespread in southern Europe, so are likely expanding with migratory birds to the northern Europe. Because establishment of GRW11 transmission in northern Europe is possible, this parasite should be considered as a possible agent of emerging avian malaria in this region in the future; this should be taken in consideration in conservation projects.

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LIST OF PUBLICATIONS ON THE DISSERTATION TOPIC

Publications in international scientific journals

1. **Palinauskas, V**., Kosarev, V., Shapoval, A., Valkiūnas, G., Bensch, S. 2007. Comparison of mitochondrial cytochrome *b* lineages and morphospecies of two avian malaria parasites of the subgenera *Haemamoeba* and *Giovannolaia* (Haemosporida: Plasmodiidae). Zootaxa 1626, 39-50.
2. **Palinauskas, V**., Valkiūnas, G., Bensch, S., Bolshakov, V. C. 2008. *Plasmodium relictum* (lineage P-SGS1): Effects on experimentally infected passerine birds. Experimental Parasitology 120, 372-80.
3. **Palinauskas, V**., Valkiūnas, G., Križanauskienė, A., Bensch, S., Bolshakov, V. C. 2009. *Plasmodium relictum* (lineage P-SGS1): Further observation of effects on experimentally infected passeriform birds, with remarks on treatment with Malarone™. Experimental Parasitology 123, 134-139.
4. Valkiūnas, G., Iezhova, T. A., Križanauskienė, A., **Palinauskas, V.** Sehgal, R. N. M., Bensch, S. 2008. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. Journal of Parasitology 94, 1395-1401.

Abstracts of scientific conferences

1. **Palinauskas, V.**, Križanauskienė, A., Markovets, M., Kosarev, V., Efremov, V., Bensch, S., Valkiūnas, G. 2007. The distribution of blood parasites of passeriform birds in some European and Asian districts. Bulletin of the IInd International Eurasian Ornithology Congress. Antalya, Turkey, p. 92.
2. **Palinauskas, V.**, Valkiūnas, G., Bensch, S., Bolshakov, V. C. 2008. The development of *Plasmodium relictum* (lineage P-SGS1) in experimentally infected passerine birds. Bulletin of the Xth European Multicolloquium of Parasitology. Cite Universitaire de Paris, Paris, France, p. 83.

3. **Palinauskas, V.**, Valkiūnas, G., Bensch, S., Bolshakov, V. C. 2008. Virulence of *Plasmodium relictum* (lineage P-SGS1) in experimentally infected passerine birds. Bulletin of the IVth Congress of the Russian Society of Parasitologists, Russian Academy of Sciences. St Petersburg, Russia, 3, p. 21-23.
4. **Palinauskas, V.,** Valkiūnas, G., Križanauskienė, A., Bensch, S., Bolshakov, C. V. 2008. Experimental infection of *Plasmodium relictum* (lineage P-SGS1) in passerine birds, with remarks on treatment with Malarone™. Bulletin of the IVth Avian Malaria Workshop, Badajoz, Spain, p. 24.
5. **Palinauskas, V.,** Valkiūnas, G., Križanauskienė, A., Bensch, S., Bolshakov, C. V. 2009. Effects of *Plasmodium relictum* (lineage P-SGS1) on experimentally infected passerine birds, with remarks on the infection treatment with Malarone™. Bulletin of the IIIrd Symposium of the Scandinavian-Baltic Society for Parasitology, Riga, Latvia, p. 39.
6. Valkiūnas, G., Iezhova, T. A., Križanauskienė, A., **Palinauskas, V.,** Sehgal, R. N. M., Bensch, S. 2008. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. Bulletin of the IVth Congress of the Russian Society of Parasitologists, Russian Academy of Sciences. St Petersburg, Russia, 1, p. 117-118.


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