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Exo-erythrocytic development of avian malaria parasites and haemoproteids: completing the cycle

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Egzoeritrocitinis paukščių maliarinių parazitų ir hemoproteidų vystymasis: gyvenimo ciklo papildymas

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ABBREVIATIONS

BCIP – 5-bromo-4-chloro-3-indolyl phosphate

DNA – deoxynucleic acid

dpe – days post exposure

H&E – haematoxylin and eosin

ISH – chromogenic *in situ* hybridization

NBT – 4-nitro blue tetrazolium chloride

PCR – polymerase chain reaction

rRNA – ribosomal ribonucleic acid

LIST OF PUBLICATIONS ON THE DISERTATION TOPIC

The dissertation is based on the following co-authored original publications in journals with an impact factor and referred in the Clarivate Analytics Web of Science database. Throughout the text, the publications are referred using the Roman numerals:

I. **Ilgūnas, M.**, Bukauskaitė, D., Palinauskas, V., Iezhova, T.A., Dinhopl, N., Nedorost, N., Weissenbacher-Lang, C., Weissenböck, H., Valkiūnas, G. 2016. Mortality and pathology in birds due to *Plasmodium (Giovannolaia) homocircumflexum* infection, with emphasis on the exoerythrocytic development of avian malaria parasites. Malaria Journal 15, 256. doi: 10.1186/s12936-016-1310-x. (Parasitology Q2; Tropical Medicine Q1)

II. Palinauskas, V., Žiegytė, R., Iezhova, T.A., **Ilgūnas, M**., Bernotienė, R., Valkiūnas, G. 2016. Description, molecular characterisation, diagnostics and life cycle of *Plasmodium elongatum* (lineage pERIRUB01), the virulent avian malaria parasite. International Journal for Parasitology 46, 697-707. doi: 10.1016/j.ijpara.2016.05.005. (Parasitology Q1)

III. Valkiūnas, G., **Ilgūnas, M.**, Bukauskaitė, D., Iezhova, T.A. 2016. Description of *Haemoproteus ciconiae* sp. nov. (Haemoproteidae, Haemosporida) from the white stork *Ciconia ciconia*, with remarks on insensitivity of established polymerase chain reaction assays to detect this infection. Parasitology Research 115, 2609-2616. doi: 10.1007/s00436-016-5007-4. (Parasitology Q2)

IV. Valkiūnas, G., **Ilgūnas, M.**, Bukauskaitė, D., Žiegytė, R., Bernotienė, R., Jusys, V., Eigirdas, V., Fragner, K., Weissenböck, H., Iezhova, T.A. 2016. *Plasmodium delichoni* n. sp.: description, molecular characterisation and remarks on the exoerythrocytic merogony, persistence, vectors and transmission. Parasitology Research 115, 2625-2636. doi: 10.1007/s00436-016-5009-2. (Parasitology Q2)

V. Valkiūnas, G., **Ilgūnas, M.**, Bukauskaitė, D., Fragner, K., Weissenböck, H., Atkinson, C.T., Iezhova, T.A. 2018. Characterization of *Plasmodium relictum*, a cosmopolitan agent of avian malaria. Malaria Journal 17, 184. doi: 10.1186/s12936-018-2325-2. (Parasitology Q2)

VI. **Ilgūnas, M.**, Bukauskaitė, D., Palinauskas, V., Iezhova, T., Fragner, K., Platonova, E., Weissenböck, H., Valkiūnas, G. 2019. Patterns of *Plasmodium homocircumflexum* virulence in experimentally infected passerine birds. Malaria Journal 18, 174. doi: 10.1186/s12936-019-2810-2. (Parasitology Q1; Tropical Medicine Q1)

VII. **Ilgūnas, M.**, Chagas, C.R.F, Bukauskaitė, D., Bernotienė, R., Iezhova, T., Valkiūnas, G. 2019. The life cycle of the avian haemosporidian parasite *Haemoproteus majoris*, with emphasis on the exo-erythrocytic and sporogonic development. Parasites & Vectors, 12, 516. doi: 10.1186/s13071-019-3773-4. (Parasitology Q1)

VIII. **Ilgūnas, M.**, Palinauskas, V., Platonova, E., Iezhova, T., Valkiūnas, G. 2019. The experimental study on susceptibility of common European songbirds to *Plasmodium elongatum* (lineage pGRW6), a widespread avian malaria parasite. Malaria Journal 18, 290. doi: 10.1186/s12936-019-2926-4. (Parasitology Q1; Tropical Medicine Q1)

AUTHOR CONTRIBUTIONS IN THE CORRESPONDING PAPERS

In dissertation publications, research on exo-erythrocytic development of parasites was done and text were prepared by M. Ilgūnas. Contribution of co-authors in each study was as described below.

I. Experimental conception and design: GV, **MI (Mikas Ilgūnas)** and VP; fieldwork: **MI**, TAI, DB, VP and GV; bird laboratory care and dissection: **MI** and DB; histology work: **MI** and TAI; ISH and interpretation of the ISH results: HW, **MI**, ND, NN, CWL and GV; paper writing: **MI** and GV. All authors read and approved the final manuscript.

II. Conceptualization: VP, GV; Formal analysis: **MI**, TI; Funding acquisition: GV; Investigation: VP, **MI**, RŽ, RB; Resources: GV; Supervision: GV; Visualization: VP, **MI**, TI, RŽ; Writing—original draft preparation: VP; Writing—review and editing: GV, **MI**, RŽ, TI, RB. All authors read and approved the final manuscript.

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V. GV designed this study and wrote the manuscript. GV, **MI**, DB, TAI did field and experimental work. TAI and GV analyzed morphological data. DB carried out vector research. **MI**, KF, HW did histological and in situ hybridization investigations. CA provided data on the Hawaiian lineage pGRW4. All authors read and approved the final manuscript.

VI. Conceptualization: **MI**, VP, GV; Formal analysis: DB; Funding acquisition: GV; Investigation: **MI**, DB, VP, TI, KF, HW, EP; Resources: HW, GV; Supervision: GV; Visualization: **MI**, TI; Writing—original draft preparation: **MI**; Writing—review and editing: DB, VP, TJ, KF, HW, EP, GV. All authors read and approved the final manuscript.

VII. Conceptualization: GV, **MI**; Funding acquisition: RB, CFRC; Investigation: **MI**, CFRC, DB, TJ, RB, GV; Resources: GV, CFRC, RB; Supervision: GV; Visualization: **MI**, TI; Writing—original draft preparation: **MI**, GV; Writing—review and editing: CFRC, DB, RB, TI. All authors read and approved the final manuscript.

VIII. Conceptualization: **MI**, VP, GV; Formal analysis: **MI**; Funding acquisition: GV, VP; Investigation: **MI**, VP, TI, EP; Resources: GV; Supervision: GV; Visualization: **MI**, TI; Writing—original draft preparation: **MI**; Writing—review and editing: VP, TJ, EP, GV. All authors read and approved the final manuscript.

LIST OF CONFERENCE PRESENTATIONS ON THE SUBJECT OF THE DISSERTATION

1. **Ilgūnas, M.**, Bukauskaitė, D., Palinauskas, V., Iezhova, T., Dinhopl, N., Nedorost, N., Weissenbacher-Lang, C., Weissenböck, H., Valkiūnas, G. 2016. The development of pathogenic malaria parasite in three experimentally infected common European bird species. *COINS 2016*. Vilnius, Lithuania.

2. **Ilgūnas, M.**, Bukauskaitė, D., Palinauskas, V., Iezhova, T., Dinhopl, N., Nedorost, N., Weissenbacher-Lang, C., Weissenböck, H., Valkiūnas, G. 2016. *Plasmodium* (*Giovannolaia*) *homocircumflexum* kills birds. *3rd International conference on Malaria and Related Haemosporidian Parasites of Wildlife*. Arbanasi, Bulgaria.

3. **Ilgūnas, M.**, Bukauskaitė, D., Palinauskas, V., Iezhova, T., Dinhopl, N., Nedorost, N., Weissenbacher-Lang, C., Weissenböck, H., Valkiūnas, G. 2016. Application of *in situ* hybridization in histopathological studies of avian malaria. *20th Laboratory Animals in Research Conference*. Vilnius, Lithuania.

4. **Ilgūnas, M.**, Bukauskaitė, D., Palinauskas, V., Platonova, E., Fragner, K., Weissenböck, H., Valkiūnas, G. 2018. Different patterns of virulence of *Plasmodium homocircumflexum* (lineage pCOLL4) infection in wild birds. *4th International conference on Malaria and Related Haemosporidian Parasites of Wildlife.* Beijing, China.

5. **Ilgūnas, M.**, Bukauskaitė, D., Palinauskas, V., Platonova, E., Fragner, K., Weissenböck, H., Valkiūnas, G. 2018. *Plasmodium homocircumflexum* (genetinė linija pCOLL4) virulentiškumas laukiniams paukščiams. *11-oji Jaunųjų mokslininkų konferencija Bioateitis: gamtos ir gyvybės mokslų perspektyvos (11th Junior Researcher Conference Biofuture: Perspectives of Life Sciences).* Vilnius, Lithuania.

AWARDS

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4. Research council of Lithuania. Support to publish a scientific paper in Q1 category journal (2016, award no. PUB-16012).
5. Research council of Lithuania. Support to publish a scientific paper in Q1 category journal (2019, award no. P-PUB-19-30).
6. Research council of Lithuania. Support of a scientific internship at the University of Veterinary Medicine Vienna, Vienna, Austria (2017, award no. P-KEL-17-364).
7. Lithuanian Academy of Sciences. Award for the best presentation at the *11-oji Jaunųjų mokslininkų konferencija Bioateitis: gamtos ir gyvybės mokslų perspektyvos (11th junior researcher conference Biofuture: perspectives of life sciences)* conference.

SCIENTIFIC PROBLEM

Parasites belonging to the genera *Plasmodium* and *Haemoproteus* are transmitted on all continents except for Antarctica and are known to cause severe diseases and even death to their vertebrate hosts (Atkinson et al., 2008; Marzal, 2012). Some species are virulent in blood-sucking insects (Valkiūnas et al., 2014; Bukauskaitė et al., 2016). These parasites have been extensively studied however, information about complete life cycles for the vast majority of haemosporidians is lacking. This is particularly true for the exo-erythrocytic stage of the life cycle (Valkiūnas and Iezhova, 2017), and is a glaring obstacle for better understanding of the epidemiology and virulence of these widespread pathogens.

Fifty-five species of avian *Plasmodium* parasites have been identified to date based on their morphological characters (Valkiūnas and Iezhova, 2018). In recent years, the issue of avian malaria virulence has attracted much attention, however, the majority of the investigations focused mainly on relatively easy to access blood stages of *Plasmodium* pathogens (Palinauskas et al., 2008; Palinauskas et al., 2009; Cellier-Holzem et al., 2010; Bichet et al., 2012; Cornet et al., 2014; Ellis et al., 2015). These studies provided valuable information about blood-related pathologies, but the understanding of true mechanisms of virulence during avian malaria infections remains limited due to insufficient knowledge about the exo-erythrocytic development. The majority of available studies investigating the exo-erythrocytic development were carried in the first half of the XXth century (Valkiūnas and Iezhova, 2017) – a period of time when molecular diagnostic techniques were not yet introduced to the field of avian malaria. Recent application of molecular diagnostic methods suggested that the inter- and the intra-species diversity of avian haemosporidian parasites was far greater than previously believed (Ricklefs et al., 2004; Bensch et al., 2009). Discovery of the great genetic diversity of *Plasmodium* parasites indicates that it might be even more difficult to understand the exo-erythrocytic development of particular malaria agents due to high intraspecies genetic variability. That calls for application of modern diagnostic tools and experimental research for better understanding of avian malaria life cycles and virulence.

Two most important causes of pathology during avian malaria infections have been identified. Mainly, blood pathology (Hayworth et al., 1987; Valkiūnas, 2005) and internal organ damage by exo-erythrocytic meronts, particularly secondary exo-erythrocytic meronts or phanerozoites (Garnham, 1966; Valkiūnas, 2005; Vanstreels et al., 2015). In birds infected

with malaria, merozoites developing in both primary exo-erythrocytic meronts (metacryptozoites) and erythrocytic meronts, can induce secondary exo-erythrocytic merogony (development of phanerozoites), a trait that is not shared with malaria parasites developing in mammals. As phanerozoites develop in the reticuloendothelial cells (macrophages, endothelial cells of capillaries, Kupffer cells), they can occur and damage various organs of the susceptible avian hosts, including brain, heart, kidney, liver, lungs and spleen (Garnham, 1966; Fix et al., 1988; Valkiūnas, 2005; Vanstreels et al., 2014; Valkiūnas and Iezhova, 2017). Due to the unclear patterns of the phanerozoite occurrence in the majority of described parasite species, pathology during avian malaria remains insufficiently investigated. It is possible this disease might be more virulent than currently believed because phanerozoite development is difficult to predict and prevent. This calls for research aimed at better understanding the exo-erythrocitic development of *Plasmodium* parasites.

A new malaria parasite *Plasmodium homocircumflexum* (lineage pCOLL4) was recently discovered and described in Europe (Pérez-Tris et al., 2007; Palinauskas et al., 2015). This parasite was isolated from a wild-caught red-backed shrike *Lanius collurio*, and an experimental study showed that this pathogen can cause mortality in domestic canaries *Serinus canaria* due to the development of phanerozoites (Palinauskas et al., 2015). Blood stages of *P. homocircumflexum* were cryopreserved, and the samples are available for experimental research at the Nature Research Centre, Vilnius, Lithuania. Even though it is clear that this agent of avian malaria can infect red-backed shrikes and cause lethal infections in experimentally exposed domestic canaries, it remains unknown if this pathogen can develop in wild European birds with whom it is present in the same ecosystems. This is an important issue which calls for investigation in order to better understand the possible impact of *P. homocircumflexum* on the local populations of European birds.

Plasmodium elongatum is a widespread avian malaria parasite which has been reported on all continents, except for Antarctica (Garnham, 1966; Valkiūnas, 2005; Bensch et al., 2009). Infection with this species has been reported in birds belonging to more than 15 avian families of 11 orders (MalAvi database, see Bensch et al., 2009). Interestingly, this parasite has never been reported in some common European passerine birds, for example common starling *Sturnus vulgaris* and common crossbill *Loxia curvirostra* (Bensch et al., 2009). The Reason for the absence of such reports in these common birds remains unclear. The lineage pGRW6 was first determined by

Beadell et al. (2004), and its *P. elongatum* identity was determined approximately 10 years ago (Valkiūnas et al., 2008). Intraspecies genetic variation of *P. elongatum* was reported (Bensch et al., 2009), but there is no information about the virulence of different lineages of the same morphospecies. There are several closely related lineages deposited in GenBank (Bensch et al., 2009), and they all could probably belong to *P. elongatum*. However, morphological evidence for such conclusion is lacking, as well as information about the exo-erythrocytic development of the known *P. elongatum* lineages. This malaria parasite has been known to cause severe disease and even death of birds living in captivity around the world during "pre-molecular" era (Fleischman et al., 1968; Herman et al., 1968; Beier and Stoskopf, 1980; Beier and Trpis, 1981; Cranfield et al., 1990; Graczyk et al., 1994). However, it is still unclear which lineages of *P. elongatum* were responsible for the bird deaths. Due to the cosmopolitan distribution of this species, it is crucial to identify what are the mechanisms of virulence during infections of different lineages of this pathogen.

Plasmodium relictum is one of the most well studied avian malaria parasites. Light microscopy-based studies conducted during the XXth century suggested that *P. relictum* is the most common avian malaria agent on the planet (Bennett et al., 1982; Bishop and Bennett, 1992; Atkinson et al., 2008; Valkiūnas, 2005). Recent molecular studies support this conclusion and suggest that there is a significant genetic diversity within the *P. relictum* species. Despite the numerous studies, exo-erythrocytic development of this parasite remains insufficiently understood. Experimental studies, which were carried out during the "pre-molecular" era, suggest that phanerozoites do develop in internal organs of the infected vertebrate hosts (Garnham, 1966; Garnham, 1980; Atkinson et al., 2008; Valkiūnas, 2005) however, information on these life stages and associated pathologies in avian hosts is still absent for all reported lineages of *P. relictum*. That is an obstacle for better understanding mechanisms of persistence during *P. relictum* infections as well as the pathogenicity of different lineages of this parasite in different avian hosts.

Avian haemosporidians belonging to the genus *Haemoproteus* are some of the most extensively studied bird blood parasites (Clark et al., 2014). Over 150 species of these pathogens have been described. However, like in the case with species of the genus *Plasmodium*, knowledge about the exo-erythrocytic development of *Haemoproteus* parasites is markedly fragmental. According to older literature, *Haemoproteus* species have been considered as relatively benign in the avian hosts (Bennett et al., 1993). However, several

recent studies have casted doubts on this assumption due to a number of well-documented cases of severe haemoproteosis in avian hosts (Cardona et al., 2002; Ferrell et al., 2007; Donovan et al., 2008; Olias et al., 2011; Dunn et al., 2013; Cannell et al., 2013; Groff et al., 2019). Severe pathologies and even mortality have been reported in non-adapted (wrong) avian hosts due to damage by exo-erythrocytic meronts, in which parasites can initiate but cannot complete their development. That leads to severe disease (Ortiz-Catedral et al., 2019). Megalomeronts of only 8 species of *Haemoproteus* parasites have been found as far (Valkiūnas and Iezhova, 2017). They were described in parrots (Miltgen et al., 1981; Olias et al., 2011; Ortiz-Catedral et al., 2019), turkeys (Atkinson, 1986; Atkinson, 1988), house sparrows *Passer domesticus* (Wenyon, 1926; Peirce, 1976; Burtikashvili, 1978), sacred kingfishers *Todiramphus sanctus* (Peirce et al., 2004), bobwhite quails *Colinus virginianus* (Cordona et al., 2002) and pigeons (Farmer, 1964; Earlé et al., 1993), however the lineages of the parasites were not determined. Despite the high species richness, the broad geographical distribution and the possible negative impact on birds, the part of the life cycle related to the exo-erythrocytic development of *Haemoproteus* parasites remain insufficiently understood.

OBJECTIVE AND MAIN TASKS OF THE STUDY

The objective was to gain new knowledge about the insufficiently investigated part of the haemosporidian (Haemosporida) life cycles - exo-erythrocytic development of malaria parasites (*Plasmodium*) and haemoproteids (*Haemoproteus*), particularly in regard of their virulence and pathologies caused in avian hosts.

The following tasks were set to achieve the objective:

1. Investigate the secondary exo-erythrocytic meronts (phanerozoites) of *Plasmodium homocircumflexum* (lineage pCOLL4) and the pathologies caused in common European passerine birds.
2. Investigate phanerozoite development of *Plasmodium elongatum* (lineages pERIRUB01 and pGRW6) in experimentally infected passerine birds.
3. Investigate exo-erythrocytic meronts of *Haemoproteus majoris* (lineages hPHYBOR04 and hPARUS1) in naturally infected avian hosts.
4. Examine organs of avian hosts experimentally infected with *Plasmodium relictum* (lineage pPHCOL01) for the presence of phanerozoites.
5. Examine organs of avian hosts experimentally infected with *Plasmodium delichoni* n. sp. (lineage pCOLL6) for the presence of phanerozoites.
6. Examine organs of a white stork naturally infected with *Haemoproteus ciconiae* n. sp. for the presence of exo-erythrocytic meronts.

STATEMENTS TO BE DEFENDED

1. *Plasmodium homocircumflexum* (lineage pCOLL4) readily develops phanerozoites in some, but not all bird species.
2. Phanerozoites of *Plasmodium homocircumflexum* (pCOLL4) cause ischaemic changes in the brain, leading to cerebral paralysis and high mortality in the affected hosts.
3. *Plasmodium elongatum* (lineage pERIRUB01) develops numerous phanerozoites in the bone marrow cells of the infected birds and interrupts erythropoiesis, resulting in severe anaemia and high mortality in avian hosts.
4. *Plasmodium elongatum* (lineage pGRW6) is relatively benign in adapted avian hosts mainly due to low ability to develop phanerozoites.
5. Megalomeronts of *Haemoproteus majoris* (lineages hPHYBOR04 and hPARUS1) were discovered; these are of unique morphology.
6. Phanerozoites were not reported in *Plasmodium relictum* (lineage pPHCOL01) and *Plasmodium delichoni* n. sp. (lineage pCOLL6) in experimentally infected birds, suggesting existence of other mechanisms of persistence than a phanerozoite stage in avian malaria parasites.

NOVELTY OF THE STUDY

1. It was proved experimentally that malaria parasite *Plasmodium homocircumflexum* (lineage pCOLL4) is an euryxenous parasite but develops markedly differently in different species of avian hosts resulting in markedly different virulence.
2. A new lineage pERIRUB01 of avian malaria parasite was discovered and attributed to *Plasmodium elongatum;* its exo-erythrocytic development was investigated, and it was shown that this infection is markedly virulent in some avian hosts, particularly due to damage of stem bone marrow cells by developing phanerozoites.
3. It was proved experimentally that the susceptibility of different bird species to *Plasmodium elongatum* (lineage pGRW6) infection is markedly variable. The global distribution of this parasite might be due to low virulence in wild adapted avian hosts, which serve as reservoirs of the infection for non-adapted birds, in whom the disease is often lethal.
4. Lineage hPHYBOR04 was discovered in Europe and attributed to *Haemoproteus majoris*.
5. Megalomeronts of *Haemoproteus majoris* (lineages hPHYBOR04 and hPARUS1) were discovered and it was shown that megalomeronts of this parasite appear not only during abortive development in non-adapted (wrong) avian hosts, but are a normal stage in the life cycle of this haemoproteid during the development in naturally infected hosts.
6. New lineage pPHCOL01 of *Plasmodium relictum* was discovered and characterized both molecularly and morphologically, contributing to the better understanding of the intraspecies genetic diversity of this cosmopolitan avian malaria agent.
7. A new species of *Plasmodium – Plasmodium delichoni* n. sp. (lineage pCOLL6) – was described, characterised molecularly, and a new hypothesis about possible mechanism of its persistence was suggested.
8. First *Haemoproteus* species, which parasitizes the white stork – *Haemoproteus ciconiae* n. sp. – was discovered and described.

1. BRIEF LITERATURE OVERVIEW

A disease with symptoms of malaria infections was already known in Ancient Greece. In their writings, Homer, Empedocles and Hippocrates mentioned that people who live in swampy areas tend to develop fever and there are noticeable changes in the spleens of the deceased individuals. Studies targeting these organisms have not begun until 1878-1879, when L. Pasteur and R. Koch uncovered the role of single cell organisms in diseases (Cox, 2010). In 1880, doctor C. L. A. Laveran noticed roundish bodies with pigment granules inside the red blood cells of several malaria-ill patients while no such structures were reported in the blood of healthy people. In 1907, C. L. A. Laveran was awarded a Nobel Prize in Physiology or Medicine for this discovery (Bruce-Chwatt, 1981). In 1885, E. Marchiafava and A. Celli described the genus *Plasmodium*, the first genus of the family Plasmodiidae and attributed agents of human malaria to it. At the same time, V. Danilewsky was studying blood pathogens of vertebrate animals in Ukraine. He was examining mainly bird and reptile blood and described and illustrated high diversity of blood parasites, which were attributed to and described as species of the genera *Plasmodium*, *Haemoproteus* and *Laucocytozoon* by other researchers latter. In 1890, D. L. Romanowsky developed a method of staining the erythrocytic stages of haemosporidian parasites using a combination of eosin and methylene blue dyes. This stain differentiates the cytoplasm in blue and the nucleus in pink colours. The stain discovery was a catalyst for morphological studies of haemosporidian parasites (Valkiūnas, 2005; Cox, 2010). In 1891, the first successful experimental infection using avian malaria was carried out. This began the era of experimental research of malaria. Such experimental work laid the foundation for the better understanding of malaria in vertebrate hosts, including human malaria. To date, one of the most prominent achievements in the field of avian malaria research is the determination of the life cycle of *Plasmodium relictum*; using this parasite it has been proven that malaria is transmitted by mosquito vectors. Sir R. Ross was awarded the Nobel Prize in Physiology or Medicine for this discovery in 1902 (Garnham, 1966). From this time forward, avian malaria parasites were used as model organisms for better understanding human malaria. Only in 1948, after the discovery of rodent malaria and simian malaria in 1966, the interest in avian malaria started decreasing in human medicine, but not in wildlife studies (Bensch et al., 2004; Bensch et al., 2009; Valkiūnas, 2005; Ricklefs et al., 2017). Although avian malaria parasites are rarely used in human malaria research today, discoveries made using these pathogens formerly are invaluable. Avian malaria model organisms were essential for the

first antimalarial drug research (Davey, 1951; Coatney et al., 1953), first methods of in vitro cultivation of these parasites (Trager, 1950; Ball and Chao, 1961) and the initial stages towards creation of an antimalarial vaccine (McGhee et al., 1977). Importantly, avian malaria model organisms remain important in research aiming better understanding difficult issues of *Plasmodium* parasite biology and virulence (Ashgar et al., 2015). In 2002, the first genome of human malaria parasite *Plasmodium falciparum* was sequenced (Gardner et al., 2002). This gave rise to the creation of databases dedicated to storage of haemosporidian parasites genetic sequences (Gardner et al., 2002; Bensch et al., 2009). Nowadays, avian malaria parasites and related haemosporidians are often used to unravel the evolutionary and phylogenetic questions and determining issues of the host-parasite relationships (Knowles et al., 2011; Marzal et al., 2011).

Avian haemosporidian parasites have complex life cycles, which consists of strictly ordered developmental stages and obligatory change of vertebrate host and arthropod vector (Garnham, 1966). Intermediate host is the bird in which exo-erythrocytic merogony (development of exo-eythrocytic meronts in cells of internal organs) and parasite development in the blood (production and maturation of gametocytes) occur. The final host is a dipteran insect, in which the sexual process and sporogony takes place. General patterns of the life cycles of avian haemosporidians were summarized by Valkiūnas (2005). In spite of the numerous available publications, details of the development of different species of haemosporidians, particularly exo-erythrocytic development, remain unknown for the vast majority of described species, as reviewed by Valkiūnas and Iezhova (2017). This dissertation study aimed to partly fill up a gap in this knowledge.

Prior to the introduction of molecular methods to the field of avian haemosporidians, these parasites were mainly investigated using light microscopic examination and electron microscopy. However, parasitaemia in the sampled birds wild is often low, and it is often difficult or even impossible to identify the observed parasites to species level (Valkiūnas, 2005). After the introduction of polymerase chain reaction (PCR)-based methods to the field of avian blood parasites, it became obvious that the diversity of these parasites has been greatly underestimated (Ricklefs et al., 2004; Besch et al., 2009; Clark et al., 2014). In the last two decades, numerous studies analysing morphology, taxonomy, ecology, evolutionary biology, genetics and genetic diversity of avian haemosporidians have been published (Clark et al., 2014; Perkins, 2014; Ricklefs et al., 2014; Outlaw et al., 2015; Sehgal, 2015; Bensch

et al., 2016; Dimitrov et al., 2016; Ricklefs et al., 2017; Videval et al., 2017; Valkiunas and Iezhova, 2018; Ellis et al., 2019; Humpfriest et al., 2019). The great majority of such works focused only on the blood stages of avian haemosporidians thus revealed only a part of the big picture of the biology of these parasites. However, several recent histological studies, combined both, microscopic examination and molecular diagnostic approaches and revealed that avian haemosporidians might cause severe pathologies and even mortality of the vertebrate hosts, especially during *Haemoproteus* infections, due to damage caused by the exo-erythrocytic meronts (Cardona et al., 2002; Ferrell et al., 2007; Donovan et al., 2008; Murata et al., 2008; Olias et al., 2011; Pacheco et al., 2011; Howe et al., 2012; Dunn et al., 2013; Cannell et al., 2013; Palinauskas et al., 2013; Vanstreels et al., 2014; Vanstreels et al., 2015; Grilo et al., 2016; Groff et al., 2019; Ortiz-Catedral et al., 2019). These are important findings as avian haemosporidians are cosmopolitan parasites which can affect populations of birds all over the world, but current understanding of their virulence is markedly scarce.

Review of the aforementioned studies called for research aimed at better understanding the host-parasite interactions during exo-erythrocytic development, and this thesis aimed to uncover some new knowledge in regard of these problems.

2. MATERIALS AND METHODS

In depth, descriptions of material collected and used in this dissertation as well ss the methods applied are provided in the published papers (**Papers I-VIII**). Below, brief information and corresponding references are given.

2.1. Study sites

Fieldwork, during which material used in this dissertation was collected, were carried out at the Biological Station "Rybachy" belonging to the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit, Kaliningrad region, Russia (https://www.zin.ru/rybachy/index_e.html) (**Papers I, II, VI, VIII**) and Ventės ragas ornithological station, Ventė, Lithuania (www.vros.lt) (**Papers III-V**). Infection experiments were conducted at the biological station "Rybachy" (**Papers I, II, VI, VIII**), Ventės ragas ornithological station (**Papers II, IV, V**) and the P. B. Šivickis laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania (www.gamtostyrimai.lt) (**Papers II, IV, V**). All material was analysed and is currently deposited at Nature Research Centre.

2.2. Sample collection

2.2.1. Blood samples

During the fieldwork, wild birds were caught using "Rybachy" type funnel traps and mists nets (**Papers I-VIII**). After ringing, the birds were bled by puncturing the brachial vein and collecting 30-50 µL of blood using a glass microcapillary. Single small drops of blood were placed on objective slides, and thin blood films were prepared. Fresh prepared blood films were air dried using a battery powered fan and fixed using absolute methanol. Fixed blood films were stained with a 10% Giemsa stain according to Valkiūnas (2005).

For molecular analysis, all of the blood remaining in the glass capillary was placed into non-lysis SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH=8.0) (Hellgren et al., 2004) and stored at − 4 °C while in the field and at − 20 °C when back in the laboratory (**Papers I-VIII**).

2.2.2. Samples for histology research

Samples of bird organs (brain, heart, kidney, liver, lungs, spleen, and a piece of the pectoral muscle) for histological analysis were collected from deceased or euthanised birds and stored in 10 % neutral formalin solution while in the field and then processed into paraffin blocks for further analysis and long term storage. Later, 4 μm histological sections were cut from the paraffin blocks using a Leica RM2245 microtome (Leica Biosystems Inc., Buffalo Grove, USA) (**Papers I-VIII**). Histological sections were stained with haematoxylin-eosin (H&E) according to Valkiūnas (2005) (**Papers I-VIII**) or processed using chromogenic *in situ* hybridization (ISH) (see *2.3. Chromogenic in situ hybridization*) (**Papers I, IV-VI**). Additionally, histological sections of 4 μm were also prepared and placed on paraffin membrane slides (MMI-MembraneSlide, Molecular Machines and Industries, Zurich, Switzerland) for laser microdissection studies (see *2.4. Laser microdissection*) (**Paper VII**).

Additionally, bone marrow smears were prepared on glass slides from each dissected birds' tibiotarsus. These preparations were fixed with absolute methanol and stained with 10% Giemsa stain using the same protocol as for blood film staining.

2.3. Chromogenic in situ hybridization

Chromogenic *in situ* hybridization was carried out as described by Dinhopl et al. (2011) (**Papers I, IV-VI**). Briefly, 3 - 4 μm paraffin embedded histological sections were treated with a proteinase K (Roche, Basel, Switzerland) solution at 37 °C for up to 60 min. After the proteolysis treatment, the slides were incubated overnight at 40 °C with a hybridization mixture containing the target probe (sequence: 5′-TTTAATAACTCGTTATATATATCAGTGTAGCAC-3′) labelled with digoxigenin at the 3′ end (Eurofins MWG Operon, Ebersberg, Germany). The probe was designed to target the 18S rRNA strand and is specific for avian *Plasmodium* spp. (Dinhopl et al., 2015). After hybridization, the slides were incubated with antidigoxigenin-AP Fab fragments (Roche) for about 60 min at room temperature. Colour substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) (Roche) were used for the visualization of the hybridized structures.

2.4. Laser microdissection

Laser microdissection was carried out using an Olympus IX71 light microscope (Olympus, Tokyo, Japan) equipped with the Olympus/MMI CellCut Plus laser system and the PTP function software (Predefined Target Position, Molecular Machines and Industries, Zurich, Switzerland) (**Paper VII**). Adjusting the contrast allowed for easy visualization of haemosporidian parasites in the histological sections on the paraffin membranes. Dissected parts of the histological sections were removed from the membranes using adhesive silicone caps of the MMI IsolationCap test tubes (Molecular Machines and Industries, Zurich, Switzerland). The microdissections were then used for haemosporidian parasite genus identification by PCR-based techniques (**Paper VII**).

2.5. Morphological analysis

Blood films were examined microscopically for screening and parasite identification purposes. An Olympus BX51 light microscope (Olympus, Tokyo, Japan) equipped with the Olympus DP12 digital camera and imaging software Olympus DP-SOFT (Olympus, Tokyo, Japan) (**Papers I, V, VI, VIII**) and an Olympus BX61 light microscope (Olympus, Tokyo, Japan) with the Olympus DP70 digital camera and the imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) (**Paper II-V**, **VII**) were used to examine the collected material. Blood films were initially examined at low magnification (x400), followed by examination at high magnification (x1000) (**Papers I-VIII**). Parasitaemia was calculated as a percentage by actual counting number of parasites per 1000 erythrocytes (or 10 000 if the intensity was light) (Godfrey et al., 1987).

2.6. Histological analysis

2.6.1. Haematoxylin-eosin stained preparations

Histological preparations stained with H&E were examined using an Olympus BX51 light microscope (Olympus, Tokyo, Japan) with the Olympus DP12 digital camera and imaging software Olympus DP-SOFT (Olympus, Tokyo, Japan) (**Papers I, II, V-VIII**) and an Olympus BX61 light microscope (Olympus, Tokyo, Japan) with the Olympus DP70 digital camera and the imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) (**Papers III, IV**). All histological preparations were

initially examined at low magnification (x200), followed by examination medium (x400) and then at high magnification (x1000) (**Papers I-VIII**). Bone marrow preparations were examined as blood films (see *2.5. Morphological analysis*).

2.6.2. Chromogenic in situ hybridization processed preparations

Chromogenic *in situ* hybridization processed preparations were examined using an Olympus BX51 light microscope (Olympus, Tokyo, Japan) with the Olympus DP12 digital camera and imaging software Olympus DP-SOFT (Olympus, Tokyo, Japan). All histological preparations were initially examined at low magnification (x200), followed by examination medium (x400) and then at high magnification (x1000) (**Papers I, IV-VI**).

2.7. Molecular analysis

2.7.1. DNA extraction from blood and polymerase chain reaction

Total deoxynucleic acid (DNA) was extracted from blood samples stored in SET buffer using an ammonium-acetate protocol (Sambrook et al., 1989) (**Papers I-VIII**). For parasite lineage identification, a nested PCR protocol (Bensch et al., 2000; Hellgren et al., 2004) was applied (**Papers I-VIII**). Partial mitochondrial cytochrome *b* gene (*cytb*) was amplified using the two primer pairs: HaemFNI/ HaemNR3 (Hellgren et al., 2004), and HaemF/HaemR2 (Bensch et al., 2000).

2.7.2. DNA extraction and PCR from single megalomeronts obtained by laser micro-dissection of histological preparations

DNA from single megalomeronts of *Haemoproteus* parasites were obtained using the laser micro-dissected material. Chelex (Bio-Rad, Hercules, California) DNA extraction protocol was used (Palinauskas et al., 2010). Briefly, 25 µl of Chelex solution (0.2 g of Chelex suspended in 1 ml, incubated at 56°C for 60 min.) was placed on the wall of each MMI IsolationCap test tube (Molecular Machines and Industries, Zurich, Switzerland) with adhesive silicone cap containing the micro-dissected material. Then, 0.7 µl of Proteinase K (10 mg/ml) was added to each drop of Chelex. The tubes were then closed and flipped upside down making sure the Chelex/Proteinase K mixture covered the entire adhesive surface of the test tube cap. Upside down tubes were incubated in a 56 °C water bath for 60 min. followed by 12 min. incubation at 95 °C. The tubes were then spun down and the adhesive silicone

caps were replaced with regular caps. The test tubes were then centrifuged at 13.400 rpm for 12 min. The supernatant was immediately used for PCR. Total volume of the performed PCR was 25 µl and it consisted of 12.5 µl of DreamTaq Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 µl of nuclease-free water, 1 µl of each primer and 2 µl of DNA template. Primer pair HQF/HQR was used for this amplification of partial *cytb* sequence and the PCR was performed as described by Ciloglu et al. (2019) (**Paper VII**).

2.7.3. DNA sequencing

Success of the carried PCRs was evaluated by running electrophoresis using 2 µl of the final product of each PCR on a 2% agarose gel (**Papers I-VIII**). Amplified positive samples were sequenced from both 3' and 5' ends with respective primers, using the dye terminator cycle sequencing (BigDye) on an ABI PRISM TM 3100 capillary sequencing robot (Applied Biosystems, USA) (**Papers I, II, IV-VIII**).

2.8. Phylogenetic analysis

Sequences we edited and analysed using the BioEdit (Hall, 1999) software (**Papers I-VIII**). Possible mix infections were identified by the presence of double peaks in the electropherograms of the sequences. Genetic lineages were identified by applying the 'Basic Local Alignment Search Tool' (megablast algorithm) (NCBI BLAST, 2019 https://blast.ncbi.nlm.nih.gov/Blast.cgi); they were double checked in the MalAvi database using the database local BLAST (Besnch et al., 2009). Bayesian phylogenetic trees were constructed using the MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003) software (**Papers II, IV, V, VII, VIII**). Best fit model for each tree was selected by the MrModeltest 2 software (Nylander, 2004) (**Papers II, IV, V, VII, VIII**). Each analysis was run for a total of 10 million generations with a sample frequency of every 100th generation. Before the consensus tree was constructed, 25% of the initial trees were discarded as the 'burn in' periods (**Papers II, IV, V, VII, VIII**). The trees were visualized using the software FigTree v1.4.3 (Rambaut, 2009) (**Papers V, VII, VIII**) and TreeView 1.6.6 (https://treeview.software.informer.com/1.6/) (**Papers II, IV**). Genetic distance between lineages was calculated by implementing the Jukes-Cantor model of substitution in the MEGA 5.0 (Tamura et al., 2011) (**Papers II, IV**) and the MEGA 7.0 (Kumar et al., 2016) (**Papers V, VII**) software.

2.9. Statistical analysis

"Statistica 7" (**Papers I, III-V**) and "R studio" software (R Core Team, 2013) (**Papers VI-VIII**) were implemented for the statistical analyses. Normality of the data distributions were tested using the Shapiro-Wilk test. Differences between the means for data which were not distributed according to normal distribution were evaluated using the Wilcoxon test. Fisher's exact test was applied to evaluate the statistical significance of the same parameters in the experimental and control groups.

2.10. Experimental design

Prior to the experimental infections (**Papers I, II, IV-VI, VIII)**, all birds were checked for possible natural infections with haemosporidian parasites using microscopic examination of blood films while in the field. None of the birds used in the conducted experimental studies were naturally infected with haemosporidian parasites. The negative infection status was later confirmed by PCR-based techniques in the laboratory. Experimental and control groups of birds were maintained at identical conditions (ambient temperature, natural photoperiod, same housing facilities and feeding conditions). None of the control birds developed parasitaemia during experiments, indicating that no natural transmission of the infections took place during this study where the birds were kept.

The experimental birds were infected using a mixture containing *Plasmodium* sp. infected blood of the donor bird, sodium citrate and 0.9% saline solution (Palinauskas et al., 2008). The freshly prepared mixture was sub-inoculated into the pectoral muscle of the recipient birds (**Papers I, II, IV-VI, VIII**). Blood of experimental and control birds was checked to follow parasitaemia at predetermined intervals (**Papers I, II, IV-VI, VIII**). Additionally, the body mass and haematocrit value were measured in all birds at the same time intervals (**Papers VI, VIII**).

2.11. Ethical statement

All procedures with birds were performed by licensed researchers. All described experimental procedures were approved by the International Research Co-operation Agreements between the Biological Station Rybachy, Kaliningrad, Russia and Nature Research Centre, Vilnius, Lithuania (№ 25-05-2010, **Papers I, II, IV, VI, VIII;** № 2015-09-04, **Paper V**); by the Forest

and Nature Protection Agency of Kaliningrad Region, Kaliningrad, Russia (№ 18, 5-05-2016, **Papers VI, VIII**); by the Lithuania and Environmental Protection Agency, Vilnius, Lithuania (permits: 2015-04-08, no. 21, **Paper IV**; 2015-04-27, no. 25, **Paper IV;** 2017-04-26, no. 23, **Paper V;** and 2018-04-13, no. 24, **Paper VII**); by the Lithuanian State Food and Veterinary Office (permit ref. no. 2012/01/04-0221, **Paper II;** and 2015-05-07, no. G2-27, **Papers IV, V**). All possible efforts were made to minimize the handling time and potential suffering of birds. None of the experimental birds suffered noticeable injury during the experimental work.

3. RESULTS AND DISSCUSSION

In depth description of the results and the discussion of data were published and are available in papers, which make up the foundation of this thesis (**Papers I-VIII**). Provided below are the summaries of the dissertation results corresponding to each task of the study.

3.1. Development of *Plasmodium homocircumfexum* (lineage pCOLL4) in common European passerine birds

Paper I described a pilot study using *Plasmodium homocircumfexum* (lineage pCOLL4) infection in birds belonging to three common European passeriform species. Single individuals of Eurasian siskin *Carduelis spinus*, common crossbill and common starling were successfully infected with *P. homocircumflexum* (lineage pCOLL4). All three individuals survived the peak of parasitaemia but died suddenly when parasitaemia had decreased (**Paper I**, Fig. 1). A post-mortem examination revealed an enlargement of the spleen and liver, as well as cardiomegaly with dark pericardial effusions in each individual. Phanerozoites of *P. homocircumflexum* developed in all three exposed birds. Numerous phanerozoites were located in the brain, heart, liver, kidney, spleen and breast muscle of each host. Importantly, phanerozoites developing in the brain followed the shape of brain capillaries, in some cases completely blocking the blood vessel and thus, interrupting the blood circulation (**Paper I**, Fig. 2).

The dynamics of the *P. homocircumflexum* (lineage pCOLL4) parasitaemia in all three exposed individuals were different, but the phanerozoite development and caused tissue pathologies were similar. This coincided with the results of the experimental study carried out using this pathogen and domestic canaries (Palinauskas et al., 2015). These results show the ability of *P. homocircumflexum* to produce phanerozoites in many species of susceptible birds. Ischaemic brain changes, leading to cerebral paralysis is one of the most severe pathologies caused by avian malaria parasites and it is the most likely reason of the reported sudden death of the exposed birds (Garnham, 1980; Valkiūnas, 2005). Mortality of the exposed birds was sudden, with no signs of deteriorating bird health.

Bird mortality during malaria has been linked to two main factors – high parasitaemia and damage due to developing phanerozoites (Coulston and Manwell, 1941; Huchzermeyer and Van der Vyver, 1991; Valkiūnas, 2005).

It is widely assumed that the decrease of parasitaemia intensity leading to the chronic state of the infection signifies an improvement of the infected host health (Atkinson et al., 2008; Castro et al., 2011; Vanstreels et al., 2015). However, this study shows that this is not always true as birds might die due to pathologies caused by phanerozoites, which occur even during light chronic infections. The role of exo-erythrocytic meronts in regard to the virulence of avian malaria is certainly underestimated. Mortality due to these phanerozoite infections is rapid and difficult to detect in the wild. While it is clear that widespread *Plasmodium* parasites can cause mortality in common wild European birds (Dinhopl et al., 2015), rates of such mortality remain insufficiently understood and require further studies.

3.2. Patterns of virulence of *Plasmodium homocircumflexum* (lineage pCOLL4) in experimentally exposed passerine birds

Data of the pilot study (**Paper I**) called for more detailed study on virulence of *P. homocircumflexum.* In **Paper VI**, an experimental study stemming from the positive results of the pilot study is described. Bird groups (8 experimental and 8 control birds in each) belonging to four species of common European passerine birds – common crossbills, common starlings, house sparrows and common chaffinches *Fringilla coelebs* were exposed to *P. homocircumflexum* (lineage pCOLL4). All exposed birds developed parasitemia (**Paper VI,** Table 1). Details on the dynamics of the average parasitaemia, average haematocrit value and average body mass during the course of the infection can be seen in Fig. 2 and Table 1 (**Paper VI)**. It is worth noting that in all four host species, the decrease in average haematocrit value coincided with the increases of parasitaemia. Interestingly, markedly different changes in body mass occurred among the different infected host species, ranging from increase (common starlings and house sparrows) to decrease (common crossbills and common chaffinches).

Phanerozoites developed in the brain, liver, kidney, spleen, heart and breast muscle of many of the exposed avian hosts, except for the starlings (**Paper VI**, Table 1). Absence of phanerozoites in the infected common starlings was confirmed by ISH tests. Phanerozoites have developed in the brain of the exposed common crossbills, house sparrows and common chaffinches which died after 18 days post exposure (dpe). Phanerozoites were not reported in the brain of the exposed individuals of the same three species which either died before 18 dpe or survived until the end of the study.

In general, *P. homocircumflexum* has been reported in 14 distantly related species of birds (Durrant et al., 2006; Pérez-Tris et al., 2007; Palinauskas et al., 2015; present study), indicating that this is an euryxenous *Plasmodium* parasite able to infect many different hosts. However, it was unclear if all these infections are competent (develop gametocytes). This experimental study shows that *P. homocircumflexum* is truly euryxenous. This is important in regard of epidemiology of this infection and development of its prevention measures.

Avian malaria can cause blood pathology due to destruction of red blood cells (Garnham, 1966; Valkiūnas, 2005) and/or damage of bone marrow cells and resulting in disruption of the erythropoiesis (Garnham, 1966; Palinauskas et al., 2015; Palinauskas et al., 2018). In the present study, phanerozoites were not observed in the bone marrow, however many birds developed relatively high parasitaemias (**Paper VI**, Fig. 2). Anaemia was caused by the direct destruction of erythrocytes by the developing parasites and the removal of the infected red blood cells in the liver and spleen. The decrease in haematocrit value in infected birds coincides with results of earlier studies on different avian *Plasmodium* parasites (Permin and Juhl, 2002; Paulman and McAllister, 2005; Williams, 2005; Palinauskas et al., 2008).

Interestingly, body mass of the exposed common starlings and house sparrows increased significantly compared to the controls. It is probable that some species of avian hosts might increase their food consumption in order to compensate for the stress caused by the symptoms of malaria. However, decrease of body mass was observed in exposed common crossbills and common chaffinches (**Paper VI**, Fig 2), indicating that this might be a species related mechanism coping with the disease. This study suggests that increase in body mass is not always an indication of improvement in bird health during avian malaria (**Paper VI**).

This is the first study reporting different exo-erythrocytic development of the same lineage of parasite in different species of avian hosts. Phanerozoites developed in individuals of three of the four exposed bird species. This study (**Paper VI**) supplements and expands the data on phanerozoite development reported in **Paper I**. Mainly, available data indicate that there is some variation in timing of the phanerozoite development, but they appear in the tissues at some point between 10 and 20 dpe. Additionally, there is a certain period of parasite development in the blood before erythrocytic merozoites develop ability to infect the reticuloendothelial cells in the internal organs. Furthermore, the obtained results support former

observations that phanerozoites developing in the brain are important cause of death during *P. homocircumflexum* infections as is the case during *P. gallinaceum* infections in domestic chickens as well as several other malaria infections (Valkiūnas, 2005).

3.3. Exo-erythrocytic development of *Plasmodium elongatum* (lineage pERIRUB01)

In **Paper II,** the experimental investigation of exo-erythrocytic development of *Plasmodium elongatum* (lineage pERIRUB01) was described. Seven domestic canaries were exposed to *P. elongatum* infection. All exposed domestic canaries were susceptible and developed parasitaemia. A relatively high parasitaemia (9%) developed in one exposed domestic canary, but the average maximum parasitaemia in other six exposed individuals was 0.2%. Five of the seven exposed domestic canaries died during this study. Histological examination of two deceased birds revealed that numerous phanerozoites developed in the bone marrow cells of both individuals which died on 14 and 46 dpe. Additionally, a few phanerozoites were reported in the liver and spleen of the examined domestic canaries (**Paper II**, Fig. 3).

Previous studies have shown that *P. elongatum* can cause severe pathologies and even mortality of avian hosts due to developing phanerozoites (Garnham, 1966; Valkiūnas, 2005) however, the genetic lineage of *P. elongatum* was not identified in those studies. The same result was observed in the present study with pERIRUB01 lineage, where maximum parasitaemia was relatively low (9% in 1 bird, but usually about 0.2%), however 70% of exposed individuals died. Massive destruction of cells of erythrocytic series in the bone marrow has been previously reported during *P. elongatum* infections (Garnham, 1966; Corradetti et al., 1968). Results of this study (**Paper II**) suggest that anaemia caused by the destruction of bone marrow cells by phanerozoites is the main reason of mortality of the avian hosts during *P. elongatum* (lineage pERIRUB01) infection.

3.4. Exo-erythrocytic development of *Plasmodium elongatum* (lineage pGRW6)

In **Paper VIII,** the experimental investigation of the exo-erythrocytic development *Plasmodium elongatum* (lineage pGRW6) was described in two species of common European passerine birds – common crossbill and common starling. All exposed common starlings were resistant to the *P.*

elogatum (lineage pGRW6) infection according to the microscopic examination and PCR-based testing, but all common crossbills were susceptible. Average parasitaemia reached a peak of 0.3% on 20 dpe (**Paper VIII**, Fig. 1). Despite the low parasitaemia, a statistically significant ($p<0.001$) short time decrease in haematocrit value was reported in the common crossbills (**Paper VIII**, Fig. 1). The decrease of the haematocrit value coincided with the increases of parasitaemia. However, the haematocrit value reached the normal level relatively soon, after 7-8 days (**Paper VIII**, Fig. 1). No significant changes in body mass of the exposed common crossbills were reported. All *P. elongatum* (lineage pGRW6) exposed common crossbills survived until the end of the study. Few phanerozoites were reported in the bone marrow of five out of 8 exposed common crossbills (< 5 parasites were seen in the majority of phanerozoite-positive birds). Besides the bone marrow, no phanerozoites were reported in other organs of the exposed common crossbills.

Plasmodium elongatum (lineage pGRW6) has never been reported in common starlings in spite of global distribution of this invasive bird species (Bensch et al., 2009). Present study (**Paper VIII**) is in accord with the results of former observations suggesting a complete or partial resistance of common starlings to some species of avian malaria parasites (Bennett et al., 1982; Bishop and Bennett, 1992; Valkiūnas, 2005; Atkinson et al., 2008; Palinauskas et al., 2008; Ilgūnas et al., 2019). The ability of this bird species to resist or tolerate cosmopolitan malaria infections might be one of the factors allowing the common starling to successfully spread globally (BirdLife International, 2016). However, mechanisms responsible for the reported resistance remain unclear. The "common starling – *P. elongatum* (lineage pGRW6)*"* host-parasite model could be used for research of the innate resistance to malaria.

Only one to five phanerozoites were reported in the bone marrow of 80% of phanerozoite-positive common crossbills. Such results indicate low secondary exo-erythrocytic merogony, and this might be beneficial to the parasite because phanerozoites often cause severe pathologies in avian hosts (Garnham, 1966; Ilgūnas et al., 2016; Palinauskas et al., 2016; Ilgūnas et al., 2019). Thus, the ability to resist the development of secondary exo-erythrocytic meronts could explain the low virulence of *P. elongatum* (lineage pGRW6) in common crossbills, a bird species known to be markedly susceptible to other malaria agents, such as *P. relictum* (lineage pSGS1) and

P. homocircumflexum (lineage pCOLL4) (Palinauskas et al., 2008; Ilgūnas et al., 2016; Palinauskas et al., 2016; Ilgūnas et al., 2019).

Results of the present study (**Paper VIII**) are in accordance to former observations, which showed that parasitaemia is often light during *P. elongatum* infections (Garnham, 1966; Corradetti et al., 1968; Valkiūnas, 2005; Palinauskas et al., 2016). This is likely a beneficial adaptation for survival of the parasite in long perspective: blood pathology decreases in cases of light parasitaemia, and that might contribute to better survival of the host and thus, the parasite (Valkiūnas, 2005; Mukhin et al., 2016; Granthon and Williams, 2017).

Decrease in haematocrit value is a well-known feature during *Plasmodium* infections in birds during the peak of parasitaemia (Permin and Juhl, 2002; Paulman and McAllister, 2005; Williams, 2005 Palinauskas et al., 2008; Ilgūnas et al., 2019). However, this is the first study to report the same observation during *P. elongatum* infection (**Paper VIII**, Fig 1). In the past, it was believed that the decrease of haematocrit value during *P. elongatum* infections was strictly related to the pathologies caused by phanerozoites in cells of the hematopoietic system, which are responsible for erythropoiesis (Garnham, 1966; Corradetti et al., 1968; Bensch et al., 2009; Palinauskas et al., 2016). This certainly is true for *P. elongatum* (lineage pERIRUB01) infection (**Paper II**). However, *P. elongatum* (lineage pGRW6) developed only few (if at all) phanerozoites in the bone marrow cells, but the haematocrit value decreased significantly if compared to the control group. This decrease in haematocrit value was short-term, but the blood pathology caused by *P. elongatum* (lineage pGRW6) still occurs and should be considered in studies aiming virulence of this lineage.

3.5. Exo-erythrocytic development of *Haemoproteus majoris* (lineages hPHYBOR04 and hPARUS1)

Paper VII describes the study on the complete life cycle of *Haemoproteus majoris* (lineages hPHYBOR04 and hPARUS1) with emphasis on the exo-erythrocytic development of this widespread avian parasite.

Large (360 µm in diameter), roundish, covered by a thick capsule-like wall exo-erythrocytic meronts at different stages of development were reported in the histological kidney preparations of a wild-caught fieldfare *Turdus pilaris* infected with *H. majoris* (lineage hPHYBOR04). The megalomeronts contained irregular-shaped cytomeres, in which merozoites

developed. Numerous morphologically similar megalomeronts were observed in the histological sections of the liver, lungs, spleen, and kidneys of great tit *Parus major* naturally infected with *H. majoris* (lineage hPARUS1). To confirm the genetic identity of the megalomeronts with the parasites visible in the blood, laser microdissection was applied, followed by DNA extraction, PCR and sequencing. Three individual megalomeronts from the infected great tit were laser-dissected (**Paper VII**, Fig. 1) and analysed using DNA sequence information. In all three cases, the detected sequences coincided with that of the hPARUS1 lineage, indicating that megalomeronts are exo-erythrocytic stage of *H. majoris*.

Exo-erythrocytic stages of avian haemoproteids were reviewed by Valkiūnas and Iezhova (2017). The morphology of the *H. majoris* megalomeronts described in this study is unique (**Paper VII,** Fig. 4 and Fig. 5) when compared to other avian haemoproteids. Due to the features like the prominent thick capsule-like wall and the markedly developed irregular-shaped cytomeres, the reported parasites are similar to some *Leucocytozoon* spp. (Valkiūnas, 2005) or even *Besnoitia* parasites (Bennett et all., 1993). However, genetic identification confirmed that the detected megalomeronts indeed belong to *H. majoris*.

Previously, megalomeronts have been reported in 8 species of avian *Haemoproteus* parasites (Valkiūnas and Iezhova, 2017) however, these were mainly case reports in non-passerine birds (Wenyon, 1926; Farmer, 1964; Peirce, 1976; Burtikashvili, 1978; Miltgen et al., 1981; Atkinson, 1986; Atkinson, 1988; Earlé et al., 1993; Cordona et al., 2002; Peirce et al., 2004; Olias et al., 2011; Ortiz-Catedral et al., 2019). Moreover, it remained unclear if megalomeronts developed only in non-adapted (wrong) avian hosts or these stages occur during the normal life cycles of *Haemoproteus* parasites, including widespread passerines. This study (**Paper VII**) shows that *Haemoproteus* megalomeronts are normal stage of the life cycle in some haemoproteid species, including widespread *H. majoris*. The presence of mature gametocytes of *H. majoris* in megalomeront-positive birds indicates complete life cycle of the parasite. Megalomeronts of *Haemoproteus* parasites are worth more attention for better understanding pathologies caused during haemoproteosis.

3.6. Exo-erythrocytic development of *Plasmodium relictum* (lineage hPHCOL01)

Paper V presents results of the study on the new lineage pPHCOL01, which was attributed to *Plasmodium relictum* and characterised both morphologically and molecularly during this study. This lineage was found in a naturally infected common chiffchaff *Phylloscopus collybita* and experimentally passaged in two domestic canaries, two European goldfinches *Carduelis carduelis*, two zebra finches *Taeniopygia guttate* and one budgerigar *Melopsittacus undulatus*. Parasitaemia developed in the domestic canaries and the European goldfinches. The two zebra finches and the one budgerigar resisted the infection. At the end of the study the donor common chiffchaff and the two recipient domestic canaries were euthanised and the brain, liver, kidney, spleen, heart and a piece of the breast muscle were collected for histological analysis. Neither the traditional histological examination, nor the ISH revealed any exo-erythrocytic meronts in the studied vertebrate hosts.

Studies conducted during the 'pre-molecular' era have reported tissue pathologies related to exo-erythrocytic development of *P. relictum* (Garnham, 1966; Garnham, 1980; Valkiūnas, 2005; Atkinson et al., 2008). However, information on the genetic identity of the lineages used in those studies is absent. To date, there is no information on phanerozoite development of different lineages of *P. relictum*. This shortcoming clouds the understanding of persistence and pathogenicity of this parasite. Results of this study suggest that the development of large multicellular, easy to find using light microscopy phanerozoites (Garnham, 1966; Ilgūnas et al., 2016) of *P. relictum* might greatly depend on the stage of the infection. These stages might persist only temporarily, so are not easy to detect. Additionally, it is possible that *P. relictum* might develop uninuclear hypnozoite stages similar to the case during human malaria *P. vivax* and *P. ovale* infections. Even though ISH is a promising method that has recently been introduced to the field of avian haemosporidian research (Dinhopl et al., 2011; Dinhopl et al., 2015; Ilgūnas et al., 2016), the available diagnostic protocols might be insufficiently sensitive to detect these single cell intracellular stages. More sensitive methodologies and techniques such as immunofluorescent diagnostic methods might need to be applied in addition to traditional histology and ISH (Atkinson et al., 2008; Dinhopl et al., 2011; Dinhopl et al., 2015; Ilgūnas et al., 2016; Valkiūnas et al., 2016) in order to answer these questions.

3.7. Exo-erythrocytic development of *Plasmodium delichoni* (lineage pCOLL6)

Paper IV presents the study in which a new species of *Plasmodium* belonging to the subgenus *Novyella* – *Plasmodium delichoni* (lineage pCOLL6) was described. This parasite was found in a naturally infected barn swallow *Delichon urbica* and was experimentally passaged into two domestic canaries and one Eurasian siskin. At the end of the study, all exposed avian hosts were euthanised and the brain, liver, kidney, spleen, heart and a piece of the breast muscle were collected for histological analysis. Neither the traditional histological examination nor ISH revealed any exo-erythrocytic meronts in the examined vertebrate birds.

It is important to note that exo-erythrocytic development of parasites belonging to the subgenus *Novyella* is investigated only fragmentally. In most available studies, only the erythrocytic stages have been described for this group of parasites (Garnham, 1966; Valkiūnas, 2005; Atkinson et al., 2008; Mantilla et al., 2013). Due to the fact that microscopic examination of histological preparations and preparations treated using ISH did not reveal any phanerozoites, it is possible that *P. delichoni* might not develop secondary exo-erythrocytic meronts. It appears possible that phanerozoites might not develop at least in some parasites of the subgenus *Novyella*. Phanerozoites were reported in *P. nucleophilum toucani*, *P. paranucleophilum*, *P. bertii* and *P. vaughani* (Garnham, 1966; Nelson, 1966; Manwell and Sessler, 1971a; Manwell and Sessler, 1971b; Gabaldon and Ulloa, 1981; Valkiūnas, 2005) however, phanerozoites were absent during the development of *P. columbae*, *P. rouxi*, *P. hexamerium* and *P. kempi* (Manwell, 1951; Garnham, 1966; Gabaldon and Ulloa, 1976; Christensen et al., 1983).

One of the key roles phanerozoites play during avian malaria infections is the persistence of the infection in the vertebrate host (Garnham, 1980; Valkiūnas, 2005; Atkinson et al., 2008). *Plasmodium malaria*, a blood parasite of humans, persists in the host due to long-lasting light parasitaemia (Sherman, 1998). It cannot be ruled out that similar mechanisms of persistence could occur in *P. delichoni* and some other *Plasmodium* spp., however, this assumption needs further investigation.

3.8. Exo-erythrocytic development of *Haemoproteus ciconiae*

Paper III describes a case study, during which a new species of *Haemoproteus* – *Haemoproteus ciconiae* from the white stork *Ciconia ciconia*

was discovered and described. A mortally wounded white stork was brought to the fieldwork station of Ventės ragas. After the veterinary examination, it was concluded that due to severe damage and week body condition the bird would be impossible to treat, and it was euthanised. Blood samples for haemosporidian parasite infection screening were collected, and samples of the liver, kidney, spleen, heart and a piece of the breast muscle were collected for histological analysis. Microscopic examination of blood films revealed a *Haemoproteus* sp. infection. This parasite was later described as a new species – *H. ciconiae* (**Paper III**, Fig. 1). No exo-erythrocytic stages were reported during the histological analysis of the tissue sections.

This study reports the first species of genus *Haemoproteus* infecting the white stork. Even though no exo-erythrocytic meronts were reported in this study, it is impossible to conclude with certainty as these stages might be difficult to detect in *H. ciconiae*. Recently, new ISH markers have been developed for detection of *Haemoproteus* parasites (Himmel et al., 2019), and are worth applying to research this question. Additional studies of the *H. ciconiae* exo-erythrocytic development is needed.

CONCLUSIONS

1. Experimental observations have proved that malaria parasite *Plasmodium homocircumflexum* (lineage pCOLL4) is an euryxenous parasite, which readily develops secondary exo-erythrocytic stages (phanerozoites) in many tested species of avian hosts.
2. Mortality of birds infected with *P. homocircumflexum* (lineage pCOLL4) malaria is mainly due to ischaemic changes caused by the developing phanerozoites in the brain, resulting in cerebral paralysis.
3. The ability of the same lineage of a *Plasmodium* parasite to produce phanerozoites in different species of avian hosts might vary, resulting in different virulence, as is the case during *P. homocircumflexum* (pCOLL4) infection.
4. Malaria parasite *Plasmodium elongatum* (new lineage pERIRUB01) is highly virulent and often lethal in domestic canaries due to severe anaemia and damage of stem bone marrow cells by numerous developing phanerozoites.
5. Malaria parasite *Plasmodium elongatum* (lineage pGRW6) is unable to develop in common starlings, which are resistant, and, due to the low ability to develop phanerozoites, is of low virulence in some adapted avian host.
6. Megalomeronts were discovered in *Haemoproteus majoris,* a cosmopolitan haemosporidian parasite. The new lineage hPHYBOR04 and the widespread lineage hPARUS1 of this species developed similar megalomeronts in naturally infected birds, indicating that the megalomeront is a normal stage of the life cycle of *H. majoris*.
7. Two new species of haemosporidian parasite were discovered and described – malaria parasite *Plasmodium delichoni* n. sp. (Plasmodiidae) and haemoproteid *Haemoproteus ciconiae* n. sp. (Haemoproteidae), which parasitize passeriform birds and the white stork, respectively.

SCIENTIFIC AND PRACTICAL SIGNIFICANCE

1. This study contributed new knowledge for better understanding mechanisms of mortality during avian malaria, indicating directions for application of data about exo-erythrocytic development in avian medicine and healthcare for disease control. In particular, it was shown that decreased parasitemia is not always an indication of recovery and improved health during avian malaria. Phanerozoites might appear and kill birds at any stage of a long-lasting light chronic parasitemia. Thus, it is crucial to develop anti-phanerozoite malaria treatment, which remains insufficiently effective.
2. The discovery of megalomeronts in natural *Haemoproteus* infections further challenges the former assumption about non-hazard nature of these cosmopolitan parasites for birds, calling for more attention to haemoproteisis in veterinary medicine and conservation biology.
3. In spite of application of sensitive diagnostic tools, phanerozoites were not found during experimental infections with *Plasmodium relictum* (lineage pPHCOL01) and *Plasmodium delichoni* (lineage pCOLL6). These results indicate directions for future research on exo-erythrocytic development of malaria pathogens. Mainly, it seems possible that some avian *Plasmodium* parasites might persist on unicellular hypnozoite-like stage (*P. relictum*) or even do not develop phanerozoites in experimental birds and persist mainly due to long-lasting parasitemia (*P. delichoni*), as is the case during infections with *Plasmodium malariae* in humans. To answer these questions, we suggest application of more sensitive diagnostic tools, which provide opportunities to detect the parasites on hypnozoite levels.
4. After conducting experimental infections it was discovered that cmmon starlings are resistant to *Plasmodium elongatum* (lineage pGRW6) infection. The "common starling – *P. elongatum* (lineage pGRW6)" host-parasite model is recommended to use in research aiming better understanding mechanisms of innate resistance during malaria.
5. This study was carried out using common European birds and cosmopolitan haemosporidian parasites. Thus, the obtained results can be applied in broader avian haemospordian biology, pathology, ecology and biodiversity research globally, particularly across Europe.

SANTRAUKA

MOKSLINĖ PROBLEMA

Hemosporodiniai parazitai, priklausantys *Plasmodium* ir *Haemoproteus* gentims, kurių transmisija vyksta visuose žemynuose išskyrus Antarktidą, yra žinomi dėl savo gebėjimo sukelti sunkias, tam tikrais atvejais mirtinas, ligas stuburiniams šeimininkams (Atkinson et al., 2008; Marzal, 2012). Kai kurios šių parazitų rūšys yra virulentiškos ir savo pernešėjams– kraujasiurbiams dvisparniams (Valkiūnas et al., 2014; Bukauskaitė et al., 2016). Nors šie patogenai yra tiriami jau daugelį metų, informacijos apie absoliučios daugumos rūšių gyvybinius ciklus trūksta. Ypatingai menkai ištirtas egzoeritrocitinis šių parazitų vystymasis (Valkiūnas ir Iezhova, 2017) yra didelė kliūtis hemosporidijų epidemiologijos ir virulentiškumo suvokimui.

Remiantis morfologiniais požymiais, *Plasmodium* gentyje yra aprašytos penkiasdešimt penkios rūšys (Valkiūnas ir Iezhova, 2018). Pastaraisiais metais paukščių maliarijos virulentiškumo klausimas sulaukė daug tyrėjų dėmesio, tačiau dauguma tyrimų buvo atlikta naudojant tiktai sąlyginai lengvai prieinamas *Plasmodium* rūšių vystymosi stadijas kraujyje (Palinauskas et al., 2008; Palinauskas et al., 2009; Cellier-Holzem et al., 2010; Bichet et al., 2012; Cornet et al., 2014; Ellis et al., 2015). Atlikti tyrimai suteikė svarbios informacijos apie hemosporidinių parazitų sukeliamas kraujo patologijas, tačiau dėl žinių apie egzoeritrocitinį vystymąsi trūkumo šių parazitų virulentiškumo mechanizmai lieka nepakankamai ištirti. Dauguma darbų, skirtų egzoeritrocitiniam hemosporidijų vystymuisi, buvo atlikti XX a. pirmoje pusėje (Valkiūnas ir Iezhova, 2017) – laikotarpiu, kai molekuliniai diagnostikos metodai dar nebuvo taikomi paukščių hemosporidinių parazitų srityje. 1995 metais, pradėjus taikyti molekulinius metodus, buvo nustatyta jog tiek tarprūšinė, tiek vidurūšinė paukščių hemosporidinių parazitų įvairovė yra daug didesnė nei manyta anksčiau (Feldman et al., 1995; Ricklefs et al., 2004; Bensch et al., 2009). Atskleidus milžinišką genetinę *Plasmodium* genties parazitų įvairovę tapo aišku, jog tam tikrų maliarijos sukėlėjų rūšių egzoeritrocitimį vystymąsi išaiškinti bus sunku, ir norint gauti atsakymus į šiuos klausimus, reikės atlikti eksperimentinius darbus bei vystyti ir taikyti modernius tyrimo metodus.

Žinomos dvi maliarijos infekcijų sukeliamų patologinių pažeidimų grupės: kraujo patologijos (Hayworth et al., 1987; Valkiūnas, 2005) ir vidaus organų pažeidimai dėl besivystančių egzoeritrocitinių merontų, ypatingai antrinės egzoeritrocitinės merogonijos merontų – fanerozoitų (Garnham,

1966; Valkiūnas, 2005; Vanstreels et al., 2015). Paukščių maliarijos sukėlėjų merozoitai, besivystantys pirminiuose egzoeritrocitiniuose merontuose (metakriptozoituose) ir eritrocitiniuose merontuose, gali indukuoti antrinę egzoeritrocitinę merogoniją (fanerozoitų vystymąsi). Fanerozoitai gali vystytis retikuloendotelinėse ląstelėse (makrofaguose, kapiliarų endotelinėse ląstelėse, Kupferio ląstelėse) įvairiuose stuburinio šeimininko organuose įskaitant smegenis, širdį, inkstus, kepenis, plaučius ir blužnį (Garnham, 1966; Fix et al., 1988; Valkiūnas, 2005; Vanstreels et al., 2014; Valkiūnas ir Iezhova, 2017). Tikėtina jog paukščių maliarija yra pavojingesnė infekcija nei manyta anksčiau, o fanerozoitų vystymosi dėsningumai nėra pakankamai ištirti. Dėl šios priežasties tyrimai, skirti geresniam *Plasmodium* genties parazitų egzoeritrocitinio vystymosi išaiškinimui, yra būtini.

Nauja maliarijos sukėlėjų rūšis *Plasmodium homocircumflexum* (genetinė linija pCOLL4) neseniai buvo aptikta ir aprašyta Europoje (Pérez-Tris et al., 2007; Palinauskas et al., 2015). Šis parazitas buvo išskirtas iš natūraliai užsikrėtusios paprastosios medšarkės *Lanius collurio*. Atliktas eksperimentinis tyrimas parodė jog dėl išsivystančių fanerozoitų šis patogenas gali būti mirtinas naminėms kanarėlėms *Serinus canaria* (Palinauskas et al., 2015). *Plasmodium homocircumflexum* užkrėstas kraujas buvo užšaldytas skystame azote ir yra saugomas Gamtos tyrimų centre Vilniuje, Lietuvoje. Nors šis parazitas geba vystytis paprastosiose medšarkėse ir sukelia mirtinas infekcijas eksperimentiškai užkrėstose naminėse kanarėlėse, nėra žinoma ar jis gali vystytis kituose laukiniuose paukščiuose, paplitusiuose Europoje. Tai yra svarbus klausimas į kurį būtina atsakyti norint geriau suprasti galimą *P. homocircumflexum* poveikį Europos paukščių populiacijoms.

Plasmodium elongatum yra vienas iš plačiausiai paplitusių maliarijos sukėlėjų (Garnham, 1966; Valkiūnas, 2005; Bensch et al., 2009). Šis parazitas buvo rastas paukščiuose iš 15 paukščių šeimų, priklausančių 11 būrių (MalAvi database, Bensch et al., 2009). Įdomu, kad per visa tyrimų istoriją *P. elongatum* niekada nebuvo rastas kelių, Europoje dažnai sutinkamų, žvirblinių paukščių rūšių individuose, pavyzdžiui iš paprastajame varnėne *Sturnus vulgaris* arba egliniame kryžiasnapyje *Loxia curvirostra* (Bensch et al., 2009). Priežastis, kodėl parazitas niekada nebuvo aptiktas šiuose paukščiuose, nėra žinoma. Genetinę liniją pGRW6 pirmą kartą išskyrė Beadell et al. (2004), o morfologiškai ji apibūdinta kaip *P. elongatum* prieš maždaug dešimt metų (Valkiūnas et al., 2008). Vidurūšinė šio parazito įvairovė taip pat buvo tirta (Bensch et al., 2009), tačiau nėra žinių apie skirtingų *P. elongatum* genetinių linijų virulentiškumą. Genų banke (NCBI GenBank, JAV) yra deponuota

keletas žinomoms *P. elongatum* genetinėms linijoms artimų genetinių sekų. Tikėtina jog šios neidentifikuotos genetinės linijos taip pat gali priklausyti *P. elongatum*, tačiau morfologinių įrodymų nėra. Jau seniai yra žinoma jog *P. elongatum* gali sukelti sunkias ligas ar net mirtį nelaisvėje gyvenantiems paukščiams (Fleischman et al., 1968; Herman et al., 1968; Beier ir Stoskopf, 1980; Beier ir Trpis, 1981; Cranfield et al., 1990; Graczyk et al., 1994), tačiau nėra aišku kurių genetinių linijų parazitai sukelia šias infekcijas. Dėl plataus *P. elongatum* paplitimo yra būtina išaiškinti skirtingų šio parazito genetinių linijų virulentiškumą ir jo mechanizmus.

Plasmodium relictum yra vienas iš geriausiai ištirtų maliarijos sukėlėjų. Šviesinės mikroskopijos metodu paremti tyrimai atlikti XX a. parodė jog *P. relictum* yra plačiausiai pasaulyje paplitęs maliarinis parazitas (Bennett et al., 1982; Bishop ir Bennett, 1992; Atkinson et al., 2008; Valkiūnas, 2005). Vėliau atlikti molekuliniai tyrimai atskleidė ir didelę vidurūšinę šio patogeno genetinę įvairovę. Nepaisant didelio atliktų tyrimų skaičiaus, egzoeritrocinis *P. relictum* vystymasis yra nepakankamai gerai ištirtas. Eksperimentiniai tyrimai, atlikti prieš pradedant taikyti molekulinius metodus paukščių maliarijos srityje, parodė jog *P. relictum* faneroizoitai vystosi užkrėstuose stuburiniuose šeimininkuose (Garnham, 1966; Garnham, 1980; Atkinson et al., 2008; Valkiūnas, 2005), tačiau informacijos apie tai, kokioms genetinėms *P. relictum* linijom jie priklauso, nėra. Šis informacijos trūkumas trukdo nustatyti kokiu būdu *P. relictum* išlieka ir kiek patogeniškos stuburiniame šeimininke yra skirtingos šio parazito genetinės linijos.

Paukščių hemosporidiniai parazitai priklausantys *Haemoproteus* genčiai yra vieni iš intensyviausiai tiriamų paukščių kraujo parazitų (Clark et al., 2014). Iki šiol, yra aprašyta daugiau kaip 150 šios genties parazitų rūšių, tačiau, lygiai kaip ir *Plasmodium* genties atveju, žinios apie egzoeritrocitinį hemoproteidų vystymąsi yra labai fragmentiškos. Senoje literatūroje teigiama jog *Haemoproteus* parazitų rūšys yra nekenksmingos užkrėstiems stuburiniams šeimininkams (Bennett et al., 1993). Tačiau, neseniai atlikti tyrimai parodė jog *Haemoproteus* genties atstovai taip pat gali sukelti sunkias ligas (Cardona et al., 2002; Ferrell et al., 2007; Donovan et al., 2008; Olias et al., 2011; Dunn et al., 2013; Cannell et al., 2013; Groff et al., 2019). Šie tyrimai atskleidė, kad užkrėstuose neadaptuotuose šeimininkuose išsivysto *Haemoproteus* egzoeritrocitinių merontų sukeltos patologijos. Tokios infekcijos atveju parazitas geba pradėti, tačiau neužbaigia savo gyvybinio ciklo stuburiniame šeimininke, dėl ko ženkliai padidėja parazito virulentiškumas (Ortiz-Catedral et al., 2019). Iki 2019 metų, tik 8

hemoproteidų rūšių megalomerontai buvo aptikti (Valkiūnas ir Iezhova, 2017). Šios gyvybinio ciklo stadijos buvo rastos papūgose (Miltgen et al., 1981; Olias et al., 2011; Ortiz-Catedral et al., 2019), kalakutuose (Atkinson, 1986; Atkinson, 1988), naminiuose žvirbliuose *Passer domesticus* (Wenyon, 1926; Peirce, 1976; Burtikashvili, 1978), šventuosiuose todiramfuose *Todiramphus sanctus* (Peirce et al., 2004), virgininėse putpelėse *Colinus virginianus* (Cordona et al., 2002) ir balandžiuose (Farmer, 1964; Earlé et al., 1993), tačiau nebuvo nustatytos šių parazitų genetinės linijos. Nepaisant didelio rūšių skaičiaus, plataus geografinio paplitimo ir galimo neigiamo poveikio paukščiams, gyvybinio *Haemoproteus* genties atstovų ciklo dalis, susijusi su egzoeritrocitiniu vystymusi, yra nepakankamai ištirta.

DARBO TIKSLAS IR UŽDAVINIAI

Darbo tikslas buvo įgyti naujų žinių apie nepakankamai ištirtą hemosporidijų (Haemosporida) – maliarinių parazitų (*Plasmodium*) ir hemoproteidų (*Haemoproteus*) – gyvybinio ciklo dalį, egzoeritrocitinį vystymąsi, bei šių parazitų virulentiškumą ir paukščiams sukeliamas patologijas.

Tikslui pasiekti buvo iškelti šie uždaviniai:

1. Ištirti antrinius *Plasmodium homocircumflexum* (genetinė linija pCOLL4) egzoeritrocitinius merontus (fanerozoitus) bei jų sukeliamas patologijas Europoje paplitusiuose žvirbliniuose paukščiuose.
2. Ištirti *Plasmodium elongatum* (genetinės linijos pERIRUB01 ir pGRW6) fanerozoitų vystymąsi eksperimentiškai užkrėstuose žvirbliniuose paukščiuose.
3. Ištirti *Haemoproteus majoris* (genetinės linijos hPHYBOR04 ir hPARUS1) egzoeritrocitninius merontus natūraliai užsikrėtusiuose stuburiniuose šeimininkuose.
4. Išanalizuoti *Plasmodium relictum* (genetinė linija pPHCOL01) eksperimentiškai užkrėstų paukščių organus siekiant surasti fanerozoitus.
5. Išanalizuoti *Plasmodium delichoni* (genetinė linija pCOLL6) eksperimentiškai užkrėstų paukščių organus siekiant surasti fanerozoitus.
6. Išanalizuoti natūraliai *Haemoproteus ciconiae* užsikrėtusio baltojo gandro organus siekiant surasti egzoeritrocitinius merontus.

GINAMIEJI TEIGINIAI

1. *Plasmodium homocircumflexum* (genetinė linija pCOLL4) išvysto fanerozoitus daugelyje ištirtų žvirblinių paukščių rūšių.
2. *Plasmodium homocircumflexum* (genetinė linija pCOLL4) sukelia išeminius pokyčius paukščių smegenyse, dėl kurių ištinka cerebralinis paralyžius, nulemiantis stuburinių šeimininkų mirtingumą.
3. *Plasmodium elongatum* (genetinė linija pERIRUB01) išvysto fanerozoitus užkrėstų paukščių kaulų čiulpuose taip sutrikdydamas eritropoezę, dėl ko išsivysto anemija, nulemianti stuburinių šeimininkų mirtingumą.
4. *Plasmodium elongatum* (genetinė linija pGRW6) yra mažai virulentiškas adaptuotiems stuburiniams šeimininkams dėl nedidelio išsivystančių fanerozoitų intensyvumo.
5. *Haemoproteus majoris* (genetinės linijos hPHYBOR04 ir hPARUS1) megalomerontai buvo atrasti pirmą kartą; jų morfologija yra unikali.
6. *Plasmodium relictum* (genetinė linija pPHCOL01) ir *Plasmodium delichoni* (genetinė linija pCOLL6) fanerozoitai nebuvo rasti eksperimentiškai užkrėstuose paukščiuose, todėl yra tikėtina, kad be fanerozoitų stadijos egzistuoja ir kiti maliarinių parazitų išlikimo stuburiniuose šeimininkuose mechanizmai.

DARBO NAUJUMAS

1. Eksperimentiškai įrodyta, kad *Plasmodium homocircumflexum* (genetinė linija pCOLL4) yra eurikseninis parazitas, tačiau skirtingose paukščių rūšyse vystosi labai skirtingai, kas lemia skirtingą šio patogeno virulentiškumą.
2. Atrasta nauja *Plasmodium elongatum* genetinė linija, pERIRUB01, ir ištirtas jos egzoeritrocitinis vystymasis bei eksperimentikai. Eksperimentiškai įrodyta, kad ši genetinė linija yra labai virulentiška kai kurioms paukščių rūšims dėl fanerozoitų sukeltų pažeidimų kaulų čiulpuose.
3. Eksperimentiškai įrodyta, kad *Plasmodium elongatum* (genetinė linija pGRW6) skirtingai paveikia įvairių paukščių rūšių astovus.

Kosmopolitinis šio parazito paplitimas gali būti susijęs su nedideliu virulentiškumu adaptuotiems laukiniams paukščiams, kurie yra parazito nešiotojai. Dėl to parazitas plinta ir neadaptuoti paukščiai būna užkrečiami, o pastaruosiuose sukelta infekcija gali būti mirtina.

4. Genetinė linija hPHYBOR04 buvo pirmą kartą rasta Europoje ir priskirta *Haemoproteus majoris* rūšiai.
5. Pirmą kartą buvo atrasti ir ištirti *Haemoproteus majoris* (genetinės linijos hPHYBOR04 ir hPARUS1) megalomerontai. Nustatyta, kad *H. majoris* megalomerontai išsivysto ne tik abortatyvaus vystymosi stuburiniame šeimininke metu, bet yra įprasta gyvybinio ciklo stadija natūraliai užsikrėtusiuose paukščiuose.
6. Atrasta nauja genetinė linija pPHCOL01 ir remiantis morfologiniais bei molekuliniais požymiais priskirta *Plasmodium relictum*, taip papildant žinias apie šio kosmopolitinio parazito vidurūšinę genetinę įvairovę.
7. Aprašyta nauja mokslui *Plasmodium* rūšis – *Plasmodium delichoni* n. sp. (genetinė linija pCOLL6), ir iškelta hipotezė apie galimą šio parazito išlikimą stuburiniame šeimininke mechanizmą.
8. Atrasta ir aprašyta pirma *Haemoproteus* genties rūšis – *Haemoproteus ciconiae* n. sp. – parazituojanti baltąjį gandrą.

LITERATŪROS APŽVALGOS APIBENDRINIMAS

Literatūros apžvalgoje glaustai pateikta informacija apie hemosporinidių parazitų gyvybinius ciklus, tyrimų istoriją, atliktų tyrimų svarbą bei detalesnių egzoeritrocitinio šių patogenų vystymosi tyrimų poreikį.

MEDŽIAGA IR METODAI

Detalus darbe taikytų metodų aprašymas yra pateiktas disertacijos tekste bei straipsniuose (**I-VIII straipsniai**), kurių pagrindu ši disertacija yra parengta.

Medžiaga tyrimams surinkta „Rybachy“ biologinėje stotyje (Kaliningrado sritis, Rusija, **I, II, VI, VIII straipsniai**), Ventės rago ornitologinėje stotyje (Ventė, Lietuva **III-V straipsniai**) bei Gamtos tyrimų centre (Vilnius, Lietuva **II, IV, V straipsniai**). Surinkta medžiaga saugoma Gamtos tyrimų centre.

Atliktų tyrimų metu buvo daromi paukščių kraujo tepinėliai bei renkami kraujo mėginiai molekuliniams parazitų tyrimams (**I-VIII straipsniai**). Histologinei analizei buvo renkami paukščių vidinių organų mėginiai bei daromi kaulų čiulpų tepinėliai (**I-VIII straipsniai**).

Kraujo tepinėliai buvo analizuojami šviesinės mikroskopijos metodu (**I-VIII straipsniai**). Histologiniai preparatai buvo analizuojami šviesiniu mikroskopu (**I-VIII straipsniai**) bei apdorojami taikant *in situ* hibridizacijos (ISH) (**I, IV-VI straipsniai**) metodą.

SET buferyje užfiksuotas užkrėstas paukščių kraujas (**I-VIII straipsniai**) bei lazerinės mikrodisekcijos metodu surinkta histologinė medžiaga (**VII straipsnis**) buvo panaudoti genetiniams parazitų tyrimams. DNR išskirta naudojant DNR nusodinimo amonio acetatu metodą (**I-VIII straipsniai**) bei taikant DNR išskyrimo naudojant „Chelex“ protokolą (**VII straipsnis**). Išskirta DNR buvo padauginta taikant polimerazės grandininės reakcijos metodą (PGR) (**I-VIII straipsniai**). Gautos DNR sekos buvo nuskaitytos ABI PRISM TM 3100 sekoskaitos robotu (**I, II, IV-VIII straipsniai**). Gautos DNR sekos buvo analizuojamos BioEdit (Hall, 1999) programa (**I-VIII straipsniai**). Filogenetiniai medžiai buvo sukonstruoti MrBayes version 3.1 (Ronquist ir Huelsenbeck, 2003) programa (**II, IV, V, VII, VIII straipsniai**). Tinkamiausias evoliucijos modelis buvo parinktas MrModeltest 2 programa (Nylander, 2004) (**II, IV, V, VII, VIII straipsniai**). Filogenetiniai medžiai vizualizuoti FigTree v1.4.3 (Rambaut, 2009) (**V, VII, VIII straipsniai**) ir TreeView 1.6.6 (https://treeview.software.informer.com/1.6/) (**II, IV straipsniai**).

Eksperimentiškai užkrėsti paukščiai buvo laikomi kontroliuojamomis sąlygomis (kambario temperatūra, natūralus fotoperiodas, maitinimas, ta pati eksperimentinės ir kontrolinės grupių laikymo vieta) (**I, II, IV-VI, VIII straipsniai)**. Eksperimentų metu paukščių kraujas buvo tikrinamas iš anksto nustatytai intervalais (**I, II, IV-VI, VIII straipsniai)**. Tais pačiais intervalais buvo tikrinamas paukščių hematokrito lygis bei matuojama kūno masė (**VI, VIII straipsniai).** Atliekant tyrimus buvo stengiamasi kaip įmanoma labiau sumažinti paukščių patiriamą stresą.

Statistinė analizė atlikta naudojant “Statistica 7” (**I, III-V straipsniai**) ir “R studio” (**VI-VIII straipsniai**) programas. Duomenų atitikimas normalųjį skirstinį buvo patikrintas taikant Šapiro-Vilko testą. Duomenų nepasiskirsčiusių pagal normalųjį skirstinį vidurkių skirtumas buvo įvertintas taikant Vilkoksono testą. Fišerio tikslusis testas buvo pritaikytas siekiant

įvertinti statistinį tų pačių parametrų reikšmingumą lyginant eksperimentinę ir kontrolinę grupes.

REZULTATAI IR JŲ APTARIMAS

Išsamūs rezultatai ir jų aptarimas yra pateikti **I-VIII straipsniuose**, kurių pagrindu yra parengta ši disertacija.

Plasmodium homocircumfexum (genetinė linija pCOLL4) vystymasis Europoje paplitusiuose žvirbliniuose paukščiuose

I straipsnyje aprašytas bandomasis tyrimas, kurio metu buvo tiriamas *Plasmodium homocircumfexum* (genetinė linija pCOLL4) vystymasis trijų Europoje paplitusių žvirblinių paukščių rūšių atstovuose. *Plasmodium homocircumfexum* buvo sėkmingai užkrėsti po vieną eurazinių alksninukų *Carduelis spinus,* eglinių kryžiasnapių ir paprastųjų varnėnų individą. Visi trys paukščiai išgyveno parazitemijos piką, tačiau infekcijos intensyvumui nukritus staiga nugaišo (**I straipsnis**, Fig. 1). Skrodimo metu buvo pastebėti blužnies ir kepenų padidėjimai. Taip pat, visuose trijuose paukščiuose užfiksuota kardiomegalija bei perikardo efuzija. *Plasmodium homocircumfexum* fanerozoitai išsivystė visuose trijuose individuose. Daugybė fanerozoitų buvo užfiksuota visų užkrėstų paukščių smegenyse, kepenyse, inkstuose, blužnyje, širdies ir krūtinės raumenyse. Verta paminėti, jog fanerozoitai, išsivystę smegenų kapiliaruose, buvo tokios formos kaip ir patys kapiliarai, kai kuriais atvejais fanerozoitai visiškai užkimšo kraujagysles taip sutrikdydami kraujo cirkuliaciją (**I straipsnis**, Fig. 2).

Plasmodium homocircumfexum (genetinė linija pCOLL4) parazitemijos dinamika buvo skirtinga visuose trijuose paukščiuose, tačiau fanerozoitų vystymasis ir sukeltos patologijos buvo panašūs. Gauti rezultatai atitinka ankstesnių tyrimų rezultatus, kuomet buvo tirtas šio parazito vystymasis naminėse kanarėlėse (Palinauskas et al., 2015). Tai liudija apie *P. homocircumfexum* gebėjimą išvystyti fanerozoitus kelių imlių rūšių paukščiuose. Išeminiai pokyčiai smegenyse, dėl kurių paukščius ištinka cerebralinis paralyžius, yra viena rimčiausių stuburiniams šeimininkams maliarijos sukeliamų patologijų ir yra labiausiai tikėtina pastebėto staigaus paukščių gaišimo priežastis (Garnham, 1980; Valkiūnas, 2005). Visi trys paukščiai nugaišo staiga, be jokių blogėjančios būklės simptomų.

Paukščiams sukeliamos patologijos maliarijos infekcijų metu dažniausiai yra susijusios su dviem faktoriais – aukšta parazitemija arba besivystančių fanerozoitų sukeliamomis pažaidomis (Coulston ir Manwell, 1941; Huchzermeyer ir Van der Vyver, 1991; Valkiūnas, 2005). Plačiai priimta manyti, jog mažėjanti parazitemija, kuri galiausiai virsta chronine, liudija apie užsikrėtusio individo būklės gerėjimą (Atkinson et al., 2008; Castro et al., 2011; Vanstreels et al., 2015). Tai nėra teisinga prielaida tam tikrų maliarijos infekcijų metu. Paukščiai gali žūti ir dėl fanerozoitų sukeltų pažeidimų, net kai parazitemija mažėja ir akivaizdūs maliarijos simptomai išnyksta. Egzoeritrocitinių merontų vaidmuo parazitų virulentiškumui yra nepakankamai įvertintas. Gaišimas dėl fanerozoitų gamtoje yra staigus, o tokiu būdu žuvusius paukščius aptikti sunku. Yra duomenų rodančių jog plačiai paplitę *Plasmodium* parazitai gali sukelti Europoje sutinkamų paukščių gaišimą (Dinhopl et al., 2015), tačiau šio reiškinio dažnumas yra nepakankamai išaiškintas.

Plasmodium homocircumfexum (genetinė linija pCOLL4) vystymosi dėsningumai eksperimentiškai užkrėstuose žvirbliniuose paukščiuose

Bandomojo darbo metu gauti rezultatai (**I straipsnis**) nurodė tolimesnių *Plasmodium homocircumfexum* (genetinė linija pCOLL4) tyrimų kryptį. **VI straipsnyje** aprašytas tyrimas, kuris buvo suplanuotas remiantis teigiamais **I straipsnyje** aprašyto tyrimo rezultatais. Keturių Europoje plačiai paplitusių rūšių, eglinių kryžiasnapių, paprastųjų varnėnų, naminių žvirblių ir paprastųjų kikilių *Fringilla coelebs* paukščiai buvo užkrėsti *P. homocircumflexum* (genetinė linija pCOLL4) (eksperimentinės grupės; 8 kiekvienos rūšies individai), arba inokuliuoti neužkrėstu krauju (kontrolinės grupės; 8 kiekvienos rūšies individai). Visuose paukščiuose, kuriems buvo inokuliuotas užkrėstas kraujas, išsivystė parazitemija (V**I straipsnis,** Table 1). Vidutinės parazitemijos vystymosi dinamika, vidutinio hematokrito bei vidutinės kūno masės pokyčiai tyrimo eigoje pateikti **VI straipsnyje**, Fig. 2 ir Table 1. Verta pastebėti, jog visose eksperimentinėse paukščių rūšių grupėse, vidutinio hematokrito sumažėjimas sutapo su parazitemijos padidėjimu. Taip pat įdomu, jog skirtingų paukščių rūšių grupėse vidutinė kūno masė pakito skirtingai, nuo padidėjimo (paprastieji varnėnai ir naminiai žvirbliai) iki sumažėjimo (egliniai kryžiasnapiai ir paprastieji kikiliai).

Fanerozoitai išsivystė daugelio užkrėstų eglinių kryžiasnapių, naminių žvirblių ir paprastųjų kikilių individų smegenyse, kepenyse, inkstuose, blužnyse, širdies bei krūtinės raumenyse (**VI straipsnis**, Table 1).

Paprastuosiuose varnėnuose fanerozoitai nebuvo rasti, o šis neigiamas rezultatas patvirtintas ISH metodu. Fanerozoitai buvo rasti eglinių kryžiasnapių, naminių žvirblių ir paprastųjų kikilių, individų nugaišusių po 18 dienos po infekcijos (dpe), smegenyse. Individų, nugaišusių iki 18 dpe, arba išgyvenusių iki tyrimo pabaigos, smegenyse fanerozoitai nebuvo rasti.

Viso, *P. homocircumflexum* infekcija buvo nustatyta 14 paukščių rūšių individuose (Durrant et al., 2006; Pérez-Tris et al., 2007; Palinauskas et al., 2015; šis tyrimas). Tai rodo jog *P. homocircumflexum* išties yra eurikseninis parazitas, gebantis užkrėsti paukščius priklausančius daugybei rūšių. Vis dėl to, nėra aišku ar visuose rūšyse, kuriose buvo užfiksuota *P. homocircumflexum* infekcija, šis patogenas geba užbaigti vystymąsi (ar išvystomi gametocitai). Šiuo tyrimu buvo patvirtinta, kad *P. homocircumflexum* tikrai yra platau specifiškumo parazitas, kas yra svarbus rezultatas tiriant šio maliarijos sukėlėjo epidemiologiją, bei kuriant kovos su šia infekcija priemones.

Paukščių maliarija sukelia kraujo patologijas, nes yra suardomi užkrėsto šeimininko eritrocitai (Garnham, 1966; Valkiūnas, 2005) ir/arba yra pažeidžiamos kaulų čiulpų ląstelės, dėl ko yra sutrikdoma eritropoezė (Garnham, 1966; Palinauskas et al., 2015; Palinauskas et al., 2018). Atlikto tyrimo metu fanerozoitų kaulų čiulpuose rasta nebuvo, tačiau paukščiuose išsivystė sąlyginai aukštos parazitemijos (**VI straipsnis**, Fig. 2). Anemija užkrėstuose individuose išsivystė dėl tiesiogiai parazito suardomų eritrocitų bei užkrėstų eritrocitų pašalinimo kepenyse ir blužnyje. Hematokrito sumažėjimo rezultatas užkrėstuose paukščiuose sutampa su ankstesnių tyrimų, atliktų naudojant kitas *Plasmodium* rūšis, rezultatais (Permin ir Juhl, 2002; Paulman ir McAllister, 2005; Williams, 2005; Palinauskas et al., 2008).

Įdomu pastebėti, kad lyginant su kontrolinėmis grupėmis, vidutinė eksperimentiškai užkrėstų paprastųjų varnėnų ir naminių žvirblių kūno masė statistiškai reikšmingai padidėjo. Tikėtina jog paukščiai, jausdami maliarijos sukeliamų simptomų stresą, padidina suvartojamo maisto kiekį, taip bandydami kompensuoti blogą savijautą. Tačiau sumažėjusi vidutinė eglinių kryžiasnapių ir paprastųjų kikilių kūno masė (**VI straipsnis**, Fig. 2) leidžia manyti, jog toks mechanizmas gali būti susijęs su stuburinio šeimininko rūšimi. Šio tyrimo rezultatai rodo, jog didėjanti paukščio kūno masė nebūtinai liudija apie gerėjančią paukščio būklę maliarijos infekcijų metu (**VI straipsnis**).

Šis darbas yra pirmasis, kurio rezultatai rodo skirtingą tos pačios parazito genetinės linijos vystymąsi skirtingų rūšių šeimininkuose. Fanerozoitai išsivystė trijų iš keturių užkrėstų paukščių rūšių atstovuose. Atlikto eksperimento rezultatai (**VI straipsnis**) papildo žinias apie fanerozoitų vystymąsi aprašytą **I straipsnyje**. Gauti duomenys rodo, kad su kai kuriomis variacijomis, fanerozoitai audiniuose išsivysto tarp 10 ir 20 dpe. Taip pat, reikalingas tam tikras laiko tarpas, po kurio parazito stadijos besivystančios kraujyje gali užkrėsti retikuloendotelines ląsteles vidaus organuose. Fanerozoitų išsivystymas smegenyse yra dažniausia gaišimo priežastis *P. homocircumflexum* infekcijų metu, lygiai kaip ir *P. gallinaceum* infekcijų naminėse vištose atveju (Valkiūnas, 2005).

Egzoeritrocitinis *Plasmodium elongatum* (genetinė linija pERIRUB01) vystymasis

II staipsnyje aprašytas eksperimentinis *Plasmodium elongatum* (genetinė linija pERIRUB01) egzoeritrocitinio vystymosi tyrimas. Septynios naminės kanarėlės buvo užkrėstos *P. elongatum*. Visose septyniose naminėse kanarėlėse išsivystė parazitemija. Gana aukšta parazitemija (9%) išsivystė viename individe, tačiau kituose individuose vidutinė maksimali parazitemija pasiekė tik 0.2%. Penkios iš septynių užkrėstų kanarėlių tyrimo metu nugaišo. Dviejų nugaišusių paukščių (žuvusių 14 ir 46 dpe) histologinė analizė atskleidė, jog fanerozoitai išsivystė abiejų individų kaulų čiulpuose. Taip pat fanerozoitai buvo aptikti užsikrėtusių individų kepenyse ir blužnyse (**II straipsnis**, Fig. 3).

Ankstesni tyrimai parodė, kad dėl besivystančių fanerozoitų *P. elongatum* stuburiniam šeimininkui gali sukelti sunkias patologijas ar net gaišimą (Garnham, 1966; Valkiūnas, 2005), tačiau genetinės *P. elongatum* linijos kurios taip paveikė paukščius publikuotuose darbuose nėra žinomos. Toks pats parazito poveikis buvo pastebėtas ir šio tyrimo metu, kuomet paukščiai buvo užkrėsti *P. elongatum* pERIRUB01 genetine linija. Nors maksimali parazitemija buvo gana žema, 70% užkrėstų stuburinių šeimininkų nugaišo. Daugybinis kaulų čiulpų ląstelių užkrėtimas pastebėtas šio tyrimo metu buvo aprašytas *P. elongatum* infekcijų atveju ir anksčiau (Garnham, 1966; Corradetti et al., 1968). Šis tyrimas (**II straipsnis**) rodo, jog anemija sukelta dėl kaulų čiulpų ląstelių suardymo, yra pagrindinė paukščių gaišimo priežastis *P. elongatum* (genetinė linija pERIRUB01) infekcijų metu.

Egzoeritrocitinis *Plasmodium elongatum* (genetinė linija pGRW6) vystymasis

VIII straipsnyje yra aprašytas eksperimentinis *Plasmodium elongatum* (genetinė linija pGRW6) egzoeritrocitinio vystymosi dviejų Europoje paplitusių paukščių rūšių atstovuose – egliniuose kryžiasnapiuose ir paprastuosiuose varnėnuose – tyrimas. Visi paprastieji varnėnai buvo atsparūs *P. elongatum* (genetinė linija pGRW6) infekcijai. Tai buvo nustatyta remiantis šviesinės mikroskopijos ir patvirtinta molekuliniais tyrimo metodais. Visuose užkrėstuose egliniuose kryžiasnapiuose parazitemija išsivystė. Vidutinė parazitemija pasiekė piką (0.3%) 20 dpe) (**VIII straipsnis**, Fig. 1). Nepaisant to, kad parazitemija egliniuose kryžiasnapiuose buvo žema, buvo pastebėtas statistiškai reikšmingas ($p<0.001$) trumpalaikis hematokrito sumažėjimas (**VIII straipsnis**, Fig. 1). Hematokrito sumažėjimas sutapo su parazitemijos padidėjimu, o vėliau (po 7-8 dienų) hematokritas pasiekė įprastą lygį (**VIII straipsnis**, Fig. 1). Reikšmingų kūno masės pokyčių tiek eksperimentinės, tiek ir kontrolinės grupės egliniuose kryžiasnapiuose nebuvo pastebėta. Visi užkrėsti egliniai kryžiasnapiai išgyveno iki tyrimo pabaigos. Keli fanerozoitai buvo rasti penkių iš aštuonių užkrėstų eglinių kryžiasnapių kaulų čiulpuose (< 5 fanerozoitai buvo rasti daugumos paukščių preparatuose). Jokiuose kituose eglinių kryžiasnapių organuose fanerozoitai neišsivystė.

Anksčiau *P. elongatum* (genetinė linija pGRW6) parazitas nebuvo rastas paprastuosiuose varnėnuose nepaisant kosmopolitinio šios rūšies paukščių paplitimo (Bensch et al., 2009). Šio tyrimo (**VIII straipsnis**) rezultatai patvirtina ankstesnių tyrimų išvadas dėl visiško ar dalinio paprastųjų varnėnų atsparumo kai kurioms maliarinių parazitų rūšims (Bennett et al., 1982; Bishop ir Bennett, 1992; Valkiūnas, 2005; Atkinson et al., 2008; Palinauskas et al., 2008; Ilgūnas et al., 2019). Šios paukščių rūšies gebėjimas atsispirti ar bent toleruoti plačiai paplitusius maliarinius parazitus gali būti vienas iš faktorių, leidžiančių paprastiesiems varnėnams sėkmingai plisti visame pasaulyje (BirdLife International, 2016). Vis dėl to, mechanizmas nulemiantis šį atsparumą infekcijai nėra žinomas. „Paprastojo varnėno – *P. elongatum* (genetinė linija pGRW6)“ parazito – šeimininko modelis galėtų būti naudojamas įgimto atsparumo maliarijos parazitams tyrimams.

Tik 1-5 fanerozoitai buvo rasti 80% eglinių kryžiasnapių individų, kuriuose fanerozoitai išsivystė kaulų čiulpuose. Šis rezultatas liudija žemo intensyvumo antrinę egzoeritrocitinę merogoniją. Eglinių kryžiasnapių gebėjimas atsispirti antrinių egzoeritrocitinių merontų vystymuisi gali paaiškinti nedidelį *P. elongatum* (genetinė linija pGRW6) virulentiškumą šiai

paukščių rūšiai, kuri yra žinoma dėl savo imlumo kitiems maliariniams patogenams – *P. relictum* (genetinė linija pSGS1) ir *P. homocircumflexum* (genetinė linija pCOLL4) (Palinauskas et al., 2008; Ilgūnas et al., 2016; Palinauskas et al., 2016; Ilgūnas et al., 2019).

VIII straipsnyje aprašyto tyrimo rezultatai neprieštarauja ankstesniems darbams, kurie parodė, jog *P. elongatum* infekcijų metu išsivystančios parazitemijos nebūna aukštos (Garnham, 1966; Corradetti et al., 1968; Valkiūnas, 2005; Palinauskas et al., 2016). Tai veikiausiai yra vienas iš parazito prisitaikymų išgyventi ilgalaikėje perspektyvoje: žemos parazitemijos atveju parazito sukeliamos kraujo patologijos taip pat būna nežymios, kas padidina stuburinio šeimininko, o tuo pačiu ir parazito, išgyvenimo tikimybę (Valkiūnas, 2005; Mukhin et al., 2016; Granthon ir Williams, 2017).

Hematokrito sumažėjimas parazitemijos piko metu yra gerai žinomas *Plasmodium* infekcijų paukščiuose simptomas (Permin ir Juhl, 2002; Paulman ir McAllister, 2005; Williams, 2005 Palinauskas et al., 2008; Ilgūnas et al., 2019). Vis dėl to, šis tyrimas (**VIII straipsnis**) yra pirmas pranešimas apie tokį reiškinį *P. elongatum* infekcijos atveju (**VIII straipsnis**, Fig. 1). Seniau buvo manoma, kad hematokrito mažėjimas *P. elongatum* infekcijų metu yra susijęs su fanerozoitų, besivystančių kaulų čiulpuose, sukeltomis patologijomis (Garnham, 1966; Corradetti et al., 1968; Bensch et al., 2009; Palinauskas et al., 2016). Tai galioja ir *P. elongatum* (genetinė linija pERIRUB01) infekcijos atveju (**II straipsnis**). Tačiau *P. elongatum* (genetinė linija pGRW6) infekcijos metu kaulų čiulpuose išsivystančių fanerozoitų skaičius nėra didelis, o hematokrito lygis pakinta statistiškai reikšmingai lyginant eksperimentinės ir kontrolinės grupių duomenis. Planuojant *P. elongatum* (genetinė linija pGRW6) virulentiškumo tyrimus rekomenduojama atkreipti dėmesį ir į šio parazito sukeliamas kraujo patologijas.

Egzoeritrocitinis *Haemoproteus majoris* (genetinės linijos hPHYBOR04 ir hPARUS1) vystymasis

VII straipsnyje aprašomas *Haemoproteus majoris* (genetinės linijos hPHYBOR04 ir hPARUS1) gyvybinio ciklo tyrimas.

Dideli (iki 360 µm diametro), apvalūs, padengti storą kapsulę primenančia sienele egzoeritrocitiniai merontai buvo rasti histologiniuose smilginio strazdo *Turdus pilaris*, natūraliai užsikrėtusio *H. majoris* (genetinė linija hPHYBOR04), inkstų preparatuose. Megalomerontuose buvo matomi

netaisyklingos formos citomerai, kuriuose vystėsi merozoitai. Panašios morfologijos megalomerontai buvo rasti ir histologiniuose didžiosios zylės *Parus major*, natūraliai užsikrėtusios *H. majoris* (genetinė linija hPARUS1), kepenų, plaučių, blužnies ir inkstų preparatuose. Siekiant patvirtinti, jog aptikti megalomerontai išties priklauso *Haemoproteus* genties parazitui, buvo pasitelkta lazerinė mikrodisekcija bei molekuliniai diagnostikos metodai. Trys megalomerontai lazeriu buvo išpjauti iš didžiosios zylės histologinių inkstų preparatų (**VII straipsnis**, Fig. 1) ir ištirti remiantis genetinių sekų informacija. Visais trimis atvejais, nuskaityta megalomeronto seka sutapo su hPARUS1 genetinės linijos genetine seka, kas leido priskirti rastus megalomerontus *H. majoris* rūšies parazitui.

Egzoeritrocitines paukščių hemoproteidų stadijas apžvelgė Valkiūnas ir Iezhova (2017). Šio tyrimo metu apibūdintų *H. majoris* megalomerontų morfologija yra unikali (**VII straipsnis**, Fig. 4 ir Fig. 5) lyginant su kitais hemoproteidais. Dėl tokių požymių kaip stora, kapsulę primenanti sienelė ir gerai išsivystę citomerai, rasti megalomerontai yra panašus į *Leucocytozoon* spp. (Valkiūnas, 2005) ar net *Besnoitia* parazitus (Bennett et all., 1993). Vis dėl to, genetinė informacija leido priskirti matytus megalomerontus *H. majoris*.

Anksčiau, buvo rasti tik 8 *Haemoproteus* rūšių parazitų megalomerontai (Valkiūnas ir Iezhova, 2017). Visais atvejais tai buvo *Haemoproteus* infekcijos ne žvirbliniuose paukščiuose (Wenyon, 1926; Farmer, 1964; Peirce, 1976; Burtikashvili, 1978; Miltgen et al., 1981; Atkinson, 1986; Atkinson, 1988; Earlé et al., 1993; Cordona et al., 2002; Peirce et al., 2004; Olias et al., 2011; Ortiz-Catedral et al., 2019). Taip pat nebuvo aišku ar megalomerontai vystosi tik neadaptuotuose stuburiniuose šeimininkuose, ar vis tik jie gali vystytis ir įprasto *Haemoproteus* genties parazitų gyvybinio ciklo metu, adaptuotuose šeimininkuose. Šis tyrimas (**VII straipsnis**) atskleidė, jog megalomerontai gali būti įprasta tam tikrų hemoproteidų gyvybinio ciklo stadija. Faktas jog subrendę *H. majoris* gametocitai buvo aptikti tuose pačiuose paukščiuose kaip ir megalomerontai rodo, jog parazitas geba užbaigti vystymosi ciklą. *Haemoproteus* genties rūšių megalomerontai turėtų būti geriau ištirti siekiant įvertinti šių parazitų paukščiams sukeliamas patologijas.

Egzoeritrocitinis *Plasmodium relictum* (genetinė linija pPHCOL01) vystymasis

V straipsnyje yra aprašytas pirmą kartą rastos genetinės linijos pPHCOL01, kuri buvo priskirta *Plasmodium relictum* rūšiai, tyrimas. Šis parazitas buvo rastas pilkojoje pečialindoje *Phylloscopus collybita*. Kraujas, užkrėstas šiuo patogenu, buvo eksperimentiškai inokuliuotas į dvi namines kanarėles, du dagilius *Carduelis carduelis*, dvi zebrines amadinas *Taeniopygia guttate* ir vieną banguotąją papūgėlę *Melopsittacus undulatus*. Parazitemija išsivystė naminėse kanarėlėse ir dagiliuose, o zebrinės amadinos ir banguotoji papūgėlė buvo atsparūs šiai infekcijai. Tyrimo pabaigoje pilkoji pečialinda ir dvi naminės kanarėlės buvo eutanazuotos ir jų smegenys, kepenys, inkstai, blužnys, širdies ir krūtinės raumenys buvo ištirti taikant histologinius tyrimo metodus. Fanerozoitai nebuvo aptikti nei tradiciniais histologiniais metodais, nei ISH metodu.

Moksliniai darbai, atlikti iki molekulinių metodų taikymo paukščių hemosporidijų tyrimuose pradžios, nustatė paukščių audinių patologijas, susijusias su *P. relictum* fanerozoitų vystymusi (Garnham, 1966; Garnham, 1980; Valkiūnas, 2005; Atkinson et al., 2008). Deja, informacijos apie tai, kokios *P. relictum* genetinės linijos buvo naudotos tuose tyrimuose, nėra. Taip pat nėra jokios informacijos apie visų žinomų *P. relictum* genetinių linijų egzoeritrocitinį vystymąsi. Šios žinios padėtų geriau suprasti šio parazito išlikimo šeimininke būdą, bei užkrėstam paukščiui sukeliamas patologijas. **V straipsnyje** aprašyto tyrimo rezultatai leidžia spėti, jog daugialąsčių, lengvai randamų šviesinės mikroskopijos metodais (Garnham, 1966; Ilgūnas et al., 2016) *P. relictum* fanerozoitų vystymasis gali priklausyti nuo infekcijos stadijos, o tokie egzoeritrocitiniai merontai gali gyvuoti laikinai. Tikėtina, kad *P. relictum* išvysto tik vienaląsčius hipnozoitus, kaip ir žmonių maliarijos *P. vivax* ir *P. ovale* sukėlėjai. ISH yra daug žadantis metodas, neseniai pradėtas taikyti paukščių hemosporidijų tyrimų srityje (Dinhopl et al., 2011; Dinhopl et al., 2015; Ilgūnas et al., 2016), tačiau net ir šis metodas gali būti nepakankamai jautrus pavienių parazito ląstelių nustatymui. Norint nagrinėti *P. relictum* egzoeritrocitinis vystymąsi tikriausiai yra reikalingi dar jautresni diagnostiniai metodai, tokie kaip imunoflorescencinis dažymas (Atkinson et al., 2008; Dinhopl et al., 2011; Dinhopl et al., 2015; Ilgūnas et al., 2016; Valkiūnas et al., 2016).

Egzoeritrocitinis *Plasmodium delichoni* (genetinė linija pCOLL6) vystymasis

IV straipsnyje aprašytas naujo mokslui *Plasmodium* genties, *Novyella* pogentės parazito – *Plasmodium delichoni* (genetinė linija pCOLL6) tyrimas. Šis patogenas buvo rastas natūraliai užsikrėtusioje langinėje kregždėje *Delichon urbica*. Šiuo parazitu eksperimentiškai buvo užkrėstos dvi naminės kanarėlės ir vienas eurazinis alksninukas. Tyrimo pabaigoje visi eksperimentiškai užkrėsti paukščiai buvo eutanazuoti ir jų smegenys, kepenys, inkstai, blužnys bei širdies ir krūtinės raumenys buvo surinkti histologinei analizei. Fanerozoitai nebuvo rasti nei tradiciniais histologiniais metodais, nei ISH metodu.

Egzoeritrocitinis *Novyella* pogentės maliarinių parazitų vystymasis yra ištirtas fragmentiškai. Dauguma publikuotų darbų apibūdino tik eritrocitines šios pogentės atstovų vystymosi stadijas (Garnham, 1966; Valkiūnas, 2005; Atkinson et al., 2008; Mantilla et al., 2013). Kadangi fanerozoitai nebuvo rasti nei tradiciniais histologiniais metodais, nei ISH metodu, gali būti, kad *P. delichoni* iš viso nevysto antrinių egzoeritrocitinių merontų. Panašu, kad net keli *Novyella* pogentės atstovai gali nevystyti fanerozoitų. Fanerozoitai buvo rasti *P. nucleophilum toucani*, *P. paranucleophilum*, *P. bertii* ir *P. vaughani* (Garnham, 1966; Nelson, 1966; Manwell ir Sessler, 1971a; Manwell ir Sessler, 1971b; Gabaldon ir Ulloa, 1981; Valkiūnas, 2005), tačiau nebuvo aptikti *P. columbae*, *P. rouxi*, *P. hexamerium* ir *P. kempi* (Manwell, 1951; Garnham, 1966; Gabaldon ir Ulloa, 1976; Christensen et al., 1983) infekcijų metu.

Vienas iš svarbiausių fanerozoitų vaidmenų paukščių maliarijos infekcijų metu yra parazito išlikimas stuburiniame šeimininke (Garnham, 1980; Valkiūnas, 2005; Atkinson et al., 2008). *P. malaria*, žmonių maliarijos sukėlėjas stuburiniame šeimininke išlieka ilgalaikės parazitemijos dėka (Sherman, 1998). Negalima atmesti galimybės, kad panašus išlikimo šeimininke mechanizmas gali būti būdingas ir kai kuriems paukščių maliarijos sukėlėjams, tačiau norint daryti tokią išvadą reikia atlikti daugiau tyrimų.

Egzoeritrocitinis *Haemoproteus ciconiae* vystymasis

III straipsnyje aprašomas tyrimas, kurio metu buvo aprašyta nauja mokslui *Haemoproteus* rūšis – *Haemoproteus ciconiae*, parazituojanti baltajame gandre *Ciconia ciconia*. Nepagydomai sužeistas baltasis gandras buvo atgabentas į Ventės rago ornitologinę stotį. Po veterinarinės apžiūros

buvo nuspręsta, jog dėl patirtų sužeidimų ir prastos būklės paukštis neišgyvens, todėl jis buvo eutanazuotas. Atlikus kraujo tepinėlių analizę buvo nustatyta, jog paukštis yra užsikrėtęs *Haemoproteus* sp. parazitu, kuris vėliau buvo apibūdintas kaip nauja mokslui rūšis *Haemoproteus ciconiae* (**III straipsnis**, Fig. 1). Histologinei analizei buvo paimti baltojo gandro kepenų, plaučių, inkstų, blužnies bei širdies ir krūtinės raumenų mėginiai. Analizės metu egzoeritrocitiniai merontai nebuvo rasti.

Atlikto tyrimo metu buvo aprašyta pirma *Haemoproteus* genties parazito rūšis parazituojanti baltajame gandre. Nors egzoeritrocitiniai merontai nebuvo rasti šio tyrimo metu, negalima teigti, jog jie tikrai neišsivysto *H. ciconiae* infekcijų metu. Neseniai buvo sukurti nauji ISH žymenys skirti *Haemoproteus* parazitų diagnostikai (Himmel et al., 2019). Šiuos žymenis galima panaudoti bandant nuodugniau ištirti *H. ciconiae* egzoeritrocitinio vystymosi klausimą.

IŠVADOS

1. Eksperimentiniais tyrimais patvirtinta, kad maliarijos sukėlėjas *Plasmodium homocircumflexum* (genetinė linija pCOLL4) yra eurikseninis parazitas, kuris išvysto antrinius egzoeritrocitinius merontus daugelyje ištirtų žvirblinių paukščių rūšių.
2. Paukščių gaišimas *P. homocircumflexum* (genetinė linija pCOLL4) infekcijos metu yra daugiausiai nulemiamas išeminių pokyčių smegenyse, atsirandančių dėl besivystančių fanerozoitų, dėl ko šeimininkus ištinka cerebralinis paralyžius.
3. Tos pačios *Plasmodium* genetinės linijos fanerozoitai skirtingose paukščių rūšyse gali vystytis labai skirtingai, dėl ko stebimas ženklus virulentiškumo skirtumas, kaip nustatyta *P. homocircumflexum* (genetinė linija pCOLL4) infekcijos atveju.
4. Maliarijos sukėlėjas *Plasmodium elongatum* (genetinė linija pERIRUB01) yra labai virulentiškas naminėms kanarėlėms dėl besivystančių fanerozoitų sukeliamų kaulų čiulpų pažaidų ir dėl to išsivystančios anemijos.
5. Maliarijos sukėlėjas *Plasmodium elongatum* (genetinė linija pGRW6) nesivysto paprastuosiuose varnėnuose, kurie yra atsparūs šiai infekcijai, ir dėl žemo antrinės egzoeritrocitinės merogonijos intensyvumo yra mažai virulentiškas kai kurioms adaptuotoms paukščių rūšims.
6. Magalomerontai vystosi plačiai paplitusio hemosporidinio parazito *Haemoproteus majoris* infekcijos metu. Naujai aprašyta genetinė linija hPHYBOR04 ir plačiai paplitusi hPARUS1 genetinė linija išvysto panašius megalomerontus natūraliai užsikrėtusiuose paukščiuose, kas rodo, jog megalomerontai yra įprasta *H. majoris* gyvybinio ciklo dalis.
7. Dvi naujos hemosporidinių parazitų rūšys buvo atrastos ir aprašytos – maliarijos sukėlėjas *Plasmodium delichoni* n. sp. (Plasmodiidae) ir hemorpoteidas *Haemoproteus ciconia* n. sp. (Haemoproteidae), parazituojančios, atitinkamai, žvirblinius paukščius ir baltuosius gandrus.

MOKSLINĖ IR PRAKTINĖ REIKŠMĖ

1. Atlikti tyrimai papildė žinias ir padėjo geriau suprasti paukščių maliarijos virulentiškumo mechanizmus, bei nurodė kryptis kaip geriau pritaikyti turimą informaciją apie egzoeritrocitinį vystymąsi veterinarinėje medicinoje bei kovoje su šia liga. Ypatingai svarbus yra pastebėjimas, jog mažėjanti parazitemija nebūtinai yra gerėjančios paukščio sveikatos indikatorius. Fanerozoitai gali išsivystyti net ir chroninės infekcijos stadijoje bei nulemti paukščio gaišimą. Dėl šios priežasties yra nepaprastai svarbu vystyti gydymą nukreiptą į fanerozoito stadiją, kuris šiai dienai nėra veiksmingas.
2. *Haemoproteus* parazitų megalomerontų atradimas kelia abejonių dėl plačiai paplitusios nuomonės, jog šios genties parazitai gamtoje nėra pavojingi. Atliktų tyrimų rezultatai skatina labiau kreipti dėmesį į *Haemoproteus* parazitus veterinarinėje medicinoje ir aplinkosaugoje.
3. Nepaisant to, kad šiame darbe buvo pritaikyti jautrūs diagnostikos metodai, *Plasmodium relictum* (genetinė linija pPHCOL01) ir *Plasmodium delichoni* (genetinė linija pCOLL6) infekcijų metu fanerozoitai nebuvo rasti. Šie rezultatai nurodo tolimesnių maliarinių parazitų egzoeritrocitinio vystymosi tyrimų kryptį. Panašu, jog kai kurios maliarijos sukėlėjų rūšys gali išsilaikyti stuburiniame šeimininke vienaląsčių, į hipnozoitus panašių, stadijų formavimo būdu (*P. relictum*) arba iš viso nevystyti fanerozoitų ir išlikti dėka ilgalaikės parazitemijos (*P. delichoni*), kaip yra *Plasmodium malariae* infekcijų žmonėse atveju. Siekiant atsakyti į šiuos klausimus, rekomenduojama taikyti jautrius, pavienes ląsteles gebančius identifikuoti, tyrimo metodus.
4. Atlikus eksperimentinį darbą nustatyta, kad paprastieji varnėnai yra natūraliai atsparūs *Plasmodium elongatum* (genetinė linija pGRW6) infekcijai. „Paprastojo varnėno - *P. elongatum* (genetinė linija pGRW6)“ šeimininko – parazito modelis yra rekomenduojamas kaip tinkamas įgimto atsparumo maliariniams parazitams tyrimams.
5. Atliktų tyrimu metu buvo naudojami plačiai Europoje paplitę paukščiai ir jų hemosporidiniai parazitai, todėl gauti rezultatai gali būti pritaikyti platesniems šių patogenų biologijos, ekologijos ir bioįvairovės bei sukeliamos patologijos tyrimams visame pasaulyje, ypatingai Europoje.

REFERENCES

1. Asghar, M., Hasselquist, D., Hansson, B., Zehtindjiev, P., Westerdahl, H., Bensch, S. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. Science 6220, 436–438.
2. Atkinson, C.T., Forrester, D.J., Greiner, E.C. 1988. Pathogenicity of *Haemoproteus meleagridis* (Haemosporina: Haemoproteidae) in experimentally infected domestic turkeys. Journal of Parasitology 74, 228–239.
3. Atkinson, C.T., Greiner, E.C., Forrester, D.J. 1986. Pre-erythrocytic development and associated host responses to *Haemoproteus meleagridis* (Haemosporina: Haemoproteidae) in experimentally infected domestic turkeys. Journal of Protozoology 33, 375–381.
4. Atkinson, C.T., Thomas, N.J., Hunter, B.C. 2008. Parasitic diseases of wild birds. Wiley-Blackwell, Oxford.
5. Ball, G.H., Chao, J. 1961. Infectivity to canaries of sporozoites of *Plasmodium relictum* developing in vitro. Journal of Parasitology 47, 787–790.
6. Beadell, J.S., Gering, E., Austin, J., Dumbacher, J.P., Peirce, M.A., Pratt, T.K., et al. 2004. Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. Molecular Ecology 13, 3829–3844.
7. Beier, J.C., Stoskopf, M.K. 1980. The epidemiology of avian malaria in black-footed penguins (*Spheniscus demersus*). Journal of Zoo Animal Medicine 11, 99–105.
8. Beier, J.C., Trpis, M. 1981. Incrimination of natural culicine vectors which transmit *Plasmodium elongatum* to penguins at the Baltimore Zoo. Canadian Journal of Zoology 59, 470–475.
9. Bennett, G.F., Peirce, M.A., Ashford, R.W. 1993. Avian haematozoa: mortality and pathogenicity. Journal of Natural History 27, 993–1001.
10. Bennett, G.F., Whiteway, M., Woodworth-Lynas, C. 1982. A host-parasite catalogue of the avian haematozoa. Memphis University of Newfoundland Occasional Papers in Biology 5, 1–243.
11. Bensch, S., Canbäck, B., DeBarry, J.D., Johansson, T., Hellgren, O., Kissinger, J.C., Palinauskas, V., Videvall, E., Valkiūnas, G. 2016. The genome of *Haemoproteus tartakovskyi* and its relationship to human malaria parasites. Genome Biology and Evolution 8, 1361–1373.
12. Bensch, S., Hellgren, O., Pérez-Tris, J. 2009. A public database of malaria parasites and related haemosporidians in avian hosts based on

mitochondrial cytochrome b lineages. Molecular Ecology Resources 9, 1353–1358.

13. Bensch, S., Perez-Tris, J., Waldenström, J., Hellgren, O. 2004. Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: Multiple cases of cryptic speciation? Evolution 58, 1617–1621.
14. Bensch, S., Stjernman M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H., Pinheiro, R.T. 2000. Host specifity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proceedings of the Royal Society B 267, 1583–1589.
15. Bichet, C., Cornet, S., Larcombe, S., Sorci, G. 2012. Experimental inhibition of nitric oxide increases *Plasmodium relictum* (lineage SGS1) parasitemia. Experimental Parasitology 132, 417–423.
16. BirdLife International 2016. Sturnus vulgaris. The IUCN Red List of Threatened Species 2016:e.T22710886A87847984. Accessed 26 Jun 2019.
17. Bishop, M.A., Bennett, G.F. 1992. Host-parasite catalogue of the avian haematozoa: supplement 1, and bibliography of the avian blood-inhabiting haematozoa. Memphis University of Newfoundland Occasional Papers in Biology 2, 1–244.
18. Bruce-Chwatt, L.J., 1981. Alphonse Laveran's discovery 100 years ago and today's global fight against malaria. Journal of the Royal Society of Medicine 74, 531-536.
19. Bukauskaitė, D., Bernotienė, R., Iezhova, T.A., Valkiūnas, G. 2016. Mechanisms of mortality in *Culicoides* biting midges due to *Haemoproteus* infection. Parasitology. 143, 1748–1754.
20. Burtikashvili, L.P. 1978. Blood parasites of wild birds in Georgia. Tbilisi: Metsniereba (In Russian).
21. Cannell, B.L., Krasnec, K.V., Campbell, K., Jones, H.I., Miller, R.D., Stephens, N. 2013. The pathology and pathogenicity of a novel *Haemoproteus* spp. infection in wild Little Penguins (*Eudyptula minor*). Veterinary Parasitology 197, 74–84.
22. Cardona, C.J., Ihejirika, A., McClellan, L. 2002. *Haemoproteus lophortyx* infection in Bobwhite Quail. Avian Diseases 46, 249–255.
23. Castro, I., Howe, L., Tompkins, D.M., Barraclough, R.K., Slaney, D. 2011. Presence and seasonal prevalence of *Plasmodium* spp. in a rare endemic New Zealand passerine (tieke or saddleback, *Philesturnus carunculatus*). Journal of Wildlife Diseases 47, 860–867.

24. Cellier-Holzem, E., Esparza-Salas, R., Garnier, S., Sorci, G. 2010. Effect of repeated exposure to *Plasmodium relictum* (lineage SGS1) on infection dynamics in domestic canaries. International Journal for Parasitology 40, 1447–1453.
25. Christensen, B.M., Barnes, H.J., Rowley, W.A. 1983. Vertebrate host specificity and experimental vectors of *Plasmodium (Novyella) kempi* sp. n. from the eastern wild turkey in Iowa. Journal of Wildlife Diseases 19, 204–213.
26. Ciloglu, A., Ellis, V.A., Bernotienė, R., Valkiūnas, G., Bensch, S. 2019. A new one-step multiplex PCR assay for simultaneous detection and identification of avian haemosporidian parasites. Parasitology Research 118, 191–201.
27. Clark, N.J., Clegg, S.M., Lima, M.R. 2014. A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): new insights from molecular data. International Journal for Parasitology 44, 329–338.
28. Coatney, G.R., Cooper, W.C., Eddy, N.B., Greenberg, J. 1953. Survey of antimalarial agents. Chemotherapy of *Plasmodium gallinaceum* infections, toxicity, correlation of structure and action. Public Health Monograph 9, 322 p.
29. Cornet, S., Bichet, C., Larcombe, S., Faivre, B., Sorci, G. 2014. Impact of host nutritional status on infection dynamics and parasite virulence in a bird-malaria system. Journal of Animal Ecology 83, 256–265.
30. Corradetti, A., Neri, I., Cavallini, C. 1968. Non identiti di *Plasmodium paddae* e di *Plasmodium praeco*x(*relictum*). Parassitologia. 10, 145–149.
31. Coulston, F., Manwell, R.D. 1941. Single-parasite infections and exoeryth- rocytic schizogony in *Plasmodium circumflexum*. American Journal of Epidemiology 34, 119–125.
32. Cox, F.E.G. 2010. History of the discovery of the malaria parasites and their vectors. Parasites & Vectors 3: 5.
33. Cranfield, M.R., Shaw, M., Beall, F., Skjoldager, M., Ialeggio, D. 1990. A review and update of avian malaria in the African penguin (*Spheniscus demersus*). Proceedings, American Association of Zoo Veterinarians 243–248.
34. Davey D.G. 1951. Chemotherapy of malaria. British Medical Bulletin 8, 37–46.
35. Dimitrov, D., Iezhova, T.A., Zehtindjiev, P., Bobeva, A., Ilieva, M., Kirilova, M., et al. 2016. Molecular characterisation of three avian haemoproteids (Haemosporida, Haemoproteidae), with the description of

Haemoproteus (Parahaemoproteus) palloris n. sp. Systematic Parasitology 93, 431–449.
36. Dinhopl, N., Mostegl, M.M., Richter, B., Nedorost, N., Maderner, A., Fragner, K., et al. 2011. Application of in situ hybridization for the detection and identifica- tion of avian malaria parasites in paraffin wax-embedded tissues from captive penguins. Avian pathology 40, 315–320.
37. Dinhopl, N., Nedorost, N., Mostegl, M.M., Weissenbacher-Lang, C., Weissenböck, H. 2015. In situ hybridization and sequence analysis reveal an association of *Plasmodium* spp. with mortalities in wild passerine birds in Austria. Parasitology Research 114, 1455–1462.
38. Donovan, T.A., Schrenzel, M., Tucker, T.A., Pessier, A.P. and Stalis, I.H. 2008. Hepatic haemorrhage, hemocoelom, and sudden death due to *Haemoproteus* infection in passerine birds: eleven cases. Journal of Veterinary Diagnostics Investigation 20, 304–313.
39. Dunn, J.C., Goodman, S.J., Benton, T.G., Hamer, K.C. 2013. Avian blood parasite infection during the non-breeding season: an overlooked issue in declining populations? BMC Ecology 13, 30.
40. Durrant, K.L., Beadell, J.S., Ishtiaq, F., Graves, G.R., Olson, S.L., Gering, E., et al. 2006. Avian hematozoa in South America: a comparison of temperate and tropical zones. Ornithological Monographs 60, 98–111.
41. Earlé, R.A., Bastianello, S.S., Bennett, G.F., Krecek, R.C. 1993. Histopathology and morphology of the tissue stages of *Haemoproteus columbae* causing mortality in Columbiformes. Avian pathology 22, 67–80.
42. Ellis, V.A., Cornet, S., Merrill, L., Kunkel, M.R., Tsunekage, T., Ricklefs, R.E. 2015. Host immune responses to experimental infection of *Plasmodium relictum* (lineage SGS1) in domestic canaries (*Serinus canaria*). Parasitology Research 114, 3627–3636.
43. Ellis, V.A., Sari, E.H.R., Rubenstein, D.R., Dickerson, R.C., Bensch, S., Ricklefs, R.E. 2019. The global biogeography of avian haemosporidian parasites is characterized by local diversification and intercontinental dispersal. Parasitology 146, 213–219.
44. Farmer, J.N. 1964. Gizzard lesions associated with *Haemoproteus sacharovi* infections of pigeons. Proceedings of Iowa Academy of Science 71, 537–542.
45. Ferrell, S.T., Snowden, K., Marlar, A.B., Garner, M., Lung, N.P. 2007. Fatal hemoprotozoal infections in multiple avian species in a zoological park. Journal of Zoo and Wildlife Medicine 38, 309–316.

46. Feldman, R.A., Freed, L.A., Cann, R.L. 1995. A PCR test for avian malaria in Hawaiian birds. Molecular Ecology 4, 663–674.
47. Fix, A.S., Waterhouse, C., Greiner, E.C., Stoskopf, M.K. 1988. *Plasmodium relictum* as a cause of avian malaria in wild-caught magellanic penguins (*Spheniscus magellanicus*). Journal of Wildlife Diseases 24, 610–619.
48. Fleischman, R.W., Squire, R.A., Sladen, W.J.L., Melby, E.C. 1968. Malaria (*Plasmodium elongatum*) in captive African penguins (*Spheniscus demersus*). Journal of the American Veterinary Medical Association 153, 928–935.
49. Gabaldon, A., Ulloa, G. 1976. Revalidación y redescripción de *Plasmodium columbae* Carini, 1912. Boletin de la Direccion de Malariolooia y Saneamiento Ambiental 16, 93–106.
50. Gabaldon, A., Ulloa, G. 1981. A new species of the subgenus *Novyella* (Haemosporina, Plasmodiidae) from *Aramides cajanea* (Gruiformes, Rallidae). In: Canning EU (ed). Parasitological topics. A presentation volume to P.C.C. Garnham, F.R.S. on the occasion of his 80th birthday, pp 100–105.
51. Gardner M.J., Hall N., Fung E., White O., Berriman M., Hyman R.W., et al. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 6906, 498–511.
52. Garnham, P.C.C. 1980. Malaria in its various vertebrate hosts. In: Kreier JP, editor. Malaria epidemiology, chemotherapy, morphology and metabolism. New York: Academic Press.
53. Garnham PCC. 1966. Malaria parasites and other haemosporidia. Oxford: Blackwell.
54. Godfrey R.D., Fedynich A.M., Pence D.B. 1987. Quantification of hematozoa in blood smears. Journal of Wildlife Diseases 23, 558–565.
55. Graczyk, T.K., Cranfield, M.R., McCutchan, T.F., Bicknese, E.J. 1994. Characteristics of naturally acquired avian malaria infections in naive juvenile African black-footed penguins (*Spheniscus demersus*). Parasitology Research 80, 634–637.
56. Granthon, C., Williams, D.A. 2017. Avian Malaria, Body Condition, and Blood Parameters In Four Species of Songbirds. The Wilson Journal of Ornithology 129, 492-508.
57. Grilo, M.L., Vanstreels, R.E.T., Wallace, R., Garcia-Parraga, D., Braga, E.M., Chitty, J., et al. 2016. Malaria in penguins – current perceptions. Avian pathology 45, 393–407.
58. Groff, T.C., Lorenz, T.J., Crespo, R., Iezhova, T., Valkiūnas, G., Sehgal, R.N.M. 2019. Haemoproteosis lethality in a woodpecker, with molecular

and morphological characterization of *Haemoproteus velans* (Haemosporida, Haemoproteidae). International Journal for Parasitology: Parasites and Wildlife 10, 93–100.
59. Hall, T.A. 1999. A user-friendly biological sequence alignment editor and analysis program for Windows 98/98/NT. Nucleic Acid Symposium Series 41, 95–98.
60. Hayworth, A.M., Riper, C.V., Weathers, W.W. 1987. Effects of *Plasmodium relictum* on the metabolic rate and body temperature in Canaries (*Serinus canarius*). Journal of Parasitology 73, 850–853.
61. Hellgren, O., Waldenström, J., Bensch, S. 2004. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium* and *Haemoproteus* from avian blood. Journal of Parasitology 90, 797–802.
62. Herman, C.M., Kocan, R.M., Snyder, E.L., Knisley, J.O. 1968. *Plasmodium elongatum* from a penguin. Bulletin of Wildlife Disease Association 4, 132.
63. Himmel, T., Harl, J., Kübber-Heiss, A., Konicek, C., Fernández, N., Juan-Sallés, C., et al. 2019. Molecular probes for the identification of avian *Haemoproteus* and *Leucocytozoon* parasites in tissue sections by chromogenic *in situ* hybridization. Parasites & Vectors 12, 282.
64. Howe, L., Castro, I.C., Schoener, E.R., Hunter, S., Barraclough, R.K., Alley, M.R. 2012. Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. Parasitology Research 110, 913–923.
65. Huchzermeyer, F.W., Van der Vyver, F.H. 1991. Isolation of *Plasmodium circumflexum* from wild guineafowl (*Numida meleagris*) and the experimental infection in domestic poultry. Avian pathology 20, 213–223.
66. Humphries, M.B., Stacy, M.T., Ricklefs, R.E. 2019. Population structure of avian malaria parasites. Ecology and Evolution 9, 7741–7751.
67. Ilgūnas, M., Bukauskaitė, D., Palinauskas, V., Iezhova, T., Fragner, K., Platonova, E., et al. 2019. Patterns of *Plasmodium homocircumflexum* virulence in experimentally infected passerine birds. Malaria Journal 18, 174.
68. Ilgūnas, M., Bukauskaitė, D., Palinauskas, V., Iezhova, T.A., Dinhopl, N., Nedorost, N., et al. 2016. Mortality and pathology in birds due to *Plasmodium (Giovannolaia) homocircumflexum* infection, with emphasis on the exoerythrocytic development of avian malaria parasites. Malaria Journal 15, 256.
69. Knowles, S.C.L., Wood, M.J., Alves, R., Wilson, G.J., Bensch, S., Sheldon, B.C. 2011. Molecular epidemiology of malaria prevalence and parasitaemia in a wild bird population. Molecular Ecology 20, 1062–1076.

70. Kumar, S, Stecher, G., Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution 33, 1870–1874.
71. Mantilla, J.S., González, A.D., Valkiūnas, G., Moncada, L.I., Matta, N.E. 2013. Description and molecular characterization of *Plasmodium (Novyella) unalis* sp. nov. from the Great Thrush (*Turdus fuscater*) in highland of Colombia. Parasitology Research 112, 4193–4204.
72. Manwell, R.D. 1951. Exoerythrocytic schizogony in *Plasmodium hexamerium*. American Journal of Hygiene 53, 244–248.
73. Manwell, R.D., Sessler, G.J. 1971a. Malaria parasites of toucans. Journal of Protozoology 18, 570–574.
74. Manwell, R.D., Sessler, G.J. 1971b. *Plasmodium paranucleophilum* n. sp. from a South American tanager. Journal of Protozoology 18, 629–632.
75. Marzal, A., Ricklefs, R.E., Valkiūnas, G., Albayrak, T., Arriero, E., Bonneaud, C., et al. 2011. Diversity, loss, and gain of malaria parasites in a globally invasive bird. PLoS ONE 6, e21905.
76. Marzal, A. 2012. Recent advances in studies on avian malaria parasites. In: Okwa OO editor Malaria parasites. InTech, Rijeka, Croatia.
77. McGhee, R.B., Singh, S.D., Weathersby, A.B. 1977. *Plasmodium gallinaceum*: vaccination in chickens. Experimental Parasitology 43, 231–238.
78. Miltgen, F., Landau, I., Ratanaworabhan, N., Yenbutra, S. 1981. *Parahaemoproteus desseri* n. sp.; Gamétogonie et schizogonie chez l'hôte naturel: *Psittacula roseata* de Thailande, et sporogonie expérimentale chez *Culicoides nubeculosus*. Annales de Parasitologie Humaine et Comparee 56, 123–130.
79. Mukhin, A., Palinauskas, V., Platonova, E., Kobylkov, D., Vakoliuk, I., Valkiūnas, G. 2016. The strategy to survive primary malaria infection: an experimental study on behavioural changes in parasitized birds. PLoS ONE 11, e0159216.
80. Murata, K., Nii, R., Sasaki, E., Ishikawa, S., Sato, Y., Sawabe, K., et al. 2008. *Plasmodium* (*Bennettinia*) *juxtanucleare* infection in a captive white eared-pheasant (*Crossoptilon crossoptilon*) at a Japanese zoo. Journal of Veterinary Medical Science 70, 203–205.
81. NCBI BLAST. Rockville Pike, Bethesda MD, USA. 2019 https://blast.ncbi.nlm.nih.gov/Blast.cgi
82. Nelson, P. 1966. Exo-erythrocytic stages of a Ceylon strain of *Plasmodium vaughani* in pigeons. Transactions of the Royal Society of Tropical Medicine and Hygiene 60, 424.

83. Nylander, J.A.A. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.
84. Olias, P., Wegelin, M., Zenker, W., Freter, S., Gruber, A.D., Klopfleisch, R. 2011. Avian malaria deaths in parrots, Europe. Emerging Infectious Diseases 17, 950–952.
85. Ortiz-Catedral, L., Brunton, D., Stidworthy, M.F., Elsheikha, H.M., Pennycott, T., Schulze C., et al. 2019. *Haemoproteus minutus* is highly virulent for Australasian and South American parrots. Parasites & Vectors 12, 40.
86. Outlaw, R.K., Counterman, B., Outlaw, D.C. 2015. Differential patterns of molecular evolution among Haemosporidian parasite groups. Parasitology 142, 612–622.
87. Pacheco, M.A., Escalante, A.A., Garner, M.M., Bradley, G.A., Aguilar, R.F. 2011. Haemosporidian infection in captive masked bobwhite quail (*Colinus virginianus ridgwayi*), an endangered subspecies of the northern bobwhite quail. Veterinary Parasitology 182, 113–120.
88. Palinauskas, V., Dolnik, O., Valkiunas, G., Bensch, S. 2010. Laser microdissection, microscopy, and single cell PCR of avian haemosporidians. Journal of Parasitology 96, 420–424.
89. Palinauskas, V., Iezhova, T.A., Križanauskienė, A., Markovets, M.Y., Bensch, S., Valkiūnas G. 2013. Molecular characterization and distribution of *Haemoproteus minutus* (Haemosporida, Haemoproteidae): a pathogenic avian parasite. Parasitology International 62, 358–363.
90. Palinauskas, V., Valkiūnas, G., Bolshakov, V.C., Bensch, S. 2008. *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. Experimental Parasitology 120, 372-380.
91. Palinauskas, V., Valkiūnas, G., Križanauskienė, A., Bensch, S., Bolshakov, C.V. 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.
92. Palinauskas, V., Žiegytė, R., Iezhova, T.A., Ilgūnas, M., Bernotienė, R., Valkiūnas, G. 2016. Description, molecular characterisation, diagnostics and life cycle of *Plasmodium elongatum* (lineage pERIRUB01), the virulent avian malaria parasite. International Journal for Parasitology 46, 697–707.
93. Palinauskas, V., Žiegytė, R., Ilgūnas, M., Iezhova, T.A., Bernotienė, A., Bolshakov, C., et al. 2015. Description of the first cryptic avian malaria parasite, *Plasmodium homocircumflexum* n. sp. with experimental data on

its virulence and development in avian hosts and mosquitoes. International Journal for Parasitology 45, 51–62.
94. Palinauskas, V., Žiegytė, R., Šengaut, J., Bernotienė, R. 2018. Different paths – the same virulence: experimental study on avian single and co-infections with *Plasmodium relictum* and *Plasmodium elongatum*. International Journal for Parasitology 48, 1089–1096.
95. Paulman, A., McAllister, M.M. 2005. *Plasmodium gallinaceum*: clinical progression, recovery, and resistance to disease in chickens infected via mosquito bite. American Journal of Tropical Medicine and Hygiene 73, 1104–1107.
96. Peirce, M.A., Lederer, R., Adlard, R.D., O'Donoghue, P.J. 2004. Pathology associated with endogenous development of haematozoa in birds from southeast Queensland. Avian pathology 33, 445–450.
97. Peirce, M.A. 1976. Haemoproteid parasites of *Passer* spp. Parasitology 73, 407–415.
98. Pérez-Tris, J., Hellgren, O., Križanauskienė, A., Waldenström, J., Secondi, J., Bonneaud, C., et al. 2007. Within-host speciation of malaria parasites. PLoS ONE 2, e235.
99. Perkins, S.L. 2014. Malaria's many mates: past, present and future of the systematics of the order Haemosporida. Journal of Parasitology 100, 11–25.
100. Permin, A., Juhl, J. 2002. The development of *Plasmodium gallinaceum* infections in chickens following single infections with three different dose levels. Veterinary Parasitology 105, 1–10.
101. R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2013. http://www.R-proje ct.org/.
102. Rambaut, A. 2009. FigTree: tree figure drawing tool version 1.4.3. 2009. http://tree.bio.ed.ac.uk/software/figtree. Accessed 15 Nov 2017.
103. Ricklefs, R.E., Medeiros, M., Ellis, V.A., Svensson-Coelho, M., Blake, J.G., Loiselle, B.A., et al. 2017. Avian migration and the distribution of malaria parasites in New World passerine birds. Journal of Biogeography 44, 1113–1123.
104. Ricklefs, R.E., Outlaw, D.C., Svensson-Coelho, M., Medeiros, M.C., Ellis, V.A., Latta, S. 2014. Species formation by host shifting in avian malaria parasites. Proceedings of the National Academy of Sciences of the United States of America 11, 14816–14821
105. Ricklefs, R.E., Fallon, S.M., Bermingham, E. 2004. Evolutionary relationships, cospeciation, and host switching in avian malaria parasite. Systematic Biology 53, 111–119.

106. Ronquist, F., Heulsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
107. Sambrook, J., Fritsch, E.F., Mamiatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press.
108. Sehgal, R.N.M. 2015. Manifold habitat effects on the prevalence and diversity of avian blood parasites. International Journal for Parasitology: Parasites and Wildlife 4, 421–430.
109. Sherman, I.W. 1998. Malaria: parasite biology, pathogenesis, and protection. American Society for Microbiology, Washington.
110. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28, 2731.
111. Trager, W. 1950. Studies on the extracellular cultivation of an intracellular parasite (avian malaria). The Journal of Experimental Medicine 92, 349–365.
112. Valkiūnas, G., Iezhova, T. 2018. Keys to avian malaria parasites. Malaria Journal 17, 212.
113. Valkiūnas, G., Iezhova, T.A. 2017. Exo-erythrocytic development of avian malaria and related haemosporidian parasites. Malaria Journal 16, 101.
114. Valkiūnas, G., Ilgūnas, M., Bukauskaitė, D., Žiegytė, R., Bernotienė, R., Jusys, V., et al. 2016. *Plasmodium delichoni* n. sp.: description, molecular characterisation and remarks on the exoerythrocytic merogony, persistence, vectors and transmission. Parasitology Research 115, 2625–2636.
115. Valkiūnas, G., Kazlauskienė, R., Bernotienė, R., Bukauskaitė, D., Palinauskas, V., Iezhova, T.A. 2014. *Haemoproteus* infections (Haemosporida, Haemoproteidae) kill bird-biting mosquitoes. Parasitology Research 113, 1011–1018.
116. Valkiūnas, G., Zehtindjiev, P., Dimitrov, D., Križanauskienė, A., Iezhova, T.A., Bensch, S. 2008. Polymerase chain reaction-based identification of *Plasmodium (Huffia) elongatum*, with remarks on species identity of haemosporidian lineages deposited in GenBank. Parasitology Research 102, 1185.
117. Valkiūnas, G. 2005. Avian malaria parasites and other haemosporidia. CRC Press, Boca Raton, FL, USA.

118. Vanstreels, R.E., da Silva-Filho, R.P., Kolesnikovas, C.K., Bhering, R.C., Ruoppolo, V., Epiphanio, S., et al. 2015. Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. Veterinary Research 46, 30.
119. Vanstreels, R.E.T., Kolesnikovas, C.K.M., Sandri, S., Silveira, P., Belo, N.O., Ferreira, F.C., et al. 2014. Outbreak of avian malaria associated to multiple species of *Plasmodium* in Magellanic penguins undergoing rehabilitation in southern Brazil. PLoS ONE 9, e94994.
120. Videvall, E., Cornwallis, C.K., Ahrén, D., Palinauskas, V., Valkiūnas, G., Hellgren, O. 2017. Transcriptome of the avian malaria parasite *Plasmodium ashfordi* displays host-specific gene expression. Molecular Ecology 26, 2939–2958.
121. Wenyon, C.M. 1926. Protozoology: a manual for medical men, veterinarians and zoologists, etc. London: Bailliére Tindal & Cox.
122. Williams, R.B. 2005. Avian malaria: clinical and chemical pathology of *Plasmodium gallinaceum* in the domesticated fowl *Gallus gallus*. Avian pathology 34, 29–47.

ADDITIONAL PAPERS PUBLISHED DURING THE PHD STUDIES (NOT INCLUDED IN THIS THESIS)

Publications in journals with an impact factor and referred in the Clarivate Analytics Web of Science database.

1. Valkiūnas, G., **Ilgūnas, M.**, Bukauskaitė, D., Palinauskas, V., Bernotienė, R., Iezhova, T. 2017. Molecular characterization and distribution of *Plasmodium matutinum*, a common avian malaria parasite. Parasitology 144, 1726–1735. doi:10.1017/S0031182017000737
2. Bukauskaitė, D., Iezhova, T.A., **Ilgūnas, M.**, Valkiūnas, G. 2018. High susceptibility of the laboratory-reared biting midges *Culicoides nubeculosus* to *Haemoproteus* infections, with review on *Culicoides* species that transmit avian haemoproteids. Parasitology 146, 333–341 doi.org/10.1017/S0031182018001373
3. Chagas, C.R.F., Bukauskaitė, D., **Ilgūnas, M.**, Iezhova, T., Valkiūnas, G. 2018. A new blood parasite of leaf warblers: molecular characterization, phylogenetic relationships, description and identification of vectors. Parasites & Vectors 11, 538. doi.org/10.1186/s13071-018-3109-9
4. Weinberg, J., Field, J.T., **Ilgūnas, M.**, Bukauskaitė, D., Iezhova, T., Valkiūnas, G., Sehgal, R.N.M. 2018. De novo transcriptome assembly and preliminary analyses of two avian malaria parasites, *Plasmodium delichoni* and *Plasmodium homocircumflexum*. Genomics S0888-7543(18)30431-2. doi: 10.1016/j.ygeno.2018.12.004
5. Chagas, C.R.F., Bukauskaitė, D., **Ilgūnas, M.**, Bernotienė, R., Iezhova, T., Valkiūnas, G. 2019. Sporogony of four *Haemoproteus* species (Haemosporida: Haemoproteidae), with report of in vitro ookinetes of *Haemoproteus hirundinis*: phylogenetic inference indicates patterns of haemosporidian parasite ookinete development. Parasites & Vectors 12, 422. doi.org/10.1186/s13071-019-3679-1
6. Himmel, T., Harl, J., Kübber-Heiss, A., Konicek, C., Fernández, N., Juan-Sallés, C., **Ilgūnas, M.**, Valkiūnas, G., Weissenböck, H. 2019. Molecular probes for the identification of avian *Haemoproteus* and *Leucocytozoon* parasites in tissue sections by chromogenic *in situ* hybridization. Parasites & Vectors 12, 282.
7. Valkiūnas, G., **Ilgūnas, M.**, Bukauskaitė, D., Chagas, C.R.F., Bernotienė, R., Himmel, T., Harl, J., Weissenböck, H., Iezhova, T. 2019. Molecular characterization of six widespread avian

haemoproteids, with description of three new *Haemoproteus* species. Acta tropica S0001-706X(19)30558-3 doi.org/10.1016/j.actatropica.2019.105051

8. Bukauskaitė, D., Chagas, C.R.F., Bernotienė, R., Žigytė, R., **Ilgūnas, M.**, Iezhova, T., Valkiūnas, G. 2019. New methodology for sporogony research of avian haemoproteids in laboratory reared *Culicoides* species, with description of complete sporogonic development of *Haemoproteus pastoris*. Parasites & Vectors *In press*

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I would also like to thank dr. Vaidas Palinauskas. You have guided my first steps in real science when you supervised me during my bachelor and master studies. Following you, I learned so much about work with wild birds, experimental work, fieldwork and many more things that are too numerous to list. Thank you for always answering my questions (big or small), always stopping what you were doing just to help me with my works. I appreciate all the serious discussions (and all the small talk) which we had. The foundation that you have laid during my first four years of working at the laboratory made the next four years, which were dedicated to my PhD project, much easier.

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Work Experience

2016 – Present Junior Researcher, P.B. Sivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania.

2012 – 2016 Laboratory Technician, P.B. Sivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania.

Main Research Areas

Specificity and virulence of avian malaria parasites. Haemosporidian parasite identification based on microscopic and PCR diagnosis. Molecular and evolutionary biology of avian pathogens. Histopathology to the vertebrate host, caused by haemosporidian parasites. Development of new methods for parasites studies.

Education and Academic Degrees

2015 – 2019 PhD, Ecology and Environmental Science, Nature Research Centre and Vilnius University, Vilnius, Lithuania.

2015 MSc, Zoology, Vilnius University, Vilnius, Lithuania.

2013 BSc, Molecular Biology, Vilnius University, Vilnius, Lithuania.

Scholarships, Awards, and Recognitions

2019 Scholarship for High Academic Achievements from Research Council of Lithuania.

2018 Lithuanian Academy of Sciences Award for the Best Oral Presentation during the Biofuture: Natural and Life Science Perspectives Conference in Vilnius, Lithuania.

2018 Scholarship from Research Council of Lithuania to attend the 4th International Conference on Malaria and Related Haemosporidian Parasites of Wildlife in Beijing, China.

2018 Scholarship for High Academic Achievements from Research Council of Lithuania.

2017 Scholarship from Research Council of Lithuania for an internship at Vienna Veterinary Medicine University in Vienna, Austria.

2017 Scholarship for High Academic Achievements from Research Council of Lithuania.

2016 Scholarship from Research Council of Lithuania to attend the 3rd International Conference on Malaria and Related Haemosporidian Parasites of Wildlife in Arbanasi, Bulgaria.

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2014 Scholarship from Ministry of Education, Culture, Sports, Science and Technology of Japan to attend a seminar and training for young researchers in Kanagawa, Japan.

2013 Scholarship from Scandinavian Baltic Society for Parasitology to attend the 5th Conference of the Scandinavian – Baltic Society for Parasytology in Copenhagen, Denmark.

Teaching Experience

2017 11 05 – 2017 11 11 Lecturer at Avian Malaria Workshop 2017 (organized by Beijing Normal University and Beijing Zoo) Beijing, China. Taught courses on PCR methods used for screening for parasites, tissue stages of avian haemoporidian parasites, molecular characterization of avian haemosporidian parasites, methods of blood collection from live animals and preservation of collected material. Led practical course of blood smear preparation and avian haemosporidian parasite identification using light microscopy.

2016 08 03 – 2016 08 05 Taught Histopathological Studies of Avian Malaria and led practical courses for Immanuel Kant Baltic Federal University, Saint Petersburg State University, and Lomonosov, and Moscow State University students at the Biological station "Rybachy", Kaligrad region, Russia.

Internships

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2015 12 14 – 2015 12 18 University of Veterinary Medicine, Vienna, Austria. Goal: Testing of IN SITU hybridization method on parasite samples gathered during fieldwork.

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2014 02 21 – 2014 03 02 Veterinary Research Center, Nihon University, Tokyo, Japan. Goal: Study molecular methods of parasite screening and participation in a seminar for young researchers.

Language Proficiency

Lithuanian (native); English (fluent); Russian (moderate).

Bachelor Student Supervision

2015 – 2017 Karolis Gimbutis, Molecular Biology.

Scientific Publications

22 publications in the following journals: Malaria Journal, International Journal for Parasitology, Parasitology Research, Experimental Parasitology, Parasites and Vectors, Genomics Zootaxa.

Scientific Conferences

Participated in 8 scientific conferences (one poster and seven oral presentations) in Lithuania, Denmark, Bulgaria and China.

Conference Organization

2013 Member of the Organizing Committee, International Conference on Malaria and Related Haemosporidian Parasites of Wildlife, Vilnius, Lithuania.

Research Grants

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External Professional Activities

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2012 – 2016 Member of Malaria Research Coordination Network.

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Prof. Herbert Weissenbock, University of Veterinary Medicine, Vienna, Austria.

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COPIES OF PUBLICATIONS

PAPER I

Mortality and pathology in birds due to *Plasmodium (Giovannolaia) homocircumflexum* infection, with emphasis on the exoerythrocytic development of avian malaria parasites.

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RESEARCH **Open Access**

Mortality and pathology in birds due to *Plasmodium (Giovannolaia) homocircumflexum* infection, with emphasis on the exoerythrocytic development of avian malaria parasites

Mikas Ilgūnas[1*], Dovilė Bukauskaitė[1], Vaidas Palinauskas[1], Tatjana A. Iezhova[1], Nora Dinhopl[2], Nora Nedorost[2], Christiane Weissenbacher-Lang[2], Herbert Weissenböck[2] and Gediminas Valkiūnas[1]

Abstract

Background: Species of avian malaria parasites (*Plasmodium*) are widespread, but their virulence has been insufficiently investigated, particularly in wild birds. During avian malaria, several cycles of tissue merogony occur, and many *Plasmodium* spp. produce secondary exoerythrocytic meronts (phanerozoites), which are induced by merozoites developing in erythrocytic meronts. Phanerozoites markedly damage organs, but remain insufficiently investigated in the majority of described *Plasmodium* spp. Avian malaria parasite *Plasmodium* (*Giovannolaia*) *homocircumflexum* (lineage pCOLL4) is virulent and produces phanerozoites in domestic canaries *Serinus canaria,* but its pathogenicity in wild birds remains unknown. The aim of this study was to investigate the pathology caused by this infection in species of common European birds.

Methods: One individual of Eurasian siskin *Carduelis spinus*, common crossbill *Loxia curvirostra* and common starling *Sturnus vulgaris* were exposed to *P. homocircumflexum* infection by intramuscular sub-inoculation of infected blood. The birds were maintained in captivity and parasitaemia was monitored until their death due to malaria. Brain, heart, lungs, liver, spleen, kidney, and a piece of breast muscle were examined using histology and chromogenic in situ hybridization (ISH) methods.

Results: All exposed birds developed malaria infection, survived the peak of parasitaemia, but suddenly died between 30 and 38 days post exposure when parasitaemia markedly decreased. Numerous phanerozoites were visible in histological sections of all organs and were particularly easily visualized after ISH processing. Blockage of brain capillaries with phanerozoites may have led to cerebral ischaemia, causing cerebral paralysis and is most likely the main reason of sudden death of all infected individuals. Inflammatory response was not visible around the brain, heart and muscle phanerozoites, and it was mild in parenchymal organs. The endothelial damage likely causes dysfunction and failure of parenchymal organs.

Conclusion: *Plasmodium homocircumflexum* caused death of experimental passerine birds due to marked damage of organs by phanerozoites. Patterns of phanerozoites development and pathology were similar in all exposed birds. Mortality was reported when parasitaemia decreased or even turned into chronic stage, indicating that the light parasitaemia is not always indication of improved health during avian malaria. Application of traditional histological and

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ISH methods in parallel simplifies investigation of exoerythrocytic development and is recommended in avian malaria research.

Keywords: Avian malaria, *Plasmodium homocircumflexum*, Virulence, Exoerythrocytic development, Phanerozoites

Background

Avian malaria parasites of the genus *Plasmodium* (Haemosporida, Plasmodiidae) are widespread on all continents, except Antarctica [1, 2]. These parasites have complex life cycles [3, 4]. Sporogony occurs in numerous species of blood-sucking mosquitoes belonging to different genera of the Culicidae, which transmit avian malaria [1, 4–6]. The following development occurs in vertebrate hosts. Susceptible birds get infected when mosquitoes inject sporozoites during their blood meal. The sporozoites develop into the first generation of primary exoerythrocytic meronts (cryptozoites), which are found in the reticular cells of the skin and some other organs. Merozoites developing in cryptozoites cannot infect red blood cells, but induce the second generation of primary exoerythrocytic meronts (metacryptozoites), which develop in macrophages and other reticular cells in many internal organs and tissues. Merozoites developing in metacryptozoites are able to infect red blood cells. Part of merozoites from metacryptozoites invade erythrocytes and develop into erythrocytic meronts and gametocytes, while another part induces the next generations of metacryptozoites. Part of merozoites from the erythrocytic meronts along with part of merozoites developed in the metacryptozoites penetrate the endothelial cells of the capillaries and other reticular cells in many organs, initiating formation of secondary exoerythrocytic meronts (phanerozoites) [4, 7].

Detection of exoerythrocytic meronts using traditional histology methods is often difficult in tissue samples of naturally infected birds due to light infection of organs and difficulties to sample in wildlife sick individuals. Moreover, fragmented nuclei within necrotic tissues of sampled dead birds might be erroneously considered as exoerythrocytic meronts [8]. To overcome this diagnostic difficulty, chromogenic in situ hybridization (ISH) protocol was developed for detection of avian *Plasmodium* spp. [8]. Application of this method simplifies research on pathology caused by *Plasmodium* spp. and provides new data for better understanding of pathogenicity of avian malaria in wild birds [9].

More than 50 species of avian malaria parasites have been described, and their number is increasing [10–14]. However, the majority of recent studies deal mainly with the morphology of blood stages, molecular characterization, evolutionary biology, and distribution of these organisms. A few recent studies address exoerythrocytic development of avian *Plasmodium* spp. [9, 15, 16], but this information is crucial for better understanding pathological events during malaria infections.

Recently, a new *Plasmodium* species, *Plasmodium* (*Giovannolaia*) *homocircumflexum* (cytochrome *b* gene lineage pCOLL4) was described [14]. This parasite was isolated from a wild-caught red-backed shrike *Lanius collurio*, and experimental studies showed that this infection is often lethal in domestic canaries *Serinus canaria* due to marked pathology caused by phanerozoites. This parasite isolate was cryopreserved and is available for experimental research at the Nature Research Centre, Vilnius, Lithuania. However, there is no information about its virulence in wild birds, the aims of this study were: (1) to investigate effects of *P. homocircumflexum* (pCOLL4) on three species of common European birds (Eurasian siskin *Carduelis spinus*, common crossbill *Loxia curvirostra* and common starling *Sturnus vulgaris*); (2) to investigate dynamics of parasitaemia and development of phanerozoites in exposed birds; and, (3) to compare sensitivity of traditional histological and ISH methods in detection of tissue stages of avian malaria parasites.

Methods

Study site and experimental design

Birds were caught using mist nets and big funnel traps [17], and experiments were carried out at the Biological Station 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 23 May and 16 July, 2014. The birds were exposed to experimental *P. homocircumflexum* (pCOLL4) infection and kept until they died. Blood samples and organs were collected and examined for blood stages and phanerozoites.

Juvenile Eurasian siskin, common crossbill and common starling were chosen for this research because they are abundant in Europe and are easy to maintain in captivity. *Plasmodium homocircumflexum* has not been reported in any of the three species of birds used in this study, so these birds can be considered as abnormal (non-adapted) hosts for this infection. It is worth noting that the range of the red-backed shrike and the experimental birds overlap in nature and transmission of malaria among them is theoretically possible.

The Eurasian siskin and the common crossbill were kept indoors in a vector-free room. The common starling was kept outside in a cage covered with a cover made of

fine-mesh bolting silk, which prevented penetration of blood-sucking insects in the cage. All birds were kept at a natural light–dark photoperiod. Before experiments, they were examined for possible presence of natural infections by microscopic examination of blood films and later by polymerase chain reaction (PCR)-based methods (see description below). All birds were non-infected with haemosporidian parasites before the experiment.

The isolate of *P. homocircumflexum* (lineage pCOLL4, GenBank accession no. KC884250), which was originally obtained from a naturally infected red-backed shrike was used. This isolate was cryopreserved and is available at the P. B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania [14]. One sample of this isolate (parasitaemia intensity of 4 %) was thawed and used to infect one Eurasian siskin, as described by Palinauskas et al. [18] with slight modifications. Briefly, the frozen tube containing infected blood was thawed and mixed with 12 % NaCl (one-third of the thawed sample amount). After equilibration for 5 min at room temperature, one volume of 1.6 % NaCl was added, followed by centrifugation at 200 g for 5 min. After centrifugation, the supernatant was removed and 1.6 % NaCl (one-third of the original sample) was added and centrifuged again. After removing the supernatant, the same procedure was repeated three times with 0.9 % NaCl solution. The final mixture was diluted with 0.9 % NaCl and sub-inoculated into one Eurasian siskin, as described by Palinauskas et al. [18].

One common crossbill and one common starling were infected using blood (parasitaemia intensity of 50 %) collected from the exposed Eurasian siskin. Briefly, the brachial vein of the donor Eurasian siskin was punctured using a needle. About 100 µl of blood was mixed with 25 µl sodium citrate and 125 µl 0.9 % saline solution. The mixture was sub-inoculated into the pectoral muscle of the experimental birds, as described by Palinauskas et al. [18]. Six wild caught non-infected Eurasian siskins were used as controls. The birds were maintained in the same room as the experimental birds.

All birds were observed until the experimental birds died [30–38 days post exposure (dpe)]. Blood was taken for microscopic examination and PCR-based testing during the course of the experiment (see Fig. 1). Approximately 50 µl of blood was collected in heparinized microcapillaries by puncturing the brachial vein. A drop of blood was used to make three blood films, which were air-dried, fixed with absolute methanol, stained with Giemsa and examined microscopically as described by Valkiūnas et al. [19]. Approximately 35 µl of the blood left in the capillary was fixed in non-lysis SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for molecular analysis; these samples were kept at room temperature in the field and at −20 °C in the laboratory.

Brain, heart, kidney, liver, lungs, spleen, and a piece of the pectoral muscle of the experimental birds were dissected after the birds' death. The samples were fixed in 10 % neutral formalin and embedded in paraffin blocks. Histological sections of 4 µm were prepared, stained with haematoxylin-eosin (H&E) [4] and examined microscopically.

Morphological analysis

An Olympus BX51 light microscope equipped with Olympus DP12 digital camera and imaging software Olympus DP-SOFT was used to examine slides and prepare illustrations. To trace the parasitaemia, each blood slide was examined for 15–20 min at medium magnification (×400), and then at least 100 fields were studied at high magnification (×1000). Intensity of parasitaemia was estimated as a percentage by actual counting of the number of parasites per 1000 erythrocytes or per 10,000 erythrocytes if infections were light [20]. Microscopic examination was also used to determine possible presence of co-infections with other haemosporidian parasites. Histological preparations were examined using similar protocol, but they were also examined at low magnification (×200) for 10–15 min, followed by examination of the preparations for 10–15 min at medium magnification (×400) and then for another 20–30 min at high magnification (×1000). Statistical analyses were carried out using the 'Statistica 7' package.

Molecular analysis

Total DNA was extracted from blood samples using the standard ammonium-acetate protocol [21] with a minor modification: instead of 250 µl of fixed blood, 125 µl was used. A nested-PCR protocol [22] was applied for the molecular analysis. For the first PCR, the primer pair HaemFNI [5′-CATATATTAAGAGAAITATGGAG-3′] and HaemNR3 [5′-ATAGAAAGATAAGAAATACCATT C-3′] was used. This is a general primer pair that amplifies the mitochondrial cytochrome *b* (cyt *b*) gene of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* species. The reaction mix for the first PCR consisted of 12.5 µl of Dreamtaq Master Mix (Fermentas, Lithuania), 8.5 µl of nuclease-free water, 1 µl of each primer and 2 µl of template DNA. The thermal conditions for the first PCR were according to Helgren et al. [22]. For the second PCR, the primer pair HAEMF [5′-ATGGTGCTTTCGATATATGC ATG-3′] and HAEMR2 [5′-GCATTATCTGGATGTGA TAATGGT-3′] was used. This primer pair amplifies a 479 bp fragment of cyt *b* gene [23]. The reaction mix for the second PCR was identical to the mix for the first PCR, only instead of extracted DNA, 2 µl of the first PCR products were used as templates. The conditions for the second PCR were the same as in the first PCR, but 35

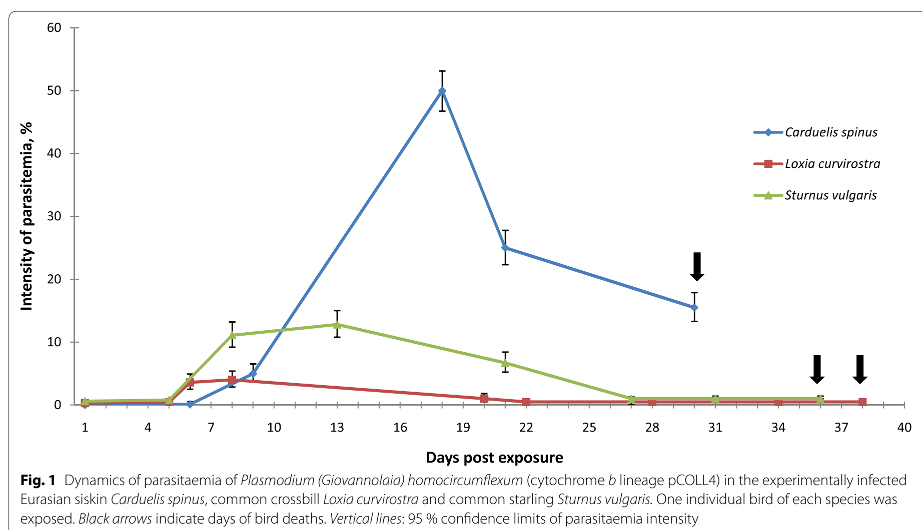

Fig. 1 Dynamics of parasitaemia of *Plasmodium (Giovannolaia) homocircumflexum* (cytochrome *b* lineage pCOLL4) in the experimentally infected Eurasian siskin *Carduelis spinus*, common crossbill *Loxia curvirostra* and common starling *Sturnus vulgaris*. One individual bird of each species was exposed. *Black arrows* indicate days of bird deaths. *Vertical lines*: 95 % confidence limits of parasitaemia intensity

cycles instead of 20 were run. Success of the amplification was evaluated by running 2 µl of the second PCR product on 2 % agarose gel. One negative control (nuclease-free water) and one positive control (a *Plasmodium* sample, which was positive by microscopic examination of blood films) were used to determine possible false amplifications. No case of false amplification was found.

The cyt *b* gene fragments were sequenced from the 5′ end using the HAEMF primer [23]. Dye terminator cycle sequencing (Big Dye) was used. Samples were loaded onto an ABI PRISM TM 3100 capillary sequencing robot (Applied Biosystems, USA). Sequences of parasites were edited and examined using the BioEdit program [24]. The 'Basic Local Alignment Search Tool' and the megablast algorithm were used to identify the cyt *b* lineages of detected DNA sequences.

In situ hybridization

Chromogenic in situ hybridization ISH was carried out according to Dinhopl et al. [8]. In brief, 3 µm paraffin wax-embedded tissue sections were subjected to proteolytic treatment with proteinase K (Roche, Basel, Switzerland) 6 µg/ml in Tris-buffered saline at 37 °C for 50 min. For hybridization, the slides were incubated overnight at 40 °C with hybridization mixture and a final probe concentration of 100 ng/ml. The used oligonucleotide probe (sequence: 5′-TTTAATAACTCGTTATATATATCAGTGTAGCAC-3′) was labelled with digoxigenin at the 3′ end (Eurofins MWG Operon, Ebersberg, Germany). The probe is aimed at 18S rRNA strand and is specific to detect avian *Plasmodium* spp. [9]. The digoxigenin-labelled hybrids were detected by incubating the slides with antidigoxigenin-AP Fab fragments (Roche) (1:200) for 1 h at RT. Visualization of the reaction was carried out using the colour substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) (Roche). Probe specificity has been extensively tested previously [8]. Tissues from a deceased wild Blackbird *Turdus merula* free of avian malaria parasites as well as application of an irrelevant oligonucleotide probe (designed for *Leishmania* spp.) on the experimental samples [see 8] were used as controls in this study.

Ethical statement

Experimental procedures of this study were approved by the International Research Co-operation Agreement between the Biological Station Rybachy of the Zoological Institute of the Russian Academy of Sciences and Institute of Ecology of Nature Research Centre (25-05-2010). All efforts were made to minimize handling time and potential suffering of animals. None of the experimental birds suffered apparent injury during experiments.

Results

All control birds survived and remained non-infected during this study. The exposed Eurasian siskin, common

crossbill and common starling got infected, and PCR-based testing confirmed presence of the parasite lineage pCOLL4 in these birds.

According to microscopic examination of blood films, the prepatent period was 6, 6 and 8 dpe in Eurasian siskin, common crossbill and common starling, respectively. Parasitaemia remained during entire observation time (Fig. 1). The highest parasitaemia developed in the Eurasian siskin, and it reached 50 % on 18 dpe. Maximum parasitaemia was 4 % (8 dpe) and 12.8 % (13 dpe) in the common crossbill and common starling, respectively. The intensity of parasitaemia markedly decreased in all exposed birds between 20 and 27 dpe.

All exposed birds survived the acute parasitaemia stage, but suddenly died when parasitaemia decreased and was 15.5, 0.2, 0.1 % in the Eurasian siskin, common crossbill and common starling, respectively; these birds died 30, 38 and 36 dpe, respectively (Fig. 1). The mortality was sudden: all birds looked healthy in the evening, but were found dead the next morning. A marked enlargement of the spleen and liver, as well as cardiomegaly with dark pericardial effusion was recorded at post-mortem examination.

Phanerozoites were observed in all dead birds. Numerous phanerozoites were detected in the brain capillaries (Fig. 2a–c); the parasites were large elongate bodies (maximum length is 34.6 µm), which followed shape of the brain capillaries. The phanerozoites blocked the capillaries resulting in the interruption of local circulation (Fig. 2a, c), which likely was the main reason of mortality. Numerous developing merozoites of roundish shape were readily visible inside phanerozoites (Fig. 2a–c). Inflammatory response was not visible around the brain phanerozoites.

Phanerozoites were also numerous in lungs (Fig. 2d–f), liver (Fig. 2g–i), spleen (Fig. 2j–l), kidney (Fig. 3a–c), heart (Fig. 3d–f), and pectoral muscle (Fig. 3g–i) of all dead birds. In these organs, the parasites appeared as roundish or oval bodies developing in endothelial cells of capillaries and in macrophages. The inflammatory response was mild in parenchymal organs; it included lymphocytes, plasma cells, heterophils, and macrophages. The endothelial damage likely causes dysfunction and failure of parasitized organs.

In the heart (Fig. 3d–f) and breast muscles (Fig. 3g–i), phanerozoites were seen in endothelial cells of capillaries. The biggest parasites reached 28.6 µm in their large diameter; they were elongated, being similar in shape to the parasites observed in the brain. Inflammatory response was not visible in the heart or the breast muscles.

Chromogenic ISH confirmed presence of phanerozoites in all examined preparations of the exposed birds. Positive ISH signals were readily visible in preparations of brain (Fig. 4d–f), heart (Fig. 4j–l), lungs (Fig. 5d–f), liver, spleen, kidney, and breast muscle of all exposed birds. They looked like black spots and could be easily counted (Fig. 4j–l) providing opportunity to estimate and compare intensity of phanerozoite infestation in different organs, which is difficult to do using microscopic examination of histological preparations stained with H&E (Figs. 4, 5). The negative controls used for the ISH assay did not give any positive signals.

At low (×200) magnification (Figs. 4a, d, g, j and 5a, d) it was nearly impossible to see phanerozoites in histological preparations stained with H&E, but they were readily visible in preparations treated for ISH (compare Figs. 4a, g and 5a with Figs. 4d, j and 5d, respectively). At medium magnification (×400) both histological (Figs. 4b, h and 5b) and ISH-treated preparations (Figs. 4e, k and 5e) allowed detection of phanerozoites, but it was difficult to determine their morphological features. At high magnification (×1000), both histological preparations (Figs. 4c, i and 5c) and those treated for ISH (Figs. 4f, l and 5f) allowed detection of phanerozoites, but structure of the parasites (shape of developing merozoites, morphology of the parasite envelope) cannot be recognized after ISH treatment (compare Fig. 4c with f). Chromogenic ISH markedly speeds up the search for phanerozoites, but morphological characters of the parasites cannot be determined using this technique.

Discussion

The key results of this study are that: (1) *Plasmodium homocircumflexum* (pCOLL4) caused lethal malaria in three species of experimental passerine birds; (2) mortality occurs suddenly during decreased or even chronic stage of parasitaemia 4–6 weeks after exposure, most likely due to damage caused by phanerozoites; and (3) dynamics of parasitaemia were different, but patterns of phanerozoites development and tissue pathology were similar in all exposed birds. It is also important to note and worth discussion that ISH is an effective method in determining tissue stages of avian malaria parasites, but traditional histology remains essential for determining the structure of exoerythrocytic meronts.

Plasmodium homocircumflexum has been reported in several species of birds. The lineage pCOLL4 of this parasite along with the synonymous lineages pU12 (GenBank nr. DQ241519) and BOBO20085 (GenBank nr. KC867664) were detected in the red-backed shrike, domestic canary, collared flycatcher *Ficedula albicollis,* red-rumped warbling finch *Poospiza lateralis,* lark-like bushrunner *Coryphistera alaudina,* curve-billed reedhaunter *Limnornis curvirostris,* chopi blackbird *Gnorimopsar chopi,* chalk-browed mockingbird *Mimus saturninus,* diademed tanager *Stephanophorus*

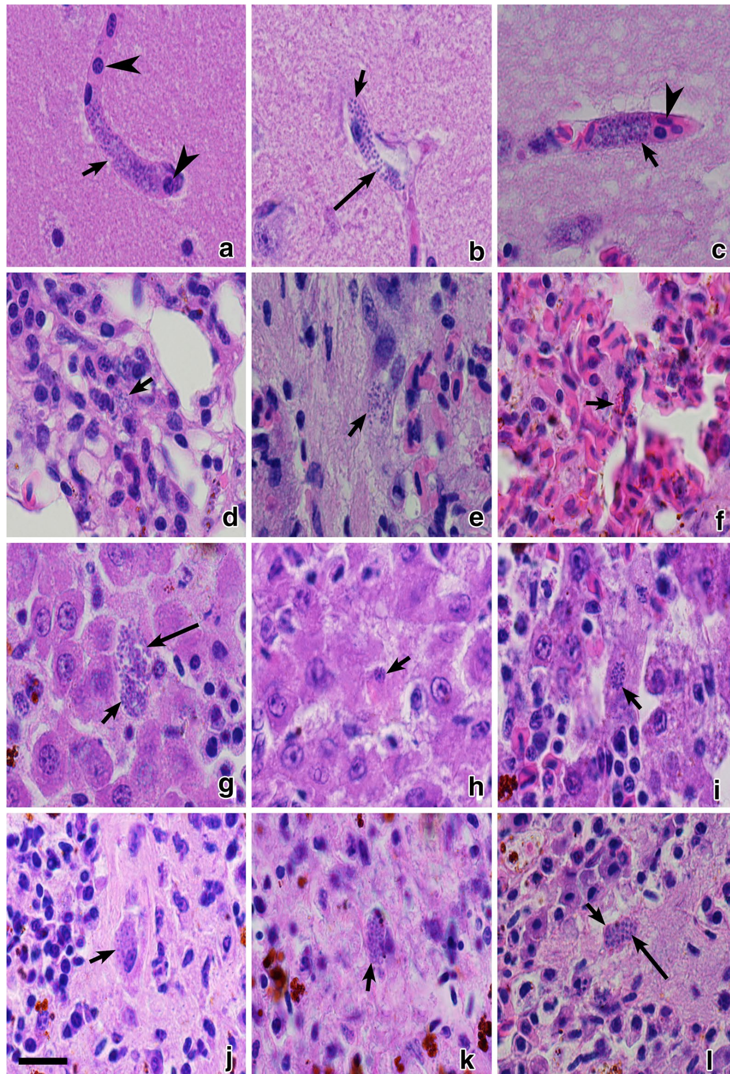

Fig. 2 Phanerozoites of *Plasmodium (Giovannolaia) homocircumflexum* (cytochrome *b* lineage pCOLL4) in histological sections of brain (**a**–**c**), lungs (**d**–**f**), liver (**g**–**i**), spleen (**j**–**l**) of experimentally infected Eurasian siskin *Carduelis spinus* (**a**, **d**, **g**, **j**), common crossbill *Loxia curvirostra* (**b**, **e**, **h**, **k**) and common starling *Sturnus vulgaris* (**c**, **f**, **i**, **l**). *Short arrows*: phanerozoites, *long arrows*: merozoites, simple *arrowheads*: red blood cells in brain capillaries. Haematoxylin-eosin stained preparations. *Scale bar* 20 µm

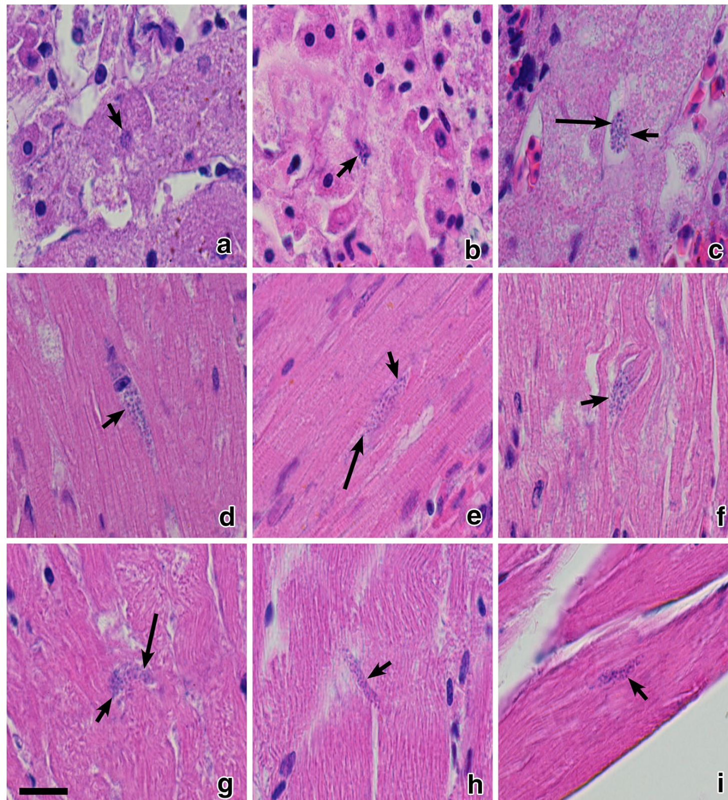

Fig. 3 Phanerozoites of *Plasmodium (Giovannolaia) homocircumflexum* (cytochrome *b* lineage pCOLL4) in histological sections of kidney (**a**–**c**), heart (**d**–**f**) and pectoral muscle (**g**–**i**) of experimentally infected Eurasian siskin *Carduleis spinus* (**a**, **d**, **g**), common crossbill *Loxia curvirostra* (**b**, **e**, **h**) and common starling *Sturnus vulgaris* (**c**, **f**, **i**). *Short arrows*: phanerozoites, *long arrows*: merozoites. Haematoxylin-eosin stained preparations. *Scale bar* 20 µm

diadematus, bobolinks *Dolichonyx oryzivorus,* Eurasian siskin, common crossbill and common starling [14, 25–27, this study]. This parasite is virulent due to development of high parasitaemia in some hosts, but particularly because of its ability to produce numerous phanerozoites in many organs. This malarial infection kills domestic canaries [14] along with individuals belonging to three species of wild birds (this study), and it might be able to parasitize other bird species and be virulent in many of them, as is the case with the generalist lineages pSGS1 and pGRW4 of *Plasmodium relictum* and the lineage pGRW2 of *Plasmodium ashfordi* [18, 28–30]. Additional studies are needed for better understanding of virulence of this parasite in different bird species.

Transmission of *P. homocircumflexum* (pCOLL4) has not been reported in Europe, where this infection has been observed only in adult far-distance migrants after their arrival from African wintering grounds [14, 26]. It seems probable that European birds get infected away from their breeding areas. Lack of susceptible mosquito vectors might be an obstacle for this parasite's transmission because sporogony was abortive in two common

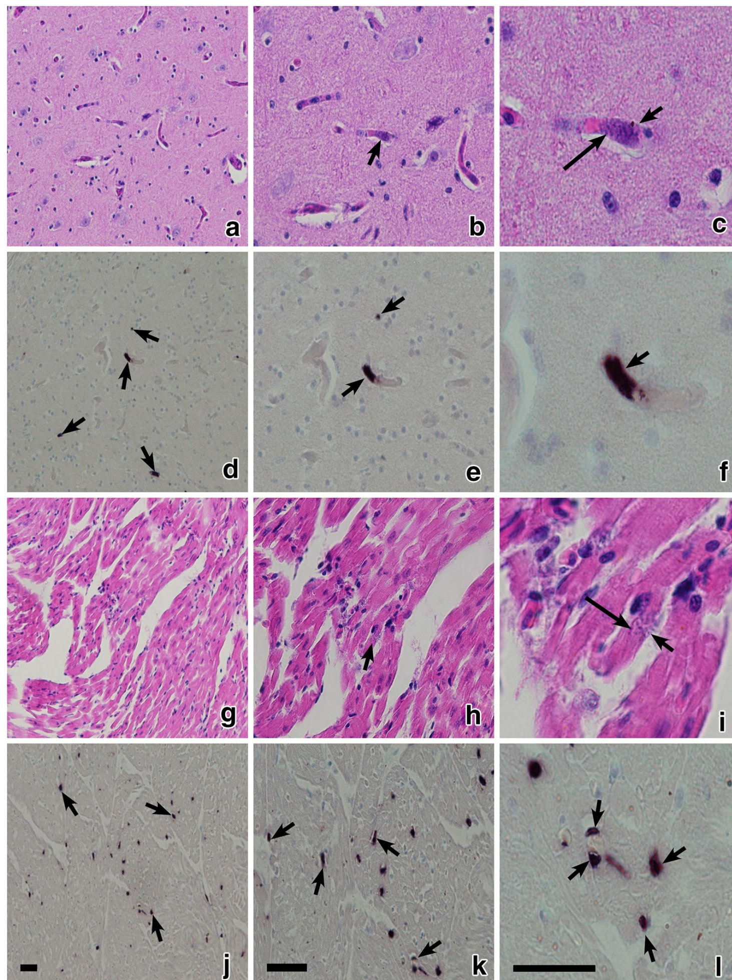

Fig. 4 Phanerozoites of *Plasmodium (Giovannolaia) homocircumflexum* (cytochrome *b* lineage pCOLL4) in histological sections of the same organs stained with haematoxylin-eosin (**a**–**c**, **g**–**i**) and processed with chromogenic in situ hybridization (**d**–**f**, **j**–**l**): brain (**a**–**f**) and heart (**g**–**l**) of common crossbill *Loxia curvirostra.* Images of same preparations are given at low (×200; **a**, **d**, **g**, **j**), medium (×400; **b**, **e**, **h**, **k**) and high (×1000; **c**, **f**, **i**, **l**) magnifications. *Short arrows*: phanerozoites; *long arrows*: merozoites; *Scale bars* 25 µm

European mosquito species, *Culex pipiens* (forms *pipiens* and *molestus*) and *Aedes vexans* [14]. Vectors of *P. homocircumflexum* remain unknown. However, it is also difficult to rule out that transmission might occur at some sites in Europe, but infected birds are dying, as was the case during the experiments. Additional experimental studies combined with post-mortem examination of naturally infected dead birds collected in wildlife are

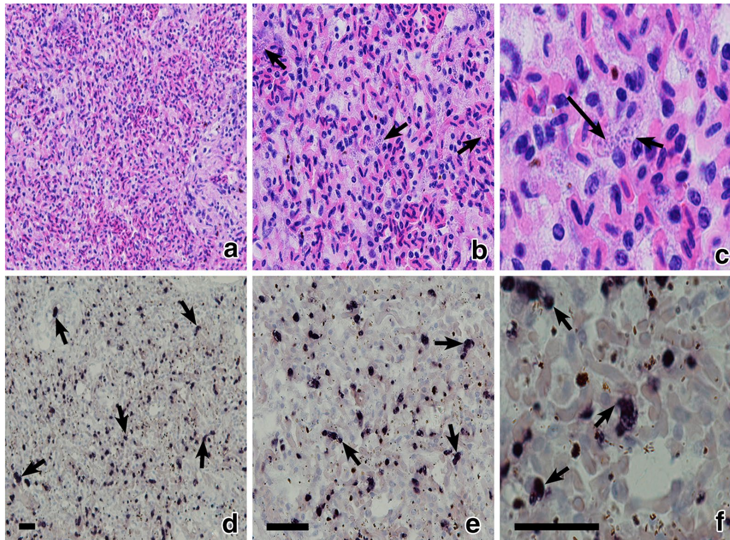

Fig. 5 Phanerozoites of *Plasmodium (Giovannolaia) homocircumflexum* (cytochrome b lineage pCOLL4) in histological sections of same organs stained with hematoxylin-eosin (**a**–**c**) and processed with chromogenic in situ hybridization (**d**–**f**): lung (**a**–**f**) of common crossbill *Loxia curvirostra*. Images of same preparations are given at low (×200; **a**, **d**), medium (×400; **b**, **e**) and high (×1000; **c**, **f**) magnifications. *Short arrows*: phanerozoites; *long arrows*: merozoites; *Scale bars* 25 µm

needed for the better understanding of epidemiology of this malaria infection.

Patterns of development of *P. homocircumflexum* phanerozoites were similar in all exposed bird species (this study) and in domestic canaries [14], indicating similar life cycles and a probable high ability of this parasite to develop secondary exoerythrocytic meronts in many species of avian hosts. Blockage of circulation in brain capillaries (Fig. 2a, c) seems to be particularly dangerous because it can lead to ischaemic brain changes, which might cause cerebral paralysis symptoms, and can explain the observed sudden death of birds [4, 7]. Because mortality of all exposed birds was observed between 30 and 38 dpe, this is the probable period when phanerozoites could develop to an extent at which they start blocking the circulation in the brain. Additional experiments are needed for better understanding of this issue. Phanerozoites causing brain pathology due to blockage of circulation in the capillaries have been described in several avian malaria parasites: *Plasmodium gallinaceum, Plasmodium cathemerium, Plasmodium durae, Plasmodium lophurae, Plasmodium matutinum, Plasmodium octamerium*, and some other species [4].

Inflammatory changes in the brain cannot be expected in avian malaria, and this is supported by many reports [3, 4, 14]. Capillary blockage is the major pathological feature in most forms of avian malaria. In the present cases (Fig. 2 and Fig. 3), circulation disturbances can explain death of the birds (due to ischaemia of the brain), pericardial effusions (due to congestion of epicardial blood vessels) and in part also hepatomegaly and splenomegaly. In the latter two organs, infiltrations of inflammatory cells are an additional feature. Marked enlargements of the spleen and liver, as well as cardiomegaly with dark pericardial effusion were recorded at post-mortem examination in all birds and likely are important pathologies during this infection.

Brain lesions leading to an ischaemic brain changes are among the most severe conditions caused by the human malaria parasite *Plasmodium falciparum*, but the underlying functional pathology in humans and birds is different [4, 7, 31, 32]. Both in avian and human malaria, cerebral pathology occurs due to blockage of circulation in brain capillaries. However, birds are dying because of development of large phanerozoites, which follow the shape of brain capillaries eventually leading to the interruption of

blood circulation (Fig. 2a, c). During *P. falciparum* malaria, severe pathology is caused by adherence of infected erythrocytes to the endothelial cells of microvascular blood vessels in the brain, leading to blockage of the circulation and resulting in ischaemic brain changes.

Mortality of birds due to *Plasmodium* infections have been reported both due to high parasitaemia and damage caused by phanerozoites [33, 34]. It is generally assumed that the decrease of parasitaemia after the acute stage of infection and the resulting chronic light parasitaemia indicate improved health in malaria infected birds [1, 35, 36]. This study shows that this is not always true because birds can die due to pathology caused by phanerozoites at chronic stage of infection when parasitaemia decreases (Fig. 1). The role of exoerythrocytic meronts in the pathology of birds is most likely underestimated during avian malaria because mortality might occur rapidly during light parasitaemia, and it is difficult to detect sick birds in wildlife. Recent studies using ISH method and DNA sequence data indicate that widespread *Plasmodium* parasite lineages are responsible for mortality in common European birds [9]; however, rates of mortality caused by malaria infections remain unknown in wildlife populations.

Better understanding of pathology caused by phanerozoites and other exoerythrocytic meronts of *Plasmodium* spp. is crucial for estimating of the true impact of these parasites on wild bird populations.

The number of "positive spots" after ISH treating is very high in some organs (Fig. 5d–f). That is in accordance with previous reports using this technique [8, 9] and certainly indicates parasites based on comparison with the negative controls, which were used both previously [8, 9] and in the present study. The application of a sensitive ISH assay for detection of exoerythrocytic meronts of avian malaria parasites and related haemosporidians markedly simplifies detection of affected organs, but it is not suitable for determining the structure of reported meronts. Histological methods using traditional staining provide additional data about morphology of tissue stages, which is important information for better understanding of the biology of malaria parasites. For example, two types of phanerozoites develop in *Plasmodium pinotti*: only roundish merozoites (micromerozoites) develop in the majority of phanerozoites, but phanerozoites containing elongate merozoites (macromerozoites) were also described [3, 37]. The role of these two types of merozoites remain unclear in the life cycle of *P. pinotti* and other avian malaria parasites, but this finding likely is important epidemiologically and might be related to peculiarities of persistence of malarial infections in avian hosts [4]. Ideally, both the traditional histology and ISH should be used in parallel in exoerythrocytic merogony research of haemosporidian parasites.

Conclusion

Plasmodium homocircumflexum (pCOLL4) caused lethal malaria in at least four species of experimentally infected passerine birds due to marked damage of organs by phanerozoites. It is likely that this infection is markedly virulent in non-adapted wild birds and it worth more attention in bird conservation projects. The patterns of parasitaemia development were different, but patterns of phanerozoites development were similar in all tested exposed birds. Mainly, sudden mortality occurred during decreased or even light chronic parasitaemia stages in all exposed birds, indicating that the chronic parasitaemia is not necessarily an indication of improved health during avian malaria. In other words, solely testing of blood samples is an insufficient method to understand avian malaria virulence, which might be underestimated in many *Plasmodium* spp. due to lack of information about their exoerythrocytic development. During investigation of exoerythrocytic merogony of haemosporidians, application of an ISH method for detection of tissue meronts in bird organs, and then processing the ISH positive organs using traditional histological methods is recommended. Application of both these tools in parallel would speed up the search for tissue meronts and also provide information about morphological characters of the parasites and their host cells.

Authors' contributions

GV, MI and VP: experimental conception and design; MI, TAI, DB, VP and GV: fieldwork; MI and DB: bird laboratory care and dissection; MI and TAI: histology work; HW, MI, ND, NN, CWL and GV: ISH and interpretation of the ISH results; MI and GV: paper writing. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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References

1. Atkinson CT, Thomas NJ, Hunter DB. Parasitic diseases of wild birds. Oxford: Wiley-Blackwell; 2008.
2. Marzal A. Recent advances in studies on avian malaria parasites. In: Okwa OO editor Malaria parasites. InTech, Rijeka, Croatia; 2012. p.135–58.

3. Garnham PCC. Malaria parasites and other Haemosporidia. Oxford: Blackwell; 1966.
4. Valkiūnas G. Avian malaria parasites and other Haemosporidia. Boca Raton: CRC; 2005.
5. Njabo K, Cornel AJ, Sehgal RNM, Loiseau C, Buermann W, Harrigan RJ, et al. *Coquillettidia* (Culicidae, Diptera) mosquitoes are natural vectors of avian malaria in Africa. Malar J. 2009;8:193.
6. Santiago-Alarcon D, Palinauskas V, Schaefer HM. Diptera vectors of avian haemosporidian parasites: untangling parasite life cycles and their taxonomy. Biol Rev Camb Philos Soc. 2012;87:928–64.
7. Garnham PCC. Malaria in its various vertebrate hosts. In: Kreier JP, editor. Malaria epidemiology, chemotherapy, morphology and metabolism. New York: Academic Press; 1980.
8. Dinhopl N, Mostegl MM, Richter B, Nedorost N, Maderner A, Fragner K, et al. Application of in situ hybridization for the detection and identification of avian malaria parasites in paraffin wax-embedded tissues from captive penguins. Avian Pathol. 2011;40:315–20.
9. Dinhopl N, Nedorost N, Mostegl MM, Weissenbacher-Lang C, Weissenböck H. In situ hybridization and sequence analysis reveal an association of *Plasmodium* spp. with mortalities in wild passerine birds in Austria. Parasitol Res. 2015;114:1455–62.
10. Ilgūnas M, Palinauskas V, Iezhova TA, Valkiūnas G. Molecular and morphological characterization of two avian malaria parasites (Haemosporida: plasmodiidae), with description of *Plasmodium homonucleophilum* n. sp. Zootaxa. 2013;3666:49–61.
11. Chagas CRF, Valkiūnas G, Nery CVC, Henrique PC, Gonzalez IHL, Monteiro EF, et al. *Plasmodium (Novyella) nucleophilum* from an Egyptian Goose in São Paulo Zoo, Brazil: microscopic confirmation and molecular characterization. Int J Parasitol Parasites Wildl. 2013;2:286–91.
12. Mantilla JS, González AD, Valkiūnas G, Moncada LI, Matta NE. Description and molecular characterization of *Plasmodium* (*Novyella*)*unalis* sp. nov. from the Great Thrush (*Turdusfuscater*) in highland of Colombia. Parasitol Res. 2013;112:4193–204.
13. Walther EL, Valkiūnas G, González AD, Matta NE, Ricklefs RE, Cornel A, et al. Description, molecular characterization, and patterns of distribution of a widespread New World avian malaria parasite (Haemosporida: plasmodiidae), *Plasmodium (Novyella) homopolare* sp. nov. Parasitol Res. 2014;113:3319–32.
14. Palinauskas V, Žiegytė R, Ilgūnas M, Iezhova TA, Bernotienė A, Bolshakov C, et al. Description of the first cryptic avian malaria parasite, *Plasmodium homocircumflexum* n. sp. with experimental data on its virulence and development in avian hosts and mosquitoes. Int J Parasitol. 2015;45:51–62.
15. Howe L, Castro IC, Schoener ER, Hunter S, Barraclough RK, Alley MR. Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. Parasitol Res. 2012;110:913–23.
16. Vanstreels RET, Kolesnikovas CKM, Sandri S, Silveira P, Belo NO, Ferreira FC, et al. Outbreak of avian malaria associated to multiple species of *Plasmodium* in Magellanic penguins undergoing rehabilitation in southern Brazil. PLoS One. 2014;9:e94994.
17. Payevsky V. Rybachy-type trap. In: Busse P, editor. Bird Station Manual. SE European Bird Migration Network. Poland: University of Gdansk; 2000. p. 20–4.
18. Palinauskas V, Valkiūnas G, Bolshakov VC, Bensch S. *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. Exp Parasitol. 2008;120:372–80.
19. Valkiūnas G, Iezhova TA, Križanauskienė A, Palinauskas V, Sehgal RNM, Bensch S. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. J Parasitol. 2008;94:1395–401.
20. Godfrey RD, Fedynich AM, Pence DB. Quantification of hematozoa in blood smears. J Wildl Dis. 1987;23:558–65.
21. Sambrook J, Fritsch EF, Mamiatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
22. Hellgren O, Waldenström J, Bensch S. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol. 2004;90:797–802.
23. Bensch S, Stjernman M, Hasselquist D, Ostman O, Hansson B, Westerdahl H, et al. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc Biol Sci. 2000;267:1583–9.
24. Hall A. BioEdit: a user-friendly biological sequence alignment editor and analysis program of Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–8.
25. Durrant KL, Beadell JS, Ishtiaq F, Graves GR, Olson SL, Gering E, et al. Avian hematozoa in South America: a comparison of temperate and tropical zones. Ornithol Monogr. 2006;60:98–111.
26. Pérez-Tris J, Hellgren O, Križanauskienė A, Waldenström J, Secondi J, Bonneaud C, et al. Within-host speciation of malaria parasites. PLoS One. 2007;2:e235.
27. Levin II, Zwiers P, Deem SL, Geest EA, Higashiguchi JM, Iezhova TA, et al. Multiple lineages of avian malaria parasites (*Plasmodium*) in the Galapagos Islands and evidence for arrival via migratory birds. Conserv Biol. 2013;27:1366–77.
28. Atkinson CT, Duset RJ, Woods KL, Iko WM. Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. J Wildl Dis. 2000;36:197–204.
29. Valkiūnas G, Zehtindjiev P, Hellgren O, Ilieva M, Iezhova TA, Bensch S. Linkage between mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites, with a description of *Plasmodium (Novyella) ashfordi* sp. nov. Parasitol Res. 2007;100:1311–22.
30. Zehtindjiev P, Ilieva M, Westerdahl H, Hansson B, Valkiunas G, Bensch S. Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler *Acrocephalus arundinaceus*. Exp Parasitol. 2008;119:99–110.
31. Holding PA, Snow RW. Impact of *Plasmodium falciparum* malaria on performance and learning: review of the evidence. In: Breman JG, Egan A, Keusch GT, editors. The Intolerable Burden of Malaria: A New Look at the Numbers: Supplement to Volume 64 of the American Journal of Tropical Medicine and Hygiene, 2001.
32. Adams Y, Kuhnrae P, Higgins MK, Ghumra A, Rowe JA. Rosetting *Plasmodium falciparum*-infected erythrocytes bind to human brain microvascular endothelial cells in vitro, demonstrating a dual adhesion phenotype mediated by distinct *P*. *falciparum* erythrocyte membrane protein 1 domains. Infect Immun. 2014;82:949–59.
33. Coulston F, Manwell RD. Single-parasite infections and exoerythrocytic schizogony in *Plasmodium circumflexum*. Am J Epidemiol. 1941;34:119–25.
34. Huchzermeyer FW, Van der Vyver FH. Isolation of *Plasmodium circumflexum* from wild guineafowl (*Numida meleagris*) and the experimental infection in domestic poultry. Avian Pathol. 1991;20:213–23.
35. Castro I, Howe L, Tompkins DM, Barraclough RK, Slaney D. Presence and seasonal prevalence of *Plasmodium* spp. in a rare endemic New Zealand passerine (tieke or saddleback, *Philesturnus carunculatus*). J Wildl Dis. 2011;47:860–7.
36. Vanstreels RET, da Silva-Filho RP, Kolesnikovas CKM, Bhering RCC, Ruoppolo V, Epiphanio S, et al. Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. Vet Res. 2015;46:30.
37. Muniz J, Soares RRL. Nota sôbre um parasita do gênero Plasmodium encontrado no Ramphastostoco Müller, 1776, 'Tugano-Açu', e diferente do Plasmodium huffi: Plasmodium pinottii n. sp. Rev Bras Malariol. 1954;6:611–7.

PAPER II

Description, molecular characterisation, diagnostics and life cycle of *Plasmodium elongatum* (lineage pERIRUB01), the virulent avian malaria parasite.

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Description, molecular characterisation, diagnostics and life cycle of *Plasmodium elongatum* (lineage pERIRUB01), the virulent avian malaria parasite

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ABSTRACT

Plasmodium elongatum causes severe avian malaria and is distributed worldwide. This parasite is of particular importance due to its ability to develop and cause lethal malaria not only in natural hosts, but also in non-adapted endemic birds such as the brown kiwi and different species of penguins. Information on vectors of this infection is available but is contradictory. PCR-based analysis indicated the possible existence of a cluster of closely related *P. elongatum* lineages which might differ in their ability to develop in certain mosquitoes and birds. This experimental study provides information about molecular and morphological characterisation of a virulent *P. elongatum* strain (lineage pERIRUB01) isolated from a naturally infected European robin, *Erithacus rubecula*. Phylogenetic analysis based on partial cytochrome *b* gene sequences showed that this parasite lineage is closely related to *P. elongatum* (lineage pGRW6). Blood stages of both parasite lineages are indistinguishable, indicating that they belong to the same species. Both pathogens develop in experimentally infected canaries, *Serinus canaria*, causing death of the hosts. In both these lineages, trophozoites and erythrocytic meronts develop in polychromatic erythrocytes and erythroblasts, gametocytes parasitize mature erythrocytes, exoerythrocytic stages develop in cells of the erythrocytic series in bone marrow and are occasionally reported in spleen and liver. Massive infestation of bone marrow cells is the main reason for bird mortality. We report here on syncytium-like remnants of tissue meronts, which slip out of the bone marrow into the peripheral circulation, providing evidence that the syncytia can be a template for PCR amplification. This finding contributes to better understanding positive PCR amplifications in birds when parasitemia is invisible and improved diagnostics of abortive haemosporidian infections. Sporogony of *P. elongatum* (pERIRUB01) completes the cycle and sporozoites develop in widespread *Culex quinquefasciatus* and *Culex pipiens pipiens* form *molestus* mosquitoes. This experimental study provides information on virulence and within species lineage diversity in a single pathogenic species of haemosporidian parasite.

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1. Introduction

Avian malaria parasites (Plasmodiidae, Haemosporida) are broadly distributed all over the world (Garnham, 1966; Valkiūnas, 2005). More than 50 avian malaria parasite species have been described. They have different life history traits and specificities to the vertebrate hosts and vectors. Some species of avian malaria are specialists and infect birds of one species or genus, but some of them are generalists and are able to infect broad ranges of avian hosts (Waldenström et al., 2002; Valkiūnas, 2005; Ishtiaq et al., 2007; Beadell et al., 2009; Dimitrov et al., 2010).

One of the most pathogenic avian malaria agents is *Plasmodium elongatum*. This species was first described more than 80 years ago and attributed to the subgenus *Huffia* (Garnham, 1966). Since then *P. elongatum* has been recorded by many authors on all continents (except Antarctica) in birds of several orders (Anseriformes, Falconiformes, Columbiformes, Sphenisciformes, Strigiformes, Passeriformes and some others) (Fleischman et al., 1968; Nayar et al., 1998; Valkiūnas, 2005; Beadell et al., 2009; Dimitrov et al., 2010; Baillie and Brunton, 2011; Howe et al., 2012; Clark et al., 2014; Vanstreels et al., 2014). According to the MalAvi database this is the most generalist species among avian malaria agents after *Plasmodium relictum* which infects more than 300 bird species of 10 orders.

Plasmodium elongatum is of particular importance due to its pathogenicity in both wild and captive birds (Atkinson et al.,

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2008; Alley et al., 2010; Castro et al., 2011; Howe et al., 2012). This parasite is able to develop in non-adapted birds such as brown kiwi and different species of penguins (Atkinson et al., 2008; Howe et al., 2012). Recent studies reveal the number of records of *P. elongatum* in introduced and endemic New Zealand birds (Alley et al., 2010; Castro et al., 2011; Baillie et al., 2012; Howe et al., 2012; Clark et al., 2014; Schoener et al., 2014). The impact of avian malaria on native New Zealand birds is well illustrated by two cases in which 60% of New Zealand dotterels, *Charadrius obscurus*, and 80% of native mohua, *Mohoua ochrocephala*, were killed by *Plasmodium* spp. parasites in Auckland Zoo, New Zealand and Orana Park, New Zealand, respectively (Derraik et al., 2008; Castro et al., 2011). The lethal impact of *P. elongatum* has also been determined for saddlebacks, *Philesturnus carunculatus carunculatus* (Alley et al., 2010; Castro et al., 2011).

One of the first reports about susceptibility of penguins to *P. elongatum* was published in 1962. Clay G. Huff and Tsugiye Shiroishi reported infection by *P. elongatum* in Humboldt's penguins, *Spheniscus humboldti*, in Washington DC Zoo, USA (Huff and Shiroishi, 1962). Later, more cases of *P. elongatum* were obtained from different zoos infecting black-footed penguins, *Spheniscus demersus*, rockhopper penguins, *Eudyptes crestatus*, and Magellanic penguins, *Spheniscus magellanicus* (Sladen et al., 1976; Vanstreels et al., 2014). Of 32 reported cases of avian malaria infections in African black-footed penguins from Baltimore Zoo, USA, 78.1% of birds were infected with *P. elongatum*, and some of those infections were fatal (Graczyk et al., 1994b). This parasite was also recorded as the most prevalent in infected black-footed penguins (Cranfield et al., 1994).

Plasmodium elongatum is a generalist, infecting a broad ranges of Culicidae mosquitoes (Santiago-Alarcon et al., 2012; Valkiūnas, 2005). However, there are conflicting data about the mosquito species that are able to transmit *P. elongatum*. In early studies, Huff (1927) found *Culex salinarius* and *Culex restuans* to be susceptible to *P. elongatum. Culex pipiens* was partially susceptible to *P. elongatum* (sporozoites were observed in 12 out of 47 infected mosquitoes), but no development in six species of genus *Aedes* and one species of *Anopheles* were recorded. Later, partial susceptibility of *Culex tarsalis* (three out of 18) and *Aedes triseriatus* (three out of nine) were also determined (Huff, 1932). In accordance, Raffaele (1934), working with an Italian strain of *P. elongatum*, obtained 100% susceptibility of *Culex quinquefasciatus* and 30% of *C. pipiens*. However, Reichenow (1932) and Micks (1949) reported no positive results for *P. elongatum* development in *C. pipiens*, *C. quinquefasciatus*, *Aedes aegypti*, *Aedes vexans* and *Anopheles quadrimaculatus* mosquitoes. Variations in susceptibility may be caused by regional differences, when studies use diverse populations of mosquitoes and probably different lineages of *P. elongatum*. These parasites may share the same morphology, but differ genetically and have different developmental abilities in certain mosquito species. PCR-based studies of the cytochrome b (cyt *b*) gene reveal that some *Plasmodium* spp. contain several lineages which might differ in infectivity to certain mosquitoes and/or vertebrate hosts. Thus some lineages of *P. elongatum* could be more generalist and others more specialist. *Plasmodium* spp. development also depends on other factors influencing the success of sporogony. Maternally inherited *Wolbachia* endosymbiontic bacteria may act as inhibitors for development of various pathogens including *Plasmodium* parasites (Kambris et al., 2009; Moreira et al., 2009; Cook and McGraw, 2010; Murdock et al., 2014a). Environmental factors such as temperature may have important impact on mosquito susceptibility to vector-borne parasites by acting both directly on the parasite and indirectly on mosquito physiology and immunity (Murdock et al., 2012, 2014a, b). Even the larval environment may influence transmission potential of vector-borne pathogens (Moller-Jacobs et al., 2014). These issues need more detailed experimental investigation and clarification. Different lineages of *P. elongatum* probably can cause expansion of dangerous disease not just to endemic species in remote islands, but also in bird populations in northern regions (Loiseau et al., 2012).

In the present study we provide information about molecular identification, morphological description and the life cycle of a virulent *P. elongatum* strain (lineage pERIRUB01) isolated from a naturally infected European robin, *Erithacus rubecula*. We describe the development of this parasite lineage in experimentally infected canaries, *Serinus canaria*, and provide a description of exoerythrocytic and blood stages, as well as information about parasite virulence in the vertebrate host. Furthermore, we describe sporogony and formation of sporozoites in widespread *C. quinquefasciatus* and *Culex pipiens pipiens* form *molestus* mosquitoes. Molecular identification of this *P. elongatum* lineage and a detailed description of its biology are of epidemiological importance and should be considered in infectious disease management.

2. Materials and methods

2.1. Plasmodium (Huffia) sp. strain and experimental design

Plasmodium (Huffia) sp. strain (mitochondrial cyt *b* gene lineage pERIRUB01) was isolated from a naturally infected European robin at the Biological station "Rybachy" of the Zoological Institute of the Russian Academy of Science in June 2014. The strain was multiplied in a robin and cryopreserved in liquid nitrogen as described by Palinauskas et al. (2015). Frozen samples were maintained at the Biobank in Nature Research Centre, Lithuania.

The study was carried out at the Nature Research Centre, Vilnius, Lithuania in 2014–2015. Domestic canaries (experimental birds) were purchased commercially under the permit no. 2012/01/04-0221 issued by the Ethical Commission of the Baltic Laboratory Animal Science Association and Lithuanian State Food and Veterinary Office. To prove that all obtained birds were free of haemosporidian parasites, blood was taken from the brachial vein for microscopy of blood films and PCR-based molecular analysis (as described in Section 2.5). All birds were kept in a vector-free room under controlled conditions (20 ± 1 °C; 50–60% relative humidity (RH)).

For the experimental setup we used one deep frozen sample of the pERIRUB01 parasite isolate. The procedure of thawing the blood sample was according to Palinauskas et al. (2015). Seven experimental canaries were inoculated with the blood solution (the dose of the asexual parasite stages was approximately 6×10^5) into the pectoral muscles by following the protocol described by Palinauskas et al. (2008). To determine the development of the infection, all birds were examined every 3–4 days post exposure (pe) by taking blood from the brachial vein as described in Section 2.2. Seven uninfected canaries were kept as a control group for the duration of the experiment.

2.2. Collection of blood and organs for microscopy and molecular analysis

Blood was taken by puncturing the brachial vein, smearing two slides for microscopic examination and placing approximately 30 μl of blood in SET buffer (0.05 M tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for PCR-based analysis. Blood slides were immediately dried, fixed with absolute methanol for 3 min and stained with Giemsa solution as described by Valkiūnas et al. (2008a). Internal organs (brain, heart, kidneys, liver, lungs, spleen) and a piece of pectoral muscle were dissected from experimental birds that died naturally during the course of the experiment. The organs were fixed in 10% neutral formalin and embedded in paraffin blocks.

Histological sections of 4 µm were obtained and stained with H&E (Valkiūnas, 2005). Smears were prepared using bone marrow obtained from the tibia bones of the dead canaries. The bones were cut at the upper joint and a syringe needle was forced inside the bone, pushing out the bone marrow. The needle was then rubbed on a microscopy glass slide, spreading the bone marrow in a thin layer. For each bird, we used different needles to avoid contamination. The preparation was dried, fixed with absolute methanol and stained with Giemsa solution using the same protocol as for blood smears.

2.3. Infection of experimental mosquitoes

To follow development of *Plasmodium* (*Huffia*) sp. (pERIRUB01) in a vector, we used *C. p. pipiens* f. *molestus* and *C. quinquefasciatus* mosquitoes.

The colonies of both species have been maintained in the laboratory of the Nature Research Centre, Lithuania for many years (Žiegytė et al., 2014; Valkiūnas et al., 2015b). All mosquitoes were kept in cages (65 × 65 × 65 cm) under standard laboratory conditions (23 ± 1 °C, 60–65% RH and 14:10 light:dark photoperiod). Cotton wool pads moistened with 5–10% saccharose solution were used for mosquito feeding.

Before exposure to an infected bird, 30 female mosquitoes were randomly chosen and placed inside a separate cage. A donor bird infected with pERIRUB01 lineage (approximate gametocytemia of 0.1–0.3%) was placed in the mosquito cage and kept for 1 h as described by Kazlauskienė et al. (2013). Briefly, the bird was placed in a plastic tube and only the legs of the bird were exposed to mosquitoes. Engorged female mosquitoes were placed into small cages (12 × 12 × 12 cm), provided with saccharose solution and maintained for approximately 25 days pe. To follow development of the parasite, mosquitoes were dissected at intervals. To make ookinete preparations, semi-digested content of midgut was extracted, mixed with a small drop of saline, and a thin smear was made. The preparations were air-dried, fixed with absolute methanol and stained with Giemsa solution using the same protocol as for blood smears. Permanent preparations of oocysts were prepared and stained with Erlich's hematoxylin as described by Kazlauskienė et al. (2013). Salivary glands of mosquitoes were dissected and preparations of sporozoites were prepared as for the ookinetes. To confirm the presence of ookinetes, preparations were made 1–6 days pe. Preparations of oocysts were made 6–25 days pe and preparations of sporozoites were made 8–25 days pe. In total, we dissected 26 *C. p. pipiens* f. *molestus* and 35 *C. quinquefasciatus* mosquito specimens.

2.4. Microscopic examination and morphological identification of parasites

For examination of blood slides, preparation of photos and measurement of parasites, we used an Olympus BX61 light microscope and AnalySIS FIVE imaging software. We examined each blood slide for 15–20 min at low magnification (×400), and approximately 100 fields at high magnification (×1000). Morphological features and identification of parasites were defined according to Valkiūnas (2005). For comparison of morphology of different parasite lineages, we also used slides of voucher material of *P. (Huffia) elongatum* (lineage pGRW6), deposited at the Nature Research Centre, Vilnius, Lithuania (Valkiūnas et al., 2008b). Intensity of parasitemia was estimated as a percentage by actual counting of the number of parasites per 1000 erythrocytes or per 10,000 erythrocytes if light infections were present (Godfrey et al., 1987).

An Olympus BX51 light microscope, equipped with an Olympus DP12 digital camera and imaging software Olympus DP-SOFT, was used to examine slides and prepare illustrations of histological specimens. They were examined at low magnification (×200) for 10–15 min, followed by examination for 10–15 min at medium magnification (×400) and then 20–30 min at high magnification (×1,000).

Vector preparations were analysed using an Olympus BX43 light microscope equipped with a digital camera Q Imaging MicroPublisher 3.3 RTV and imaging software QCapture Pro 6.0, Image-Pro Plus. We examined the slides for 15–20 min at low magnification (×100, ×200 and ×600) and then at high magnification (×1000).

A Student's *t*-test for independent samples was used for pairwise comparison of measurements of sporozoites and ookinetes (length, width and area), and oocysts (diameter) between two mosquito species. $P \leqslant 0.05$ was considered significant.

2.5. Genetic and phylogenetic analysis

DNA extraction from blood samples was performed using the standard ammonium-acetate protocol (Sambrook et al., 1989). We used a nested-PCR protocol with primer pairs HaemFNI and HaemNR3 for the first PCR, and HAEMF and HAEMR2 primers for the second PCR, which amplified a 479 bp fragment of the mitochondrial cyt *b* gene (Bensch et al., 2000; Hellgren et al., 2004). Thermal conditions for DNA amplifications and the number of cycles were the same as defined by Hellgren et al. (2004). For the PCRs we used 12.5 µl of Dream Taq Master Mix (0.4 mM of each nucleotide, 4 mM $MgCl_2$, 2× Dream Taq buffer, Dream Taq DNA Polymerase) (Thermo Fisher Scientific Baltics, Lithuania), 8.5 µl of nuclease-free water, 1 µl of each primer and 2 µl of template DNA. The amplification success was evaluated by using a MultiNa elctrophoresis system (Shimadzu, Japan). We used one negative control (nuclease-free water) and one positive control (*P. relictum* DNA) every seven samples to control for false amplifications. No case of false amplification was detected. Obtained fragments were sequenced from the 5′ and 3′ends with the primers HAEMF and HAEMR, respectively, as described by Bensch et al. (2000). We used dye terminator cycle sequencing (Big Dye) and loaded samples onto an ABI PRISM TM 3100 capillary sequencing robot (Applied Biosystems, USA). Sequences of parasites were edited and aligned using the BioEdit programme (Hall, 1999).

A Bayesian phylogeny was constructed using 33 cyt *b* gene sequences (479 bp) and the programme mrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The General Time Reversible Model including invariable sites and variation among sites (GTR+I +G) was suggested by the software mrModeltest 2.2 (software available from http://www.abc.se/~nylander/mrmodeltest2/MrModelblock). Two simultaneous runs were conducted with a sample frequency of every 100th generation over 10 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 visualised using Tree View 1.6.6. (software available from http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

The presence of possible haemosporidian co-infections was determined by visual "double bases" in the electropherogram using the programme BioEdit. The sequence divergence between the different lineages was calculated with the use of a Jukes-Cantor model of substitutions implemented in the programme MEGA 5.0 (Tamura et al., 2011).

2.6. Ethical statement

The experiments described herein comply with the current laws of Lithuania and Russia. The procedures of this study were approved by the International Research Co-operation Agreement between the Biological Station "Rybachy" of the Zoological Institute of the Russian Academy of Sciences and Nature Research

Centre (25-05-2010), Ethical Commission of the Baltic Laboratory Animal Science Association and the Lithuanian State Food and Veterinary Office (ref. no. 2012/01/04-0221).

3. Results

3.1. Identification of the parasite

Taxonomic summary

Plasmodium (Huffia) elongatum Huff, 1930

DNA sequence: Partial sequence of mitochondrial cyt *b* gene (479 bp), MalAvi database lineage pERIRUB01, GenBank accession number **KT282462**.

Vertebrate hosts: European robin *E. rubecula* (Passeriformes, Muscicapidae). This lineage of *P. elongatum* has been recorded for the first time. Canary *S. canaria* is a competent experimental host.

Site of infection: Erythrocytic meronts develop in polychromatic erythrocytes and erythroblasts, gametocytes develop in mature erythrocytes. Numerous phanerozoites develop in stem cells of the erythrocytic series in bone marrow; a few of them were seen in liver and spleen.

Vectors: Natural vectors are unknown. Experimental vectors are *C. p. pipiens* form *molestus* and *C. quinquefasciatus*.

Distribution: The lineage was recorded on the Curonian Spit in the Baltic Sea. No other data.

Specimens: Voucher specimens (exoerythrocytic meronts: nos. 48899–48901 NS, *S. canaria*, bone marrow, collected by M. Ilgūnas in 12 December 2014; blood stages: nos. 48902, 48903 NS, *E. rubecula*, intensity of parasitemia 0.3%, 15 of June, 48904, 48905 NS, *S. canaria*, intensity of parasitemia 0.4%, 12 December and 48936–48945 NS, *S. canaria,* intensity of parasitemia 9.0%, 18 December collected by V. Palinauskas in 2014; ookinetes, oocysts and sporozoites: nos. 48906–48911 NS, *C. p. pipiens* f. *molestus* and *C. quinquefasciatus*, collected by R. Žiegytė in January–March, 2015) were deposited in Nature Research Centre, Vilnius, Lithuania.

Description of parasite: Trophozoites and erythrocytic meronts (Fig. 1A–G) develop only in immature red blood cells, especially seen often in polychromatic erythrocytes, and were also present in erythroblasts. This feature was reported in the original description of *P. elongatum*, and it was reported in *P. elongatum* (lineage pGRW6) (Fig. 1Q, R). Growing trophozoites possess outgrowths and minute one or two pigment granules. The parasite sometimes slightly displaces the nucleus of infected erythrocyte. Vacuoles, which were frequently recorded in growing trophozoites of the lineage pGRW6 (Fig. 1Q) were rare during development of the lineage pERIRUB01 in European robins, but frequently recorded in canaries infected with the same parasite lineage. It seems that this character is host-dependent and cannot be used in identification of these two lineages. Morphological features of erythrocytic meronts are the same as reported for *P. elongatum* by Valkiūnas (2005). The meronts are of variable form, usually rounded or oval and contain 6–12 merozoites which are more or less elongated (Fig. 1E–G). Merozoites sometimes are arranged as fans (Fig. 1E). Pigment granules are small and usually are aggregated into one group. Meronts usually deform infected red blood cells and displace their nuclei.

Macrogametocytes (Fig. 1H–L) develop in mature erythrocytes and are of elongate form from the early stages of their development (Fig. 1I). Fully grown gametocytes are thin and usually of amoeboid outline, they are located in a lateral position to nuclei of erythrocytes and do not fill poles of the host cells completely (Fig. 1J–L). The parasite nucleus is submedian or subpolar in position. Pigment granules are small or of medium size (<1 µm), scattered in the cytoplasm or can be clamped. The number of pigment granules usually does not exceed 20. Mature gametocytes do not displace or only slightly displace the nuclei of the erythrocytes laterally.

The main morphological features of microgametocytes (Fig. 1M–P) are as for macrogametocytes with the usual sexual dimorphic characters.

Exoerythrocytic merogony: The first generations of exoerythrocytic meronts (cryptozoites and metacryptozoites), for which development is induced by sporozoites, were not investigated. Phanerozoites, which were induced by merozoites developing in erythrocytic meronts, were observed in the experimentally infected canaries (Figs. 2 and 3). Numerous phanerozoites were observed in the bone marrow taken from the tibia bone (Fig. 2A, B) 45 days pe and 14 days pe. They developed in erythroblasts and other precursor cells of the erythrocytic series. Mature phanerozoites contained a variable number of merozoites, usually ranging between 22 and 30. It should be noted that free of host cells, syncytium-like structures were numerous in bone marrow preparations (Fig. 2C, D). These structures are phanerozoite remnants, which can slip out into the circulating blood, where they were occasionally seen (Fig. 2E, F). Phanerozoites were also recorded in the liver (Fig. 3A) and spleen (Fig. 3B) however the host cells were not identified. In these organs, phanerozoites were small, roundish or oval in shape (Fig. 3A, B).

3.2. Parasitemia in experimentally infected canaries

All infected canaries were susceptible to *P. elongatum* (pERIRUB01) and developed parasitemia. Maximum parasitemia reached 9% in one canary 15 days pe, but the average maximum intensity of parasitemia for the other six birds was light (0.2%). Infection by the pERIRUB01 lineage was lethal in five of seven birds that died within 46 days pe. Parasitemia was light (approximately 0.001%) in two birds that died 14 and 46 days pe. Post-mortem investigation of both these individuals confirmed massive infestation of exoerythrocytic meronts in the bone marrow. Malaria parasites were not detected in control birds; they survived to the end of this study.

3.3. Development in mosquitoes

Sporogony was completed in *C. p. pipiens* f. *molestus* and *C. quinquefasciatus* mosquitoes (Fig. 4). Microscopy and sequencing confirmed the presence of the corresponding parasite lineage in experimentally infected mosquitoes. In *C. p. pipiens* f. *molestus* mosquitoes, ookinetes were detected in the midgut 1 day pe (Fig. 4A). The zygotes and ookinetes were seen in two out of three exposed *C. p. pipiens* f. *molestus* females. The oocysts were recorded in the midgut of infected mosquitoes 12 and 18 days pe (Fig. 4C). Sporogonic development was completed and sporozoites were observed in salivary glands 20 and 22 days pe; they were seen in two of 15 exposed mosquitoes (Fig. 4E).

Sporogonic development was also completed in *C. quinquefasciatus* mosquitoes (Fig. 4B, D, F). Mature ookinetes were detected in the midgut preparations 12 h pe and 1 day pe. Oocysts were seen in the midguts 9 and 16 days pe; they were detected in four out of 12 exposed mosquitoes. Complete sporogonic development was confirmed due to sporozoites in the salivary glands of two *C. quinquefasciatus* mosquitoes 18 and 20 days pe. Sporozoites were recorded in two of 16 exposed mosquitoes.

The shape and size of *P. elongatum* (pERIRUB01) ookinetes were similar in both mosquito species ($P > 0.1$ for length, width and area) (Table 1). These were elongated bodies containing slightly off centre located nuclei. Occasionally, a few pigment granules were discernable in the cytoplasm of the ookinetes (Fig. 4A, B). Maturing oocysts varied in size and contained pigment granules (Fig. 4C, D). Fusiform sporozoites possessed centrally located

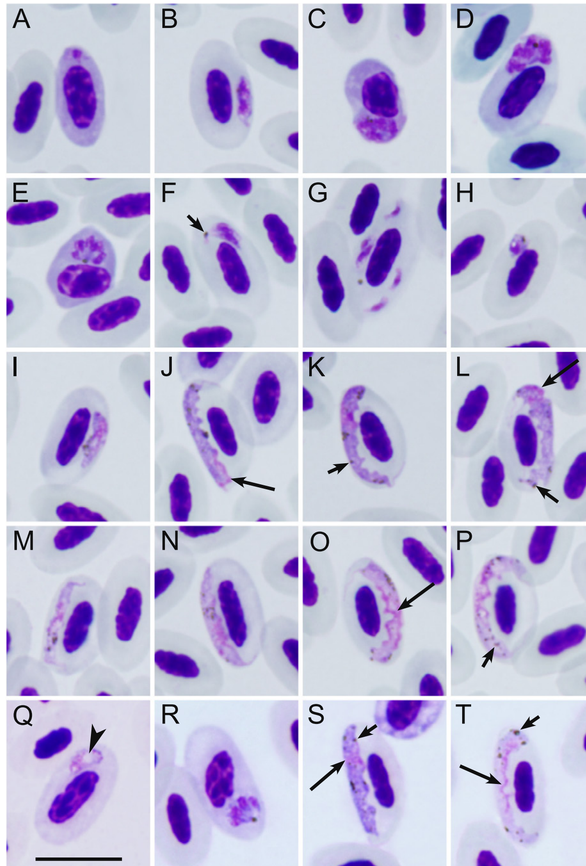

Fig. 1. Blood stages of *Plasmodium (Huffia) elongatum*: the lineage pERIRUB01 (A–P) from peripheral blood of the naturally infected European robin, *Erithacus rubecula*, and the lineage pGRW6 (Q–T) from the experimentally infected great reed warbler, *Acrocephalus arundinaceus*. (A, Q) trophozoites; (B–G, R) erythrocytic meronts; (H–L, S) macrogametocytes; (M–P, T) microgametocytes. Arrowheads indicate vacuoles, short arrows show granules and long arrows show nuclei of parasites. Giemsa stained blood films. Scale bar = 10 µm.

nuclei. Sizes of sporozoites did not differ significantly in any of the measured parameters (length, width, area) between mosquito species ($P > 0.3$, for each parameter) (Table 1).

3.4. Phylogeny of P. elongatum lineages

The lineage pERIRUB01 clusters together with *P. elongatum* (lineage pGRW6) and five other closely related cyt *b* gene lineages that were recorded in different regions and hosts by a number of authors in recent years (Fig. 5A). The genetic difference between pERIRUB01 and pGRW6 lineages is 0.3%. The genetic difference among all lineages from clade A (Fig. 5) is <0.9%, and they likely belong to *P. elongatum* or at least parasites belonging to the same *Huffia* subgenus, although this needs further clarification. Two *P. elongatum* lineages from clade A (Fig. 5), genetically differ from other morphologically described species belonging to subgenera *Haemamoeba*, *Giovannolaia* and *Novyella* by more than 6.8%, 5.9% and 7.5%, respectively.

3.5. Summary remarks

Plasmodium elongatum (pERIRUB01) strain belongs to the subgenus *Huffia*. This parasite, similar to other species belonging to this subgenus, develops exoerythrocytic stages in hematopoietic organs (Figs. 2 and 3) and forms erythrocytic meronts in polychromatic erythrocytes and erythroblasts in the peripheral

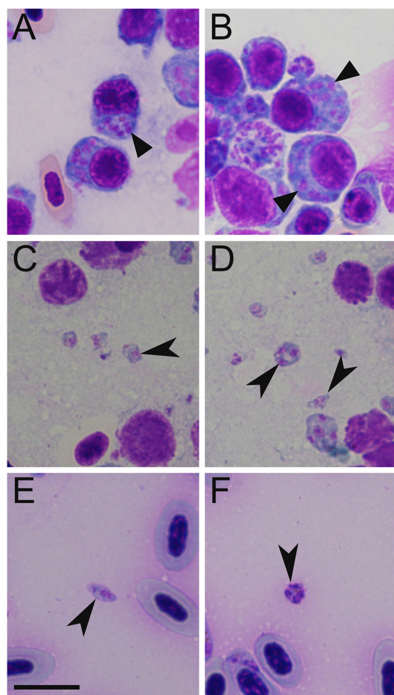

Fig. 2. Phanerozoites of *Plasmodium (Huffia) elongatum* (lineage pERIRUB01) in bone marrow (A, B), syncytium-like structures in bone marrow (C, D) and peripheral blood (E, F) of an experimentally infected domestic canary, *Serinus canaria* (45 days post exposure). Triangular arrowheads indicate merozoites in phanerozoites and other arrowheads show syncytium-like structures. Giemsa-stained blood films. Scale bar = 10 µm. Syncytia are washed out of bone marrow by circulating blood, and they were readily visible in the peripheral blood films (E, F).

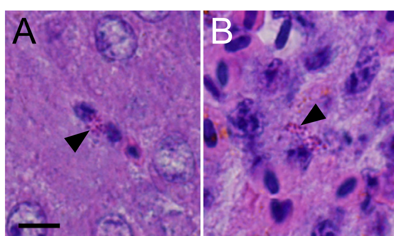

Fig. 3. Phanerozoites of *Plasmodium (Huffia) elongatum* (lineage pERIRUB01) in histological sections of liver (A) and spleen (B) of an experimentally infected domestic canary, *Serinus canaria*. Arrowheads indicate merozoites. H&E stained preparations. Scale bar = 10 µm.

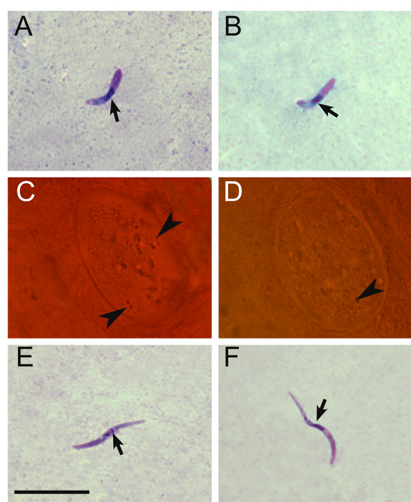

Fig. 4. Sporogonic stages of *Plasmodium (Huffia) elongatum* (lineage pERIRUB01) in mosquitoes, (A, C, E) *Culex pipiens pipiens* form *molestus* and (B, D, F) *Culex quinquefasciatus*. (A, B) ookinetes; (C, D) oocysts; (E, F) sporozoites. Arrows indicate nuclei of parasites, arrowheads point to pigment granules. (A, B, E, F) Giemsa-stained preparations; (C, D) midgut preparation stained with Erlich's hematoxylin. Scale bar = 10 µm.

Table 1
Morfometry of ookinetes, oocysts and sporozoites of *Plasmodium elongatum* (pERIRUB01 lineage) in the mosquitoes *Culex pipiens pipiens* form *molestus* and *Culex quinquefasciatus*.

Feature	Measurements[a] (µm)	
	C. p. pipiens f. *molestus*	*C. quinquefasciatus*
Ookinete		
Length	5.2–9.2 (7.4 ± 1.2)	6.7–10.3 (8.3 ± 1.3)
Width	1.2–1.7 (1.4 ± 0.2)	1.1–1.5 (1.3 ± 0.1)
Area	6.2–11.5 (8.8 ± 1.8)	6.1–9.8 (8.4 ± 1.4)
Oocyst		
Minimum diameter	16.4–42.3 (23.4 ± 8.0)	9.4–37.2 (19.3 ± 8.5)
Maximum diameter	18.9–48.8 (33.0 ± 10.0)	13.4–39.3 (24.8 ± 10.0)
Sporozoite		
Length	11.7–16.1 (14.4 ± 1.1)	11.9–16.7 (14.3 ± 1.2)
Width	0.7–1.2 (0.9 ± 0.2)	0.8–1.2 (0.9 ± 0.1)
Area	7.5–11.9 (9.2 ± 1.1)	6.8–13.9 (9.7 ± 1.8)

[a] Measurements of ookinetes ($n = 7$, methanol-fixed preparations 1 day post exposure (pe)), oocysts ($n = 12$, formalin-fixed preparations of mature parasites at 9–16 days pe) and sporozoites ($n = 21$, methanol-fixed preparations 20–22 days pe) are given in µm. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and S.D.

blood. Morphological features of gametocytes and meronts of pERIRUB01 are the same as in the original description of *P. elongatum*. The most similar parasite, which also develops meronts in young erythrocytes and exoerythrocytic stages in hematopoietic organs, is *Plasmodium hermani*. However, some morphological features of *P. hermani* (thick gametocytes, which cause marked displacement of erythrocyte nuclei, and the presence of numerous rosette-like

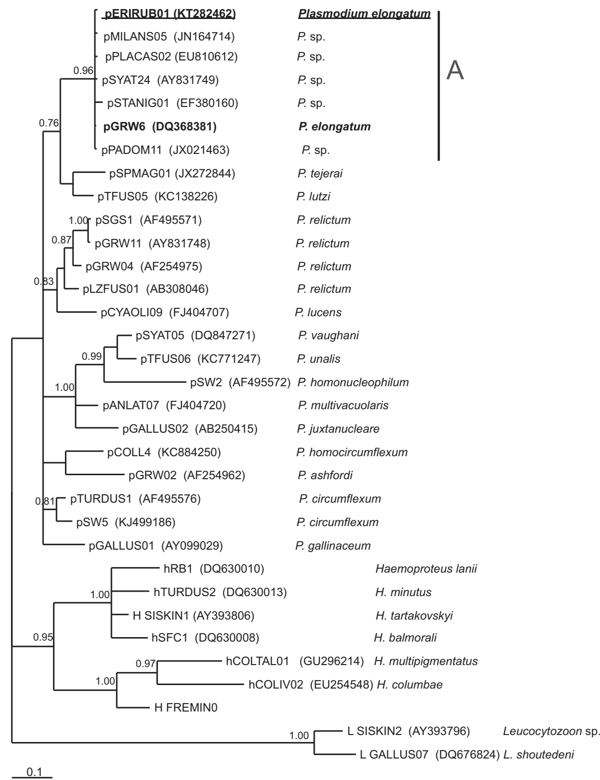

Fig. 5. Bayesian phylogenetic tree built using mitochondrial cytochrome *b* gene fragments of 24 *Plasmodium* spp. and seven *Haemoproteus* spp. lineages. Two *Leucocytozoon* spp. lineages were used as outgroups. Posterior probabilities $\geqslant$0.7 are indicated on the tree. MalAvi codes of lineages are given, followed by the GenBank accession number in parentheses and parasite species names. Bold font indicates a previously identified *Plasmodium elongatum* lineage and underlined bold font denotes *P. elongatum* lineage pERIRUB01 which was identified in the present study. Vertical bar (A) indicates *P. elongatum* and closely related lineages.

erythrocytic meronts) are absent from our *P. elongatum* (pERIRUB01). That helps to distinguish these infections.

It is important to note that morphology of the studied parasite did not change after blood passages in experimentally infected canaries compared with the parasites seen in the naturally infected European robins.

4. Discussion

Molecular characterisation, based on combination of morphological characters of blood stages and a fragment of mitochondrial cyt *b* gene, attributed the lineage pGRW6 to the species *P. elongatum* (Valkiūnas et al., 2008b). This lineage has been recorded in birds belonging to 10 orders and 26 families (according to MalAvi database, 20 January 2016). However, due to the lack of material showing complete development of this malaria parasite in vertebrate hosts, in most of these cases it is unclear whether it completes development and forms infective stages (micro- and macrogametocytes) in red blood cells. In other words, it remains unclear whether all positive PCR reports deal with competent *P. elongatum* (pGRW6) infections because abortive malaria infections seem to be common in birds (Levin et al., 2013). Abortive development happens when a parasite invades a host, in which it can develop only partially, and cannot complete its full life cycle, resulting in the absence of infective stages, i.e. gametocytes (in birds) or sporozoites in vectors (Olias et al., 2011; Valkiūnas et al., 2014).

The lineages pERIRUB01 and pGRW6 of *P. elongatum*, together with several other lineages available in the MalAvi database, form one cluster, with genetic differences ranging between 0.3% and 0.9% among them (Fig. 5, clade A). These data are in accord with our morphological analysis. Identical morphological features of blood stages, similar exoerythrocytic stages and sporogonic

development in vectors, together with genetic similarities with pGRW6, prove that the lineage pERIRUB01 belongs to *P. elongatum*. Ideally, identification of haemosporidian parasites should be based on a combination of morphological and phylogenetic information, together with data about developmental patterns in hosts (Perkins, 2000; Palinauskas et al., 2015). The geographic distribution of *P. elongatum* (pERIRUB01) and areas of its transmission remain unclear.

Plasmodium elongatum can cause severe pathology in birds due to the development of secondary exoerythrocytic stages (phanerozoites) (Garnham, 1966; Valkiūnas, 2005). The same is true for the lineage *P. elongatum* (pERIRUB01), which is markedly virulent in experimentally infected canaries: over 70% of infected birds died during this study. As in previous experimental studies with *P. elongatum* (Micks, 1949; Valkiūnas et al., 2008b), the peak parasitemia did not reach 10% in infected birds, but the devastating impact on host fitness was readily visible. It should be noted that massive destruction of immature erythrocytic cells and cells of the haematopoietic system in the bone marrow have been documented in infections with *P. elongatum,* and these characteristics are the main diagnostic features of subgenus *Huffia* (Garnham, 1966; Corradetti et al., 1968). In non-adapted hosts, this parasite also probably causes damage in other organs by phanerozoites, which lead to death of the host even during low parasitemia (Valkiūnas, 2005). Interestingly, the donor bird (*E. rubecula*) infected with this lineage had a relatively high natural parasitemia (0.5%). This may be related to the status of host fitness and the immune system, which might be weakened during migration, or a different mode of development and impact of this parasite in European robins.

Post mortem analysis of histological preparations of various organs in experimentally infected canaries showed that bone marrow cells were heavily infected (Fig. 2A, B). In previous studies, phanerozoites were also observed in the bone marrow of *P. elongatum*-infected hosts (Garnham, 1966; Valkiūnas, 2005). However, the lineage of the parasite remains unknown. Phanerozoites of pERIRUB01 were occasionally observed also in other hematopoietic organs (spleen and liver) (Fig. 3), however, they were scarce compared with bone marrow.

This study provides information for better understanding positive PCR-based records of haemosporidian infections in cases where parasites are absent from the blood cells (Levin et al., 2013). We report syncytium-like extracellular parasites, which were common in the bone marrow of deceased canaries (Fig. 2C, D). They were remnants of phanerozoites which did not complete multiplication, probably due to the rupture of host cells containing several developing parasites (Fig. 2B). Such syncytia were variable in size and shape, each possessed a portion of cytoplasm and one or several nuclei (Fig. 2C, D). Syncytia can slip out of the bone marrow into the peripheral circulation (Fig. 2E, F) and provide a template for PCR-based amplification, resulting in positive PCR signals even in the absence of developing intracellular blood stages. We believe this experimental study provides first known morphological evidence for syncytia both in the bone marrow and peripheral blood of dead birds in parallel (Fig. 2C–F). At present it is unclear how often syncytia appear in the circulation during infection of other haemosporidian parasites in wildlife. This information is important for diagnosis of disease and for the evaluation of true host range and specificity of parasites. Positive PCR amplifications may result from the DNA of syncytia (an abortive stage in the vertebrate host), and not gametocytes (the final stage of development in avian hosts) that are infective to vectors. This observation is important for a better understanding of abortive development in haemosporidians and diagnostic methods of such infections by PCR. For example, 15 out of 2923 passerine birds (20 species) were PCR-positive for *Plasmodium* spp. in the Galapagos Islands. However, gametocytes were not observed in blood films of any PCR-positive samples, indicating possible abortive infections (Levin et al., 2013). Positive PCR-based results could be also due to amplification of DNA from circulating sporozoites in the blood stream (Valkiūnas et al., 2009). Wildlife specificity studies should be accompanied with observations of gametocytes in the circulation before drawing conclusions about competent hosts of malaria and other haemosporidian parasites. These data indicate an essential need to combine PCR-based and microscopy tools in epidemiological studies of avian haemosporidian parasites.

Development of exoerythrocytic stages in cells of the haematopoietic system is a characteristic feature of the *Huffia* subgenus to which *P. elongatum* belongs (Garnham, 1966). However, according to several studies, *P. elongatum* phanerozoites were reported to develop also in the heart, lungs, brain, kidney and muscles of penguins, which are unusual hosts for malaria parasites because they evolved under conditions of ecological isolation from mosquitoes transmitting this infection (Fleischman et al., 1968; Sladen et al., 1976; Cranfield et al., 1994). The cause of such differences in development remains unclear, however it is difficult to rule out that reported phanerozoites in these organs may not belong to *P. elongatum*, but to *P. relictum*, which has been often reported in co-infection with the former species. Further experimental investigation and clarification is needed. According to Graczyk et al. (1994a,b) active transmission of different *Plasmodium* spp. occurred in Baltimore Zoo, USA in parallel, but co-infections in blood smears were absent. *Plasmodium elongatum* was determined in 78% of infected African black-footed penguins and *P. relictum* in 16% of birds (Graczyk et al., 1994b). Haemosporidian parasites, including agents of malaria, exist in co-infections, which predominate in the wild (Valkiūnas et al., 2003; Valkiūnas, 2005; van Rooyen et al., 2013). During active transmission of *P. elongatum* and *P. relictum*, co-infections should be present unless there are some mechanisms that prevent development of one of the parasites during the co-infections. Such parasite interactions are poorly understood in avian malaria. Former experimental studies show that cross-immunity does not develop and distantly related avian malaria parasites occur in co-infections (Manwell, 1938; Garnham, 1966). A recent study by Palinauskas et al. (2011) supports these data by showing that during co-infections by two *Plasmodium* parasites belonging to different subgenera, both these infections developed in the same individual host in parallel. There is a possibility that due to parasite–parasite interactions, some delays or an atypical developmental pattern of certain parasite species might occur. This needs further experimental investigation.

Another interesting pattern of *P. elongatum* development is a partial life cycle when the parasite forms exoerythrocytic stages without further development of gametocytes in some infected birds. In several studies with different species of penguins (Black-footed penguin, Magellanic penguin, Humbildt's penguin) natural infections of *P. elongatum, P. relictum, Plasmodium tejerai* and *Plasmodium juxtanucleare* developed gametocytes, which were visualised in peripheral blood (Huff and Shiroishi, 1962; Grim et al., 2003; Silveira et al., 2013; Vanstreels et al., 2014). However, in some cases the development of *Plasmodium* infections was abortive at the stage of exoerythrocytic development. For example, in the Fleischman et al. (1968) study, gametocytes of *P. elongatum* were absent from peripheral blood in five out of six infected penguins. In the Levin et al. (2013) study, gametocytes were not observed in 13 PCR-positive Galapagos penguins, *Spheniscus mendiculus*, naturally infected with *Plasmodium* spp. Records of *Plasmodium* spp. infecting penguins illustrate partial development of haemosporidians when exoerythrocytic stages are formed, but intracellular blood stages are absent, including the absence of gametocytes, which are the infective stages for mosquitoes. Such host–parasite interactions might occur when the parasite and host

did not co-evolve or when the host has not evolved a competent immune defence against a novel pathogen. If the parasite can easily circumvent host defences, it may develop within host cells and the infection may be largely uncontrolled. The elimination of the host may not be beneficial for the parasite, thus unbalanced host–parasite interactions may be costly for the parasite. From a long-term perspective, frequent exposure of hosts to parasites and their partial development in the host may contribute to evolution of the ability of the parasites to penetrate erythrocytes and develop gametocytes. However, underlying molecular mechanisms are not completely understood.

It worth mentioning that abortive haemosporidian infections likely are common in wildlife because the MalAvi database (Bensch et al., 2009, http://mbio-serv2.mbioekol.lu.se/Malavi/index.html) contains numerous records of haemosporidian lineages in unusual avian hosts. A problem is that the great majority of current avian malaria studies are based solely on PCR-based detection using general primers. This method does not provide information regarding from which parasite stage (infective or not) the PCR signal came and it markedly underestimates haemosporidian co-infections (Bernotienė et al., 2016). Consequently, the available information about genetic heterogeneity in bird–malaria interactions, if solely PCR was used in research, may not be complete even after accounting for putative vector feeding patterns, which were determined using the same methodology (Medeiros et al., 2013).

Plasmodium elongatum (pERIRUB01) developed sporozoites in 13.3% of infected *C. p. pipiens* f. *molestus* and 12.5% of *C. quinquefasciatus* mosquitoes. In the past, there were a number of studies trying to determine vectors of *P. elongatum* (mosquito species belonging to genera *Aedes*, *Anopheles*, *Culex* and *Culiseta*) as reviewed by Valkiūnas (2005) and Santiago-Alarcon et al. (2012). However, the results were contradictory and sometimes uncertain. In some studies it was shown that *C. pipiens* were fractionally susceptible to *P. elongatum* (Huff 1927), while in others complete development in up to 100% of exposed *C. pipiens* and *C. quinquefasciatus* mosquitoes was documented (Raffaele, 1934; Micks, 1949). However, Micks (1949) obtained less than three oocysts per midgut and did not observe any sporozoites in *C. quinquefasciatus*, indicating abortive development of *P. elongatum*. It is likely that incomplete (abortive) sporogonic development in blood sucking dipterans is rather common in haemosporidian parasites (Valkiūnas et al., 2013; Palinauskas et al., 2015). Discrepancies in results from different studies raise the question as to whether all groups worked with the same *P. elongatum* lineage. The ability to complete sporogony and develop sporozoites could be also caused by different environmental conditions between mosquito populations and composition of microorganisms in each mosquito within a population (Cook and McGraw, 2010; Murdock et al., 2012, 2014; Moller-Jacobs et al., 2014). According to Cantrell and Jordan (1946), successful infection of vectors also depends on the period of gametocytemia in the donor bird. These authors demonstrated that reduction in nutrient levels in the blood after high parasitemia could influence the ability of gametocytes to develop into gametes and subsequent sporogonic development. Valkiūnas et al. (2015a) demonstrated experimentally that viability of gametocytes of *Haemoproteus* sp., the sister genus of *Plasmodium*, markedly changes during the course of parasitemia and that might influence infectivity of gametocytes to vectors. Susceptibility of mosquitoes can be also rapidly increased by selection within several generations in some mosquito species (Micks, 1949).

For haemosporidians, successful transmission requires that sporogonic development should be completed and mature sporozoites of the parasite should be present in salivary glands. Interaction with gut microorganisms is important for haemosporidian parasite development in vectors (Sinden, 1999; Bahia et al., 2014). The abundance and composition of microorganisms in the mosquito midgut changes during the life of the mosquito (Wang et al., 2011; Bahia et al., 2014). Thus, partial susceptibility of some mosquito species also could be explained by these factors. After motile ookinetes are formed, they reach the inner membrane of the midgut and activate the mosquito's innate immune system, involving both cellular and humoral defence mechanisms (Dimopoulos, 2003). Huge losses of parasites are observed during formation of oocysts, even in susceptible mosquitoes (Alavi et al., 2003). Sporogonic development during the oocyst stage may be critical for successful sporogony. There are data about encapsulation and melanisation of oocysts in midguts of mosquitoes infected both with *Plasmodium* and *Haemoproteus* parasites (Collins et al., 1986; Schwartz and Koella, 2002; Dimopoulos, 2003; Sinden et al., 2004; Valkiūnas et al., 2013; Palinauskas et al., 2015). This defence mechanism is often recorded because it can be readily visualised in midgut preparations. There are other mechanisms that act against the oocysts, such as phagocyte activity or superoxide anion production and suppression of oocyst development (Weathersby and Mccall, 1968; Lanz-Mendoza et al., 2002). The absence of oocysts in some of our infected mosquitoes was also observed. Haemolymph and haemocytes also contain large quantities of immune components, thus more than 80% of sporozoites can be cleared during migration to salivary gland (Dimopoulos, 2003). It seems that successful sporogonic development in the vector is costly for the parasite and depends on many factors such as fitness of mosquito, microbiota of the midgut and success of gametocyte development in the vertebrate host, and others.

In conclusion, this study provides information about development of a virulent *P. elongatum* (pERIRUB01) parasite in vertebrate hosts and blood sucking insects. Different lineages of this species are distributed worldwide and are of particular interest due to the high virulence and mortality of birds caused by destruction of stem cells responsible for erythropoiesis in bone marrow. We showed that light parasitemia, which is commonly observed in wild birds, is not always a measure of bird health because this infection may have detrimental effects on a bird's fitness due to interruption of erythropoiesis by exoerythrocytic stages in bone marrow and, occasionally, in other hematopoietic organs. We showed that syncytium-like structures of developing phanerozoites slip out of the bone marrow into peripheral blood and provide templates for PCR amplification. *Plasmodium elongatum* (lineage pERIRUB01) develops and completes sporogony in *C. p. pipiens* f. *molestus* and *C. quinquefasciatus* mosquitoes. However, these mosquitoes exhibit only partial susceptibility to *P. elongatum*, and this vector–parasite system could serve as a model for defining underlying mechanisms of this phenomenon. The obtained information is important for better understanding the epidemiology of *P. elongatum* transmission and diagnostic methods for avian malaria infections.

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References

Alavi, Y., Arai, M., Mendoza, J., Tufet-Bayona, M., Sinha, R., Fowler, K., Billker, O., Franke-Fayard, B., Janse, C.J., Waters, A., Sinden, R.E., 2003. The dynamics of interactions between *Plasmodium* and the mosquito: a study of the infectivity of *Plasmodium berghei* and *Plasmodium gallinaceum*, and their transmission by *Anopheles stephensi*, *Anopheles gambiae* and *Aedes aegypti*. Int. J. Parasitol. 33, 933–943. http://dx.doi.org/10.1016/S0020-7519(03)00112-7.

Alley, M., Hale, K., Cash, W., Ha, H., Howe, L., 2010. Concurrent avian malaria and avipox virus infection in translocated South Island saddlebacks (*Philesturnus carunculatus carunculatus*). N. Z. Vet. J. 58, 218–223. http://dx.doi.org/10.1080/00480169.2010.68868.

Atkinson, C.T., Thomas, N.J., Hunter, D.B. (Eds.), 2008. Parasitic Diseases of Wild Birds. Wiley-Blackwell, Oxford, UK. http://dx.doi.org/10.1002/9780813804620.fmatter.

Bahia, A.C., Dong, Y., Blumberg, B.J., Mlambo, G., Tripathi, A., BenMarzouk-Hidalgo, O.J., Chandra, R., Dimopoulos, G., 2014. Exploring *Anopheles* gut bacteria for *Plasmodium* blocking activity. Environ. Microbiol. 16, 2980–2994. http://dx.doi.org/10.1111/1462-2920.12381.

Baillie, S.M., Brunton, D.H., 2011. Diversity, distribution and biogeographical origins of *Plasmodium* parasites from the New Zealand bellbird (*Anthornis melanura*). Mol. Ecol. 138, 1843–1851. http://dx.doi.org/10.1017/S0031182011001491.

Baillie, S.M., Gudex-Cross, D., Barraclough, R.K., Blanchard, W., Brunton, D.H., 2012. Patterns in avian malaria at founder and source populations of an endemic New Zealand passerine. Parasitol. Res. 111, 2077–2089. http://dx.doi.org/10.1007/s00436-012-3055-y.

Beadell, J.S., Covas, R., Gebhard, C., Ishtiaq, F., Melo, M., Schmidt, B.K., Perkins, S.L., Graves, G.R., Fleischer, R.C., 2009. Host associations and evolutionary relationships of avian blood parasites from West Africa. Int. J. Parasitol. 39, 257–266. http://dx.doi.org/10.1016/j.ijpara.2008.06.005.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc. Biol. Sci. 267, 1583–1589. http://dx.doi.org/10.1098/rspb.2000.1181.

Bensch, S., Hellgren, O., Pérez-Tris, J., 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol. Ecol. Resour. 9, 1353–1358. http://dx.doi.org/10.1111/j.1755-0998.2009.02692.x.

Bernotienė, R., Palinauskas, V., Iezhova, T., Murauskaitė, D., Valkiūnas, G., 2016. Avian haemosporidian parasites (Haemosporida): a comparative analysis of different polymerase chain reaction assays in detection of mixed infections. Exp. Parasitol. 163, 31–37. http://dx.doi.org/10.1016/j.exppara.2016.01.009.

Cantrell, W., Jordan, H.B., 1946. Changes in the infectiousness of gametocytes during the course of *Plasmodium gallinaceum* infections. J. Infect. Dis. 78, 153–159.

Castro, I., Howe, L., Tompkins, D.M., Barraclough, R.K., Slaney, D., 2011. Presence and seasonal prevalence of *Plasmodium* spp. In a rare endemic New Zealand passerine (tieke or saddleback, *Philesturnus carunculatus*). J. Wildl. Dis. 47, 860–867.

Clark, N.J., Clegg, S.M., Lima, M.R., 2014. A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): new insights from molecular data. Int. J. Parasitol. 44, 329–338. http://dx.doi.org/10.1016/j.ijpara.2014.01.004.

Collins, F., Sakai, R., Vernick, K., Paskewitz, S., Seeley, D., Miller, L., Collins, W., Campbell, C., Gwadz, R., 1986. Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. Science 234, 607–610. http://dx.doi.org/10.1126/science.3532325.

Cook, P.E., McGraw, E.A., 2010. *Wolbachia pipientis*: an expanding bag of tricks to explore for disease control. Trends Parasitol. 26, 373–375. http://dx.doi.org/10.1016/j.pt.2010.05.006.

Corradetti, A., Neri, I., Scanga, M., Cavallini, C., 1968. I cicli pre-eritrocitico e sporogonico di *Plasmodium (Huffia) elongatum*. Parasitologia 10, 133–143.

Cranfield, R., Graczyk, K., Beall, B., Shaw, L., Skjoldager, M.L., Ialeggio, M., 1994. Subclinical avian malaria infections in African black-footed penguins (*Spheniscus demersus*) and induction of parasite recrudescence. J. Wildl. Dis. 30, 372–376.

Derraik, J.G.B., Tompkins, D.M., Alley, M.R., Holder, P., Atkinson, T., 2008. Epidemiology of an avian malaria outbreak in a native bird species (*Mohoua ochrocephala*) in New Zealand. J. R. Soc. New Zeal. 38, 237–242. http://dx.doi.org/10.1080/03014220809510558.

Dimitrov, D., Zehtindjiev, P., Bensch, S., 2010. Genetic diversity of avian blood parasites in SE Europe: Cytochrome b lineages of the genera *Plasmodium* and *Haemoproteus* (Haemosporida) from Bulgaria. Acta Parasitol. 55, 201–209. http://dx.doi.org/10.2478/s11686-010-0029-z.

Dimopoulos, G., 2003. Microreview insect immunity and its implication in mosquito – malaria. Cell. Microbiol. 5, 3–14.

Fleischman, R.W., Squire, R.A., Sladen, W.J.L., Moore, J., 1968. Pathologic confirmation of malaria (*Plasmodium elongatum*) in African penguins (*Spheniscus demersus*). Bull. Wildl. Dis. Assoc. 4, 133–135.

Garnham, P.C.C., 1966. Malaria Parasites and other Haemosporidia. Blackwell Scientific Publications Ltd., Oxford, UK.

Godfrey, R.D., Fedynich, A.M., Pence, D.B., 1987. Quantification of hematozoa in blood smears. J. Wildl. Dis. 23, 558–565.

Graczyk, T.K., Cranfield, M.R., McCutchan, T.F., Bicknese, E.J., 1994a. Characteristics of naturally acquired avian malaria infections in naive juvenile African black-footed penguins (*Spheniscus demersus*). Parasitol. Res. 80, 634–637.

Graczyk, T.K., Shaw, M.L., Cranfield, M.R., Beall, F.B., Journal, T., Apr, N., 1994b. Hematologic characteristics of avian malaria cases in African black-footed penguins (*Spheniscus demersus*) during the First Outdoor Exposure Season. J. Parasitol. 80, 302–308.

Grim, K.C., Van der Merwe, E., Sullivan, M., Parsons, N., McCutchan, T.F., Cranfield, M., 2003. *Plasmodium juxtanucleare* associated with mortality in black-footed penguins (*Spheniscus demersus*) admitted to a rehabilitation center. J. Zoo Wildl. Med. 34, 250–255.

Hall, A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program of Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hellgren, O., Waldenström, J., Bensch, S., 2004. A new PCR assay for simultaneous studies of *Leucocytozoon, Plasmodium*, and *Haemoproteus* from avian blood. J. Parasitol. 90, 797–802. http://dx.doi.org/10.1645/GE-184R1.

Howe, L., Castro, I.C., Schoener, E.R., Hunter, S., Barraclough, R.K., Alley, M.R., 2012. Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. Parasitol. Res. 110, 913–923. http://dx.doi.org/10.1007/s00436-011-2577-z.

Huff, C.G., 1927. Studies on the infectivity of Plasmodia of birds for mosquitoes, with special reference to the problem of immunity in the mosquito. Am. J. Hyg. 7, 706–734.

Huff, C.G., 1932. Further infectivity experiments with mosquitoes and bird malaria. Am. J. Hyg. 15, 751–754.

Huff, C.G., Shiroishi, T., 1962. Natural infection of Humboldt's penguin with *Plasmodium elongatum*. J. Parasitol. 48, 495.

Ishtiaq, F., Gering, E., Rappole, J.H., Rahmani, A.R., Jhala, Y.V., Dove, C.J., Milensky, C., Olson, S.L., Peirce, M.A., Fleischer, R.C., 2007. Prevalence and diversity of avian hematozoan parasites in Asia: a regional survey. J. Wildl. Dis. 43, 382–398. http://dx.doi.org/10.7589/0090-3558-43.3.382.

Kambris, Z., Cook, P.E., Phuc, H.K., Sinkins, S.P., 2009. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science 326, 134–136. http://dx.doi.org/10.1126/science.1177531.

Kazlauskienė, R., Bernotienė, R., Palinauskas, V., Iezhova, T.A., Valkiūnas, G., 2013. *Plasmodium relictum* (lineages pSGS1 and pGRW11): Complete synchronous sporogony in mosquitoes *Culex pipiens pipiens*. Exp. Parasitol. 133, 454–461. http://dx.doi.org/10.1016/j.exppara.2013.01.008.

Lanz-Mendoza, H., Hernández-Martínez, S., Ku-López, M., Rodríguez, M.D.C., Herrera-Ortiz, A., Rodríguez, M.H., 2002. Superoxide anion in *Anopheles albimanus* hemolymph and midgut is toxic to *Plasmodium berghei* ookinetes. J. Parasitol. 88, 702–706. http://dx.doi.org/10.1645/0022-3395(2002) 088[0702:SAIAAH]2.0.CO;2.

Levin, I.I., Zwiers, P., Deem, S.L., Geest, E.A., Higashiguchi, J.M., Iezhova, T.A., Jiménez-Uzcátegui, G., Kim, D.H., Morton, J.P., Perlut, N.G., Renfrew, R.B., Sari, E. H.R., Valkiūnas, G., Parker, P.G., 2013. Multiple lineages of Avian malaria parasites (*Plasmodium*) in the Galapagos Islands and evidence for arrival via migratory birds. Conserv. Biol. 27, 1366–1377. http://dx.doi.org/10.1111/cobi.12127.

Loiseau, C., Harrigan, R.J., Cornel, A.J., Guers, S.L., Dodge, M., Marzec, T., Carlson, J.S., Seppi, B., Sehgal, R.N.M., 2012. First evidence and predictions of *Plasmodium* transmission in Alaskan bird populations. PLoS One 7, e44729. http://dx.doi.org/10.1371/journal.pone.0044729.

Manwell, D.R., 1938. Reciprocal immunity in the avian malarias. Am. J. Hyg. 27, 196–211.

Medeiros, M.C.I., Hamer, G.L., Ricklefs, R.E., 2013. Host compatibility rather than vector-host-encounter rate determines the host range of avian *Plasmodium* parasites. Proc. Biol. Sci. 280, 20122947. http://dx.doi.org/10.1098/rspb.2012.2947.

Micks, D.W., 1949. Investigations on the mosquito transmission of *Plasmodium elongatum*. J. Natl. Malar. Soc. 8, 905–906.

Moller-Jacobs, L.L., Murdock, C.C., Thomas, M.B., 2014. Capacity of mosquitoes to transmit malaria depends on larval environment. Parasit. Vectors 7, 593. http://dx.doi.org/10.1186/s13071-014-0593-4.

Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G., Pyke, A.T., Hedges, L.M., Rocha, B.C., Hall-Mendelin, S., Day, A., Riegler, M., Hugo, L.E., Johnson, K.N., Kay, B.H., McGraw, E.A., van den Hurk, A.F., Ryan, P.A., O'Neill, S.L., 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with *Dengue*, *Chikungunya*, and *Plasmodium*. Cell 139, 1268–1278. http://dx.doi.org/10.1016/j.cell.2009.11.042.

Murdock, C.C., Paaijmans, K.P., Cox-Foster, D., Read, A.F., Thomas, M.B., 2012. Rethinking vector immunology: the role of environmental temperature in shaping resistance. Nat. Rev. Microbiol. 10, 869–876. http://dx.doi.org/10.1038/nrmicro2900.

Murdock, C.C., Blanford, S., Hughes, G.L., Rasgon, J.L., Thomas, M.B., 2014a. Temperature alters *Plasmodium* blocking by *Wolbachia*. Sci. Rep. 4, 3932. http://dx.doi.org/10.1038/srep03932.

Murdock, C.C., Blanford, S., Luckhart, S., Thomas, M.B., 2014b. Ambient temperature and dietary supplementation interact to shape mosquito vector competence for malaria. J. Insect Physiol. 67, 37–44. http://dx.doi.org/10.1016/j.jinsphys.2014.05.020.

Nayar, J.K., Knight, J.W., Telford, S.R., 1998. Vector ability of mosquitoes for isolates of *Plasmodium elongatum* from raptors in Florida. J. Parasitol. 84, 542–546.

Olias, P., Wegelin, M., Zenker, W., Freter, S., Gruber, A.D., Klopfleisch, R., 2011. Avian malaria deaths in parrots, Europe. J. Infect. Dis. 17, 950–952. http://dx.doi.org/10.1086/605025.

Palinauskas, V., Valkiūnas, G., Bolshakov, C.V., Bensch, S., 2008. *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. Exp. Parasitol. 120, 372–380. http://dx.doi.org/10.1016/j.exppara.2008.09.001.

Palinauskas, V., Valkiūnas, G., Bolshakov, C.V., Bensch, S., 2011. *Plasmodium relictum* (lineage SGS1) and *Plasmodium ashfordi* (lineage GRW2): the effects of the co-infection on experimentally infected passerine birds. Exp. Parasitol. 127, 527–533. http://dx.doi.org/10.1016/j.exppara.2010.10.007.
Palinauskas, V., Žiegytė, R., Ilgūnas, M., Iezhova, T.A., Bernotienė, R., Bolshakov, C., Valkiūnas, G., 2015. Description of the first cryptic avian malaria parasite, *Plasmodium homocircumflexum* n. sp., with experimental data on its virulence and development in avian hosts and mosquitoes. Int. J. Parasitol. 45, 51–62. http://dx.doi.org/10.1016/j.ijpara.2014.08.012.
Perkins, S.L., 2000. Species concepts and malaria parasites: detecting a cryptic species of *Plasmodium*. Proc. Biol. Sci. 267, 2345–2350. http://dx.doi.org/10.1098/rspb.2000.1290.
Raffaele, G., 1934. Un ceppo italiano du *Plasmodium elongatum*. Riv. Malariol. 13, 3–8.
Reichenow, E., 1932. Die antwicklung von *Proteosoma circumflexum* in *Theobaldia annulata* nebst beobachtungen uber das verhalten anderer vogelsplasmodien in mucken. Jenaische Zeitschrift fur Naturwiss 67, 434–451.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574. http://dx.doi.org/10.1093/bioinformatics/btg180.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, USA, doi: 574.873224 1/1989.
Santiago-Alarcon, D., Palinauskas, V., Schaefer, H.M., 2012. Diptera vectors of avian Haemosporidian parasites: untangling parasite life cycles and their taxonomy. Biol. Rev. Camb. Philos. Soc. 87, 928–964. http://dx.doi.org/10.1111/j.1469-185X.2012.00234.x.
Schoener, E., Banda, M., Howe, L., Castro, I., Alley, M., 2014. Avian malaria in New Zealand. N. Z. Vet. J. 62, 189–198. http://dx.doi.org/10.1080/00480169.2013.871195.
Schwartz, A., Koella, J.C., 2002. Melanization of *Plasmodium falciparum* and C-25 sephadex beads by field-caught *Anopheles gambiae* (Diptera: Culicidae) from southern Tanzania. J. Med. Entomol. 39, 84–88. http://dx.doi.org/10.1603/0022-2585-39.1.84.
Silveira, P., Belo, N.O., Lacorte, G.A., Kolesnikovas, C.K.M., Vanstreels, R.E.T., Steindel, M., Catão-Dias, J.L., Valkiūnas, G., Braga, É.M., 2013. Parasitological and new molecular-phylogenetic characterization of the malaria parasite *Plasmodium tejerai* in South American penguins. Parasitol. Int. 62, 165–171. http://dx.doi.org/10.1016/j.parint.2012.12.004.
Sinden, R.E., 1999. *Plasmodium* differentiation in the mosquito. Parasitologia 41, 139–148.
Sinden, R.E., Alavi, Y., Raine, J.D., 2004. Mosquito-malaria interactions: a reappraisal of the concepts of susceptibility and refractoriness. Insect Biochem. Mol. Biol. 34, 625–629. http://dx.doi.org/10.1016/j.ibmb.2004.03.015.
Sladen, W.J.L., Gailey-Phipps, J.J., Divers, B.J., 1976. Medical problems and treatments of penguins at the Baltimore Zoo. Int. Zoo Yearbook 19, 202–209.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739. http://dx.doi.org/10.1093/molbev/msr121.
Valkiūnas, G., 2005. Avian Malaria Parasites and other Haemosporidia. CRC Press, Boca Raton, USA.
Valkiūnas, G., Iezhova, T.A., Shapoval, A.P., 2003. High prevalence of blood parasites in hawfinch *Coccothraustes coccothraustes*. J. Nat. Hist. 37, 2647–2652. http://dx.doi.org/10.1080/002229302100001033221.
Valkiūnas, G., Iezhova, T.A., Križanauskienė, A., Palinauskas, V., Sehgal, R.N.M., Bensch, S., 2008a. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. J. Parasitol. 94, 1395–1401. http://dx.doi.org/10.1645/GE-1570.1.
Valkiūnas, G., Zehtindjiev, P., Dimitrov, D., Križanauskienė, A., Iezhova, T.A., Bensch, S., 2008b. Polymerase chain reaction-based identification of *Plasmodium (Huffia) elongatum*, with remarks on species identity of haemosporidian lineages deposited in GenBank. Parasitol. Res. 102, 1185–1193. http://dx.doi.org/10.1007/s00436-008-0892-9.
Valkiūnas, G., Iezhova, T.A., Loiseau, C., Sehgal, R.N.M., 2009. Nested cytochrome B polymerase chain reaction diagnostics detect sporozoites of hemosporidian parasites in peripheral blood of naturally infected birds. J. Parasitol. 95, 1512–1515. http://dx.doi.org/10.1645/GE-2105.1.
Valkiūnas, G., Kazlauskienė, R., Bernotienė, R., Palinauskas, V., Iezhova, T.A., 2013. Abortive long-lasting sporogony of two *Haemoproteus* species (Haemosporida, Haemoproteidae) in the mosquito *Ochlerotatus cantans*, with perspectives on haemosporidian vector research. Parasitol. Res. 112, 2159–2169. http://dx.doi.org/10.1007/s00436-013-3375-6.
Valkiūnas, G., Kazlauskienė, R., Bernotienė, R., Bukauskaitė, D., Palinauskas, V., Iezhova, T.A., 2014. *Haemoproteus* infections (Haemosporida, Haemoproteidae) kill bird-biting mosquitoes. Parasitol. Res. 113, 1011–1018. http://dx.doi.org/10.1007/s00436-013-3733-4.
Valkiūnas, G., Iezhova, T.A., Palinauskas, V., Ilgūnas, M., Bernotienė, R., 2015a. The evidence for rapid gametocyte viability changes in the course of parasitemia in *Haemoproteus* parasites. Parasitol. Res. 114, 2903–2909. http://dx.doi.org/10.1007/s00436-015-4491-2.
Valkiūnas, G., Žiegytė, R., Palinauskas, V., Bernotienė, R., Bukauskaitė, D., Ilgūnas, M., Dimitrov, D., Iezhova, T.A., 2015b. Complete sporogony of *Plasmodium relictum* (lineage pGRW4) in mosquitoes *Culex pipiens pipiens*, with implications on avian malaria epidemiology. Parasitol. Res. 114, 3075–3085. http://dx.doi.org/10.1007/s00436-015-4510-3.
van Rooyen, J., Lalubin, F., Glaizot, O., Christe, P., 2013. Avian haemosporidian persistence and co-infection in great tits at the individual level. Malar. J. 12, 40. http://dx.doi.org/10.1186/1475-2875-12-40.
Vanstreels, R.E.T., Kolesnikovas, C.K.M., Sandri, S., Silveira, P., Belo, N.O., Ferreira Junior, F.C., Epiphanio, S., Steindel, M., Braga, E.M., Luiz Catao-Dias, J., 2014. Outbreak of avian malaria associated to multiple species of *Plasmodium* in magellanic penguins undergoing rehabilitation in Southern Brazil. PLoS One. http://dx.doi.org/10.1371/journal.pone.0103670.
Waldenström, J., Bensch, S., Kiboi, S., Hasselquist, D., Ottosson, U., 2002. Cross-species infection of blood parasites between resident and migratory songbirds in Africa. Mol. Ecol. 11, 1545–1554. http://dx.doi.org/10.1046/j.1365-294X.2002.01523.x.
Wang, Y., Gilbreath, T.M., Kukutla, P., Yan, G., Xu, J., 2011. Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. PLoS One 6, e24767. http://dx.doi.org/10.1371/journal.pone.0024767.
Weathersby, A.B., Mccall, J.W., 1968. The development of *Plasmodium gallinaceum* Brumpt in the Hemocoels of refractory *Culex pipiens pipiens* Linn. and susceptible *Aedes aegypti* (Linn.). J. Parasitol. 54, 1017–1022.
Žiegytė, R., Bernotienė, R., Bukauskaitė, D., Palinauskas, V., Iezhova, T.A., Valkiūnas, G., 2014. Complete sporogony of *Plasmodium relictum* (lineages pSGS1 and pGRW11) in mosquito *Culex pipiens pipiens* form molestus, with implications to avian malaria epidemiology. J. Parasitol. 100, 878–882. http://dx.doi.org/10.1645/13-469.1.

PAPER III

Description of *Haemoproteus ciconiae* sp. nov. (Haemoproteidae, Haemosporida) from the white stork *Ciconia ciconia*, with remarks on insensitivity of established polymerase chain reaction assays to detect this infection.

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Description of *Haemoproteus ciconiae* sp. nov. (Haemoproteidae, Haemosporida) from the white stork *Ciconia ciconia*, with remarks on insensitivity of established polymerase chain reaction assays to detect this infection

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Abstract

Haemoproteus ciconiae sp. nov. (Haemosporida, Haemoproteidae) was found in the white stork *Ciconia ciconia* (Ciconiiformes, Ciconiidae) after spring migration in Lithuania. This organism is illustrated and described based on the morphology of its blood stages. The new species can be readily distinguished from all other haemoproteids parasitizing ciconiiform birds due presence of large number (approximately 20 on average) small (< 1 µm) pigment granules in its mature gametocytes. Growing and mature gametocytes of *H. ciconiae* were readily visible in all blood films (parasitemia of 0.001%). However, experienced researchers were unable to detect sequences of its mitochondrial cytochrome *b* (cyt *b*) or apicoplast genes from the microscopically positive sample by using five established assays for polymerase chain reaction (PCR)-based detection of avian haemosporidian parasites. The white stork cyt *b* sequence was readily detectable, indicating the well-optimised PCR protocols and the good quality of total DNA in the sample containing the new species. The failure to amplify this parasite DNA indicates insufficient sensitivity of the currently used PCR-based assays in diagnostics of avian haemosporidian infections. We suggest possible explanations of this observation. To minimize number of the false negative PCR reports, we call for the continued use of optical microscopy in parallel with molecular diagnostics in studies of haemosporidian parasites, particularly in wildlife.

Keywords: *Haemoproteus*; new species; birds; *Cicocia ciconia;* PCR; optical microscopy.

Introduction

The white stork *Ciconia ciconia* is a widespread Palearctic bird belonging to the Ciconiidae (order Ciconiiformes) (Cramp and Simpson 1977). This common big bird is well-recognisable and often considered as an indicator of ecologically healthy environment in agricultural landscapes where it breeds singly in big solitary trees, on roofs of houses and telegraph-poles. Currently, population of the white stork is increasing throughout its entire breeding range, particularly markedly in the Eastern part of Europe, probably due to changes in farming practices and foraging conditions at wintering areas (Vaitkuvienė and Dagys 2015). North European population of the white stork winters in Africa where these birds spend the great part of a year and are exposed to parasite infections. However, haemosporidians (Haemosporida) have not been reported in the white stork in Europe (Valkiūnas et al. 2002), but were described in other species of ciconiiform birds in sub-Saharan Africa (Tendeiro 1947; Peirce and Cooper

1977; Valkiūnas 2005). It remains unclear if white storks are infected with blood parasite at African breeding grounds and if they transport these infections to European breeding areas.

In spring 2015, one damaged adult white stork was delivered to the Ornithological Station, Ventės Ragas in Lithuania. Blood samples were collected and examined by microscopic examination of blood films and polymerase chain reaction (PCR)-based methods. One previously undescribed species of *Haemoproteus* (Haemoproteidae, Haemosporida) was reported in blood films, but it was undetectable by five well-established PCR assays. This parasite is named and described here based on morphology of its blood stages. It is the first haemosporidian species described from the white stork (Bennett et al. 1982; Bishop and Bennett 1992; Valkiūnas 2005). We compare the new species with haemoproteids reported in other ciconiiform birds and discuss failures in detection of its DNA sequences by the currently broadly used PCR-based protocols.

Materials and methods

Study site, collection of samples and their microscopic examination

On 20 May 2015, one female of the white stork with both broken legs was found in Šilutė District of Lithuania and delivered to the Ornithological Station, Ventės Ragas. Blood was taken by puncturing the brachial vein. A blood sample (~50 µL) was collected using heparinized microcapillaries and stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) at ambient temperature while in the field, and then preserved at -20°C in the laboratory. We prepared 10 blood films on ready-to-use glass slides and air-dried them within 5 sec after preparation using a battery-operated fan. The blood films were fixed in absolute methanol for 1 min on the day of their preparation. The fixed smears were air-dried and stained in a 10% working solution of a commercially purchased stock solution of Giemsa stain for 1 hr. All blood films were stained within 14 days after their fixation. Details of preparation and staining of blood films were described by Valkiūnas et al. (2008).

The white stork was markedly atonic, and the veterinary observation concluded that it is impossible to treat due to the marked leg damage and weak body condition. The bird was euthanized, and pieces of organs (liver, lungs, heart, kidneys, bone marrow) and pectoral muscles were fixed in 10% neutral formalin, embedded in paraffin and processed using traditional histology methods (Valkiūnas 2005). Histological sections of 4 µm were obtained, stained with hematoxylin-eosin, mounted in Biomount (BioGnost, Croatia) and examined microscopically.

An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used to examine blood films and histological preparations, to prepare illustrations and to take measurements. Approximately 150 fields were examined at low magnification (400×), and then at least 100 fields were viewed studied at high magnification (1000×). The morphometric features studied (Table 1) are those defined by Valkiūnas (2005). Intensity of parasitemia was estimated as a percentage by counting of the number of parasites per 100,000 red blood cells. The morphometric analysis (Table 1) was carried out using the 'Statistica 7' package.

Table 1 Morphometry of host cells and mature gametocytes of *Haemoproteus ciconiae* sp. nov. from the white stork *Ciconia ciconia*

Feature	Measurements (µm)[a]
Uninfected erythrocyte	
Length	13.3-15.3 (14.5±0.6)
Width	6.4-7.9 (7.4±0.4)
Area	76.9-95.5 (85.2±5.9)
Uninfected erythrocyte nucleus	
Length	5.6-7.5 (6.5±0.5)
Width	1.9-2.8 (2.4±0.2)
Area	10.3-15.5 (12.7±1.2)
Macrogametocyte	
Infected erythrocyte	
Length	14.3-17.5 (15.9±0.8)
Width	5.7-8.7 (7.4±0.8)
Area	82.3-108.5 (95.3±7.5)
Infected erythrocyte nucleus	
Length	5.7-8.5 (6.9±0.6)
Width	1.7-2.7 (2.2±0.3)
Area	9.2-17.5 (12.4±1.9)
Gametocyte	
Length	11.5-16.8 (14.1±1.3)
Width	2.4-3.8 (3.0±0.4)
Area	32.7-51.7 (38.3±4.3)
Gametocyte nucleus	
Length	2.4-4.7 (3.3±0.8)
Width	0.8-3.1 (1.9±0.5)
Area	3.0-9.0 (5.1±1.7)
Pigment granules	20.0-29.0 (23.7±2.3)
NDR[b]	0.4-1.0 (0.7±0.2)
Microgametocyte	
Infected erythrocyte	
Length	13.7-18.7 (16.3±1.1)
Width	6.8-8.7 (7.6±0.5)
Area	83.1-116.7 (101.3±9.0)
Infected erythrocyte nucleus	
Length	6.2-7.7 (7.0±0.4)
Width	1.8-3.1 (2.4±0.3)
Area	9.2-19.5 (14.0±2.7)
Gametocyte	
Length	12.7-18.8 (15.6±1.7)
Width	2.2-4.0 (3.0±0.5)
Area	35.5-58.6 (46.2±6.0)
Gametocyte nucleus	
Length	-
Width	-
Area	-
Pigment granules	11.0-23.0 (18.1±2.6)
NDR	0.3-1.0 (0.7±0.2)

[a] All measurements (n=21) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.
[b] NDR = nucleus displacement ration, which was calculated according to Bennett and Campbell (1972).

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from blood samples using the standard ammonium-acetate protocol (Sambrook and Russell 2001) with minor modification: instead of 250 μL of fixed blood, we used 125 μL. Quantification of the DNA was performed by using nanodrop spectrophotometer (IMPLEN Nanophotometer P330). All reactions were performed in 25 μl total volumes, including 50 ng of a total genomic DNA template (2 μL), 12.5 μL of DreamTag Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 μL nuclease-free water and 1 μL of each primer. One negative control (nuclease-free water) and one positive control (a blood sample, which was positive by microscopic examination of blood films) were used to control for false amplifications. All used PCR essays (Table 2) correctly determined both these control samples. If the white stork blood sample was classified as a false negative, we tested it at least three times by each PCR essay to ensure that the negative results were not caused by the PCR failure.

Five established PCR assays (Table 2), which are broadly used in wildlife avian haemosporidian research, were applied for detection of *Haemoproteus* infection from the white stork blood sample. This sample was *Haemoproteus* parasite positive by microscopic examination of blood films. Four of these PCR assays used primer sets, which amplify fragments of the mitochondrial genome: three of cytochrome *b* (cyt *b*) gene (primer set HaemNFI/NR3 and HAEMF/R2 (Bensch et al. 2000; Hellgren et al. 2004), primers 621F/983R (Richard et al. 2002), and primers 3760F/4292Rw2 (Beadell et al. 2004), one of cytochrome oxidase subunit I (COI) gene (primer set COIF/R and COIF2/R2, Martinsen et al. 2008), and one target apicoplast genome (primer set ClpcF/R and ClpcF2/R2, Martinsen et al. 2008). Because all these PCR essays were insensitive in detection of *Haemoproteus* infection in the microscopy positive blood sample (see below), we ruled out a possibility of insufficient quality of the total DNA in our sample by application of primers, which amplify fragments of cyt *b* gene of birds (primers Avian-3 and Avian-8, Ejiri et al. 2011).

Table 2 Six polymerase chain reaction (PCR) assays used in detection of bird and haemosporidian parasite partial DNA sequences from blood samples

Gene	Primer code	Primer sequence	Reference
Apicoplast [a]	Outer ClpcF	5`-AAACTGAATTAGCAAAAATATTA-3`	Martinsen et al. 2008
	ClpcR	5`-CGWGCWCCATATAAAGGAT-3`	
	Inner ClpcF2	5`-GATTTGATATGAGTGAATATATGG-3`	
	ClpcR2	5`-CATATAAAGGATTATAWG-3`	
Cytochrome oxidase subunit I [a]	Outer COIF	5`-CTATTTATGGTTTTCATTTTTATTTGGTA-3`	
	COIR	5`-AGGAATACGTCTAGGCATTACATTAAATCC-3`	
	Inner COIF2	5`-ATGATATTTACARTTCAYGGWATTATTATG-3`	
	COIR2	5`-GTATTTTCTCGTAATGTTTTACCAAAGAA-3`	
Cytochrome *b* [a]	Outer HaemNFI	5’-CATATATTAAGAGAAITATGGAG-3’;	Bensch et al. 2000; Hellgren et al. 2004
	HaemNR3	5’-ATAGAAAGATAAGAAATACCATTC-3’	
	Inner HAEMF	5’-ATGGTGCTTTCGATATATGCATG-3’	
	HAEMR2	5’-GCATTATCTGGATGTGATAATGGT-3’	
Cytochrome *b*	3760F	5′-GAGTGGATGGTGTTTTAGAT-3′	Beadell et al. 2004
	4292Rw2	5`-TGGAACAATATGTARAGGAGT-3′	
Cytochrome *b*	621F	5`-AAAAATACCCTTCTATCCAAATCT-3`	Richard et al. 2002
	983R	5`-CATCCAATCCATAATAAAGCAT-3`	
Cytochrome *b*	Avian-3	5`-GACTGTGAYAAAATYCCMTTCCA-3`	Ejiri et al. 2011
	Avian-8	5`-GYCTTCAITYTTTGGYTTACAAGAC-3`	

[a] Nested PCR.

The amplification success was evaluated after run out of 2 µL of final PCR product on 2% agarose gels. PCR products (21 µL) were purified by adding 11 µL of NH_4Ac, 37 µL of 96% and 150 µL of 70 % ethanol. After centrifugation, air-dried DNA pellets were dissolved in 15 µL of ddH_2O. To identify sequences, samples that showed positive amplification were sequenced using Big Dye Terminator V3.1 Cycle Sequencing Kit and then loaded on an ABI PRISM ™ 3100 capillary sequencing robot (Applied Biosystems, Foster City, California). The sequences were edited and aligned using BioEdit software (Hall, 1999). The BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare detected sequences with those deposited in GenBank.

Results

Microscopic examination revealed an undescribed species of *Haemoproteus* in the white stork. Intensity of parasitemia was 0.001%. Over 100 gametocytes and parasitized red blood cells were examined. Exoerythrocytic meronts were not reported in histological sections of organs. Other blood parasites were not seen.

None of the applied PCR assays (Table 2) detected new *Haemoproteus* infection in the sample, which was microscopy positive. However, amplifications of (1) the control positive samples and (2) the bird DNA were successful, indicating the well-optimised PCR protocols and the good quality of the total DNA in the same sample containing the new species. The sequencing and application of the BLAST algorithm confirmed that we obtained bird cyt *b* sequence belonging to the white stork (GenBank accession no. U70822.1).

Parasite description

***Haemoproteus (Parahaemoproteus) ciconiae* n. sp.** (Fig. 1, Table 1)

Macrogametocytes (Fig. 1a-h, Table 1) Develop in mature erythrocytes. Earliest forms were not seen in the type material. Growing gametocytes assume lateral positions to the nuclei of infected erythrocytes, they often do not touch either envelop or nuclei, or both these structures of the host cells (Fig. 1a). Cytoplasm is finely-granular in appearance, sometimes contains several small vacuoles (Fig. 1e,g); volutin granules were not seen. Gametocytes extend along the nuclei of erythrocytes; they do not enclose or only slightly enclose the nuclei with their ends (Fig. 1a-d). Fully grown gametocytes do not fill the erythrocytes up to their poles (Fig. 1e-h); they have no permanent position in erythrocytes: gametocytes touching both the nuclei and envelop are common (Fig. 1g), but the parasites, which do not touch either envelop (Fig. 1e) or nuclei (Fig. 1f,h) also present. Outline usually is entire (Fig. 1g,h), sometimes slightly wavy (Fig. 1f). Parasite nucleus (Fig. 1b,h) is compact, markedly variable in form, median or submedian in position; it has no single predominant position in relation to gametocyte pellicle (compare Fig. 1a and Fig. 1e). Nucleolus was not seen. Pigment granules (Fig. 1d,e) are mainly small (< 0.5 µm), occasionally of medium (0.5-1.0 µm) size, roundish, usually randomly scattered throughout the cytoplasm (Fig. 1a,e-g), but also might be grouped (Fig. 1c,e,h). Nuclei of infected erythrocytes are not displaced (Fig. 1f,g) or slightly (Fig. 1a-e,h) displaced laterally. Parasitized erythrocytes are enlarged in length (Table 1).

Microgametocytes (Fig. 1i-p, Table 1) The general configuration is as for macrogametocytes with the usual haemosporidian sexually dimorphic characters, which are the pale staining of the cytoplasm and large diffuse nuclei. Boundaries of the parasite nuclei are irregular and obscure (Fig. 1i-p) making the nuclei difficult to measure. Gametocyte outline varies from complete even (Fig. 1i,j,l,m,n,p) to highly amoeboid (Fig. 1k,o). Fully grown microgametocytes are larger than macrogametocytes (Table 1), and they slightly enclose erythrocyte nuclei with their ends (Fig. 1n,p). Gametocytes with highly amoeboid outline are present. Other characters are as for macrogametocytes.

Taxonomic summary

Type host White stork *Ciconia ciconia* L. (Ciconiiformes, Ciconiidae).
Type locality Ventės ragas, Lithuania (55°20'28.1"N 21°11'25.3"E, 1 m above sea level).
Site of infection Mature erythrocytes; no other data.
Type specimens Hapantotype (accession nos. 48912-48915 NS), intensity of parasitemia is 0.001%, *Ciconia ciconia*, Ventės Ragas, collected by M. Ilgūnas, 20 May 2015) was deposited in the Nature Research Centre (NRC), Vilnius, Lithuania. Parahapantotypes (accession nos. G466178, G466179, other data as for the hapantotype) were deposited in the Queensland Museum, Queensland, Australia. Parasite gametocytes were marked with circles on the hapantotype and parahapantotype slides.
Additional material Voucher blood films (accession nos. 48916-48919 NS) and the sample of whole blood from the type host (original field number 211/15R) were deposited in NRC, Vilnius, Lithuania.
Etymology The species name is derived from the host genus *Ciconia*, to which the type host of the parasite belongs.
Taxonomic remarks Four species of *Haemoproteus* parasitize birds belonging to the order Ciconiiformes: *H. crumenium* (Peirce 1987; Valkiūnas 2005), *H. herodiadis* (de Mello 1935a; Valkiūnas 2005), *H. plataleae* (de Mello 1935b; Bennett et al. 1975) and *H. pelouroi* (Tendeiro 1947; Valkiūnas 2005). These species can be distinguished from *H. ciconiae* due to the following readily discernable characters. In *H. crumenium,* macrogametocyte nuclei assume strictly central position and they adhere to the nuclei of infected erythrocytes. Additionally, the cytoplasm often contains prominent circular vacuoles. In *H. plataleae*, the number of pigment granules in macrogametocytes is approximately half as many in microgametocytes. In *H. herodiadis,* pigment granules are not numerous (approximately 10 on average), small (< 0.5 µm) and dust-like in appearance. In *H. pelouroi*, both macro- and microgametocytes are markedly amoeboid and possess about ten pigment granules on average. None of these features are characters of *H. ciconiae* (Fig. 1, Table 1).

Among the haemoproteids of ciconiiform birds, *H. ciconiae* is most similar to *H. herodiadis*, and it can be distinguished from the latter species, primarily on the basis of (1) the greater number of pigment granules and (2) larger size of pigment granules in its gametocytes.

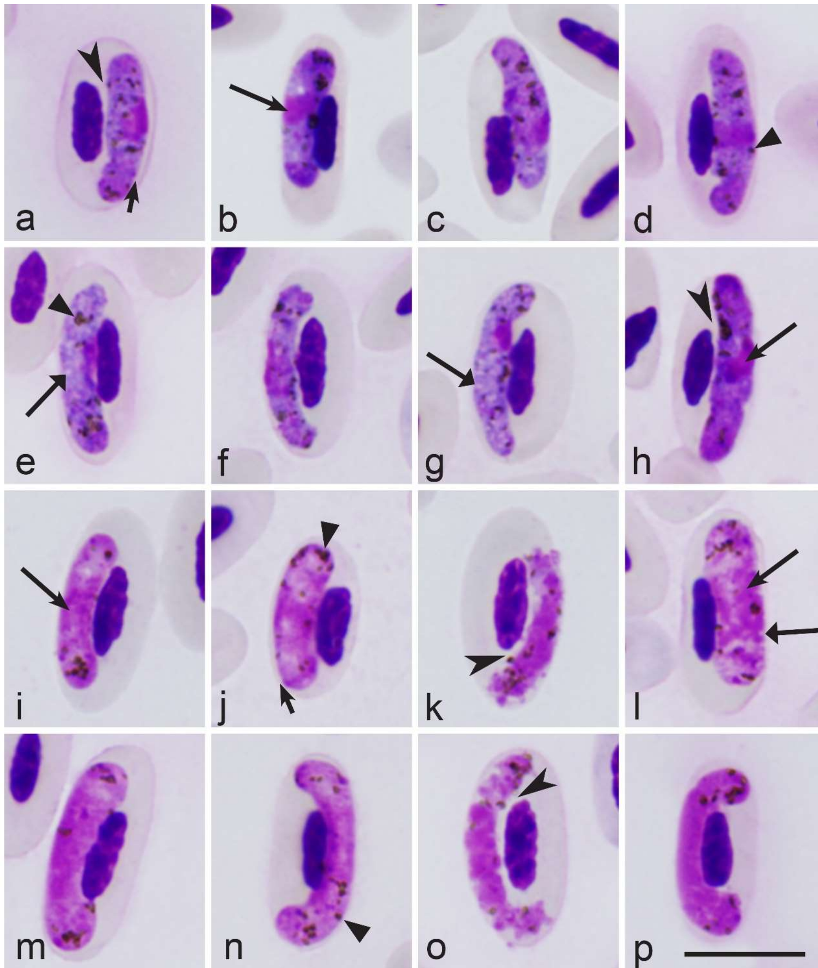

Fig. 1 Macrogametocytes (a-h) and microgametocytes (i-p) of *Haemoproteus ciconiae* sp. nov. from the blood of the white stork *Ciconia ciconia*. Giemsa-stained thin blood films. Long simple arrows – nuclei of parasites; short simple arrows – unfilled spaces between gametocytes and erythrocyte envelope; simple arrowheads – unfilled spaces between gametocytes and erythrocyte nuclei; long triangle arrows – vacuoles; triangle arrowheads – pigment granules. Note that length of mature macrogametocytes is less than in mature microgametocytes (compare f-h with n-p). Scale bar = 10 µm

Discussion

The key result of this study is the description of the first *Haemoproteus* parasite inhabiting the white stork. One may ask whether it is justifiable to describe a new species of *Haemoproteus* on the basis of a single set of blood films prepared from one individual host. Several valid species of haemosporidians with readily distinct morphological characters have been described on single records in naturally infected reptiles, birds, and mammals (Garnham 1966; Valkiūnas

2005; Telford 2009; Zehtindjiev et al. 2012). The morphological characters of blood stages of *H. ciconiae* are distinct to differentiate this parasite from other known *Haemoproteus* species. The recorded parasites and their host cells were consistent in morphology and with the interpretation that they belong to *H. ciconiae.* The type material is deposited in two museums and is available for taxonomic examination. Importantly, the original sample of whole blood from the type host also is available for future examination, thus this study is readily repeatable. Because of difficulties in blood sampling from adult white storks (see below), this information might be lost if the new species is not described.

Avian *Haemoproteus* parasites belong to two subgenera, i. e., *Parahaemoproteus* and *Haemoproteus*. Traditional subgenetic classification of these haemoproteids is based mainly on the differences in their sporogony and distribution by vectors and avian hosts (Garnham 1966; Atkinson et al. 2008; Valkiūnas 2015). Sporogony of *Parahaemoproteus* spp. occurs in biting midges (Ceratopogonidae). These parasites inhabit mainly terrestrial birds of numerous avian orders, including ciconiiform birds, as is the case with *H. ciconiae*. Species of *Haemoproteus* have been reported mainly in birds belonging to the order Columbiformes and some marine birds (Charadriiformes, Pelecaniformes); they are transmitted by louse flies of the Hippoboscidae (Levin et al. 2012; Merino et al. 2012). Vectors of *H. ciconiae* remain unknown. Phylogenies based on mitochondrial gene sequences can be used for prediction of vectors of *Haemoproteus* parasites (Bukauskaitė et al. 2015) however, the sequence information about *H. ciconiae* currently is absent. We attribute this parasite to subgenus *Parahaemoproteus* due to its ability parasitize birds of the Ciconiiformes. However, investigation of sporogony is needed to prove subgeneric classification of this parasite.

In spite of wide distribution, adult white storks are difficult to catch using traditional ornithological methods of netting and trapping, resulting in a few studies addressing its blood parasites (Rousselot 1953; Berson 1964; Jovani et al. 2001). There is no information about blood parasites of this bird species in Europe (Bennett et al. 1982; Bishop and Bennett 1992; Jovani et al. 2001; Valkiūnas 2005). Valkiūnas et al. (2002) collected and examined blood samples from 22 nestlings of the white stork on the Curonian Spit in the Baltic Sea. The birds were taken from nests at the age of approximately 5 weeks during implementation of an ornithological migration program and raised by hand in the vector-opened aviary. The blood of these birds was samples and examined microscopically twice when the nestlings were of 6 and 8 weeks old. Blood parasites were not reported. Absence of *Haemoproteus* parasites in juvenile birds indicates that white storks get infected with *H. ciconiae* away from breeding areas, bring this parasite to Europe, but transmission does not occurs. The obstacles for transmission might be insufficient abiotic conditions for development of the African origin parasite in the northern Europe or lack of susceptible vector species, or both. Experimental studies are needed to answer these questions.

Description of new species is in accord with the recent PCR-based research showing remarkable genetic diversity of avian haemosporidian parasites (Bensch et al. 2004; Clark et al. 2014; Perkins 2014; Outlaw and Ricklefs 2014; Valkiūnas 2015). Because natural haemosporidian infections are often light and are difficult to identify to species using microscopic examination of blood films, it is essential to develop molecular markers to aid in diagnosis of natural malarial and other haemosporidian infections (Dimitrov et al. 2014).

Additionally, such markers are essential for parasite species diagnostics both on the tissue stage in avian hosts and the sporogony stage in vectors (Valkiūnas et al. 2014; Clark et al. 2015).

We used five PCR essays, which target both long (approximately 1000 bp) and short (approximately 350 bp) DNA fragments and are broadly used in diagnostic and phylogenetic research of avian haemosporidian parasites in wildlife (Table 2). Applying more than one PCR protocol, targeting shorter fragments and more conserved gene regions often increase the sensitivity of haemosporidian detection (Valkiūnas et al. 2008; Perkins 2014), but that was not a case with *H. ciconiae* in this study. It worth noting that short DNA fragments (< 300 bp) usually are of low value in haemosporidian phylogenetic research and molecular characterisation of parasites (Fallon et al. 2003; Dinhopl et al. 2015); they can be recommended to use mainly for estimation prevalence of haemosporidians belonging to certain genera. Additionally, for reliable diagnostics of parasites it is important to optimize PCR protocols because they may not work similarly well across laboratories depending on DNA extraction methods, taq-DNA and other reagent supplier, PCR machine, etc (Freed and Cann 2006). All applied PCR protocols (Table 2) were optimised and worked well in our laboratory (Bernotienė et al. 2016). It is often deemed that PCR-based diagnostics is more sensitive tool in determining light infections of haemosporidian parasites (Jarvi et al. 2002; Richards et al. 2002; Durrant et al. 2006; Clark et al. 2014). However, all used PCR essays showed false negative results in this study: gametocytes of *H. ciconiae* were visible by microscopic examination in all blood films using an established microscopy protocol (Valkiūnas et al. 2008), but PCR amplification of the parasite DNA was negative. There might be following explanations of this observation.

Firstly, it is probable that light *Haemoproteus* spp. parasitemia (of ≤0.001%) might be often undetectable by the PCR amplification due to insufficient starting material of parasite DNA in comparison to huge amounts of bird DNA in each blood sample (Hellgren et al. 2004). This conclusion is in accord with observations by Jarvi et al. (2002), who showed that the application of serological diagnostic methods provided better results in detection of avian malaria infections in comparison to PCR. It worth mentioning however, that microscopic examination of blood films was of poor diagnostic sensitivity in the study by Jarvi et al. (2002) probably because these authors examined only ~50000 red blood cells for parasite detection, and that is not enough for determining haemosporidian parasite positive samples if parasitemia is ≤0.001%, which is a common case in wildlife (Valkiūnas 2005).

Secondly, primer sets, which were used in the PCR essays (Table 2), have been originally developed using DNA sequences of *Plasmodium* or *Haemoproteus* parasites inhabiting passeriform birds. These primers might be insufficiently sensitive in amplification of DNA of some distantly related haemoproteids developing in non-passerine birds.

Thirdly, several recent studies reported lack of amplification of haemosporidian parasite DNA from blood samples, in which blood stages were readily visible and even present at high parasitemia, as confirmed by the microscopic examination of blood film (Zehtindjiev et al. 2012; Schaer et al. 2015). It was speculated that some haemosporidians might lose their cyt *b* gene. This hypothesis is unexpected given our present knowledge about unique haemosporidian parasite morphology and their mitochondrial DNA organization (Wilson and Williamson 1997; Valkiūnas 2005), but it worth the attention of researchers from an evolutionary perspective. However, even if this hypothesis is correct in some haemosporidian parasites, the DNA of new

parasite should have amplified using apicoplast gene primers (Table 2). Because that was not the case in this study, we believe that the first and the second explanations are more likely.

This study provides new data indicating that a combination of advanced molecular and microscopic approaches remains essential for reliable comparative research of haemosporidian parasites, particularly during parasite biodiversity investigations (Dimitrov et al. 2014; Clark et al. 2015).

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References

1. Atkinson CT, Thomas NJ, Hunter DB (2008) Parasitic diseases of wild birds. Wiley-Blackwell, Oxford
2. Beadell JS, Gering E, Austin J, Dumbacher JP, Peirce MA, Pratt TK, Atkinson CT,
3. Fleischer RC (2004) Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. Mol Ecol 13:3829–3844
4. Bennett GF, Campbell AG (1972) Avian Haemoproteidae. I. Description of *Haemoproteus fallisi* n. sp. and a review of the haemoproteids of the family Turdidae. Can J Zool 50:1269-1275
5. Bennett GF, Greiner EC, Campbell AG (1975) Avian Haemoproteidae. 5. The haemoproteids of the family Threskiornithidae. Can J Zool 53:634-638
6. Bennett GF, Whiteway M, Woodworth-Lynas C (1982) A host-parasite catalogue of the avian haematozoa. Occasional Papers in Biology. Memorial University of Newfoundland
7. Bensch S, Pérez-Tris J, Waldenström J, Hellgren O (2004) Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation? Evolution 58:1617–1621
8. Bensch S, Stjenman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, Torres-Pinheiro R (2000) Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc R Soc B 276:1583–1589
9. Bernotienė R, Palinauskas V, Iezhova T, Murauskaitė D, Valkiūnas G (2016) Avian haemosporidian parasites (Haemosporida): a comparative analysis of different polymerase chain reaction assays in detection of mixed infections. Exp Parasitol 158: in press
10. Berson JP (1964) Les protozoaires parasites des hématies et du système histiocytaire des oiseaux. Essai de nomenclature. Res Elev Ned Vet Pays Trop 17:43-96

11. Bishop MA, Bennett GF (1992) Host-parasite catalogue of the avian haematozoa: Supplement 1, and Bibliography of the avian blood-inhabiting haematozoa: Supplement 2. Mem Univ of Nfld Occ Pap Biol 15:1-244
12. Bukauskaitė D, Žiegytė R, Palinauskas V, Iezhova TA, Dimitrov D, Ilgūnas M, Bernotienė R, Markovets MY, Valkiūnas G (2015) Biting midges (*Culicoides*, Diptera) transmit *Haemoproteus* parasites of owls: evidence from sporogony and molecular phylogeny. Parasit Vectors 8:303
13. Clark NJ, Adlard RD, Clegg SM (2015) Molecular and morphological characterization of *Haemoproteus (Parahaemoproteus) ptilotis*, a parasite infecting Australian honeyeaters (Meliphagidae), with remarks on prevalence and potential cryptic speciation. Parasitol Res 114:1921-1928
14. Clark NJ, Clegg SM, Lima MR (2014) A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): New insights from molecular data. Int J Parasitol 44:329-338
15. Cramp S, Simmons KEL (1977) The birds of the Western Palearctic, vol. 1. Oxford University Press, Oxford, New York
16. de Mello IF (1935a) A contribution to the study of the blood parasites of some Indian birds. Proc Indian Acad Sci (Sec B) 1:349-358
17. de Mello IF (1935b) New haemoproteids of some Indian birds. Proc Indian Acad Sci (Sec B) 2:469-475
18. Dimitrov D, Zehtindjiev P, Bensch S, Ilieva M, Iezhova T, Valkiūnas G (2014) Two new species of *Haemoproteus* Kruse, 1890 (Haemosporida, Haemoproteidae) from European birds, with emphasis on DNA barcoding for detection of haemosporidians in wildlife. Syst Parasitol 87:135-151
19. Dinhopl N, Nedorost N, Mostegl MM, Weissenbacher-Lang C, Weissenböck H (2015) In situ hybridization and sequence analysis reveal an association of *Plasmodium* spp. with mortalities in wild passerine birds in Austria. Parasitol Res 114:1455-1462
20. Durrant KL, Beadell JS, Ishtiaq F, Graves GR, Olson SL, Gering E, Peirce MA, Milensky CM, Schmidt BK, Gebhard C, Fleischer RC (2006) Avian haematozoa in South America: a comparison of temperate and tropical zones. Ornithol Monogr 60:98-111
21. Ejiri H, Sato Y, Kim KS, Hara T, Tsuda Y, Imura T, Murata K, Yukawa M (2011) Entomological study on transmission of avian malaria parasites in a zoological garden in Japan: bloodmeal identification and detection of avian malaria parasite DNA from blood-fed mosquitoes. J Med Entomol 48:600-607
22. Fallon SM, Ricklefs RE, Swanson BL, Bermingham E (2003) Detecting avian malaria: an improved polymerase chain reaction diagnostic. J Parasitol 89:1044-1047
23. Freed LA, Cann RL (2006) DNA quality and accuracy of avian malaria PCR diagnostics: a review. Condor 108:459-473
24. Garnham PCC (1966) Malaria parasites and other Haemosporidia. Blackwell, Oxford
25. Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symp Ser (Oxf) 41:95-98

26. Hellgren O, Waldenström J, Bensch S (2004) A new PCR assay for simultaneous studies of *Leucocytozoon, Plasmodium,* and *Haemoproteus* from avian blood. J Parasitol 90:797-802
27. Jarvi SI, Schultz JJ, Atkinson CT (2002) PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. J Parasitol 88:153-158
28. Jovani R, Tella JL, Blanco G, Bertellotti M (2001) Absence of haematozoa on colonial White Storks *Ciconia ciconia* throughout their distribution range in Spain. Ornis Fennica 78:1-4
29. Levin II, Valkiūnas G, Iezhova TA, O'Brien SL, Parker PG (2012) Novel *Haemoproteus* species (Haemosporida: Haemoproteidae) from the swallow-tailed gull (Lariidae), with remarks on the host range of hippoboscid-transmitted avian hemoproteids. J Parasitol 98:847-854
30. Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol 47:261-273
31. Merino S, Hennicke J, Martínez J, Ludynia K, Torres R, Work TM, Stroud S, Masello JF, Quillfeldt P. (2012) Infection by *Haemoproteus* parasites in four species of frigatebirds and the description of a new species of *Haemoproteus* (Haemosporida: Haemoproteidae).
32. J Parasitol 98:388-397
33. Outlaw DC, Ricklefs RE (2014) Species limits in avian malaria parasites (Haemosporida): How to move forward in the molecular era. Parasitology 141:1223-1232
34. Peirce MA (1987) Synonymy of haemoproteids of Ciconiidae. J Parasitol 73:677
35. Peirce MA, Cooper JE (1977) Haematozoa of East African birds. IV. Redescription of *Haemoproteus crumenium*, a parasite of the marabou stork. E Afr Wildl J 15:169-172
36. Perkins SL (2014) Malaria's many mates: past, present and future of the systematics of the order Haemosporida. J Parasitol 100:11-25
37. Richard FA, Sehgal RNM, Jones HI, Smith TB (2002) A comparative analysis of PCR-based detection methods for avian malaria. J Parasitol 88:819-822
38. Rousselot R (1953) Parasites du sang des animaux. Notes de Parasitologie Tropicale. Part 1. Vigot frères, Paris
39. Sambrook J, Russell DW (2001) Molecular clonning: A laboratory manual. 3rd ed., Cold Spring Harbor, New York
40. Schaer J, Reeder DM, Vodzak ME, Olival KJ, Weber N, Mayer F, Matuschewski K,
41. Perkins SL (2015) *Nycteria* parasites of Afrotropical insectivorous bats. Int J
42. Parasitol 45:375-384
43. Telford SR (2009) The hemoparasites of the reptilian. CRC Press, Boca Raton
44. Tendeiro J (1947) Acerca dos hematozoários de algumas aves da Guiné Portuguesa. Rev Med Vet (Lisbon) 42:287-350
45. Vaitkuvienė D, Dagys M (2015) Two-fold increase in White Stork (*Ciconia ciconia*) population in Lithuania: a consequence of changing agriculture? Turk J Zool 39:144-152

46. Valkiūnas G (2005) Avian malaria parasites and other haemosporidia. CRC, Boca Raton
47. Valkiūnas G (2015) *Haemoproteus* species. In: H. Mehlhorn (ed.), Encyclopedia of Parasitology, 4th edn. Springer-Verlag, Berlin, Heidelberg
48. Valkiūnas G, Bolshakov CV, Chernetsov N, Kosarev V (2002) Lack of haematozoa in the White Stork *Ciconia ciconia* in the Kaliningrad Region of Russia. Avian Ecol Behav 6:63-66
49. Valkiūnas G, Iezhova TA, Križanauskienė A, Palinauskas V, Sehgal RNM, Bensch S (2008) A comparative analysis of microscopy and PCR-based detection methods for blood parasites. J Parasitol 94:1395-1401
50. Valkiūnas G, Palinauskas V, Ilgūnas M, Bukauskaite D, Dimitrov D, Bernotienė R, Zehtindjiev P, Ilieva M, Iezhova TA (2014) Molecular characterization of five widespread avian haemosporidian parasites (Haemosporida), with perspectives on the PCR-based detection of haemosporidians in wildlife. Parasitol Res 113:2251-2263
51. Wilson RJM, Williamson DH (1997) Extrachromosomal DNA in the Apicomplexa. Microbiol Mol Biol R 61:1-16
52. Zehtindjiev P, Križanauskienė A, Bensch S, Palinauskas V, Asghar M, Dimitrov D, Scebba S, Valkiūnas G (2012) A new morphologically distinct avian malaria parasite that fails detection by established polymerase chain reaction-based protocols for amplification of the cytochrome *b* gene. J Parasitol 98:657-665

PAPER IV

Plasmodium delichoni n. sp.: description, molecular characterisation and remarks on the exoerythrocytic merogony, persistence, vectors and transmission.

Valkiūnas, G., Ilgūnas, M., Bukauskaitė, D., Žiegytė, R., Bernotienė, R., Jusys, V., Eigirdas, V., Fragner, K., Weissenböck, H., Iezhova, T.A.

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Plasmodium delichoni n. sp.: Description, molecular characterisation and remarks on the exoerythrocytic merogony, persistence, vectors and transmission

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Abstract

Malaria parasite *Plasmodium (Novyella) delichoni* n. sp. (Haemosporida, Plasmodiidae) was found in a widespread Eurasian songbird, the common house martin *Delichon urbicum* (Hirundinidae). It is described based on the morphology of its blood stages and segments of the mitochondrial cytochrome *b* and apicoplast genes, which can be used for molecular identification of this species. Erythrocytic meronts and gametocytes are strictly nucleophilic, and mature gametocytes possess pigment granules of markedly variable size, including large ones (1 μm in length). Due to these features, *P. delichoni* can be readily distinguished from all described species of avian malaria parasites belonging to subgenus *Novyella*. Additionally, mature erythrocytic merozoites contain a dense clump of chromatin, a rare character in avian malaria parasites. Erythrocytic merogony is asynchronous. Illustrations of blood stages of the new species are given, and phylogenetic analysis identifies DNA lineages closely related to this parasite. Domestic canary *Serinus canaria* and Eurasian siskin *Carduelis spinus* were infected after subinoculation of infected blood obtained from the house martin. Parasitemia was long lasting in both these hosts, but it was high (up to 70%) in Eurasian siskins and low (up to 1%) in canaries. Mortality was not observed, and histological examination and chromogenic in situ hybridization did not reveal secondary exoerythrocytic meronts (phanerozoites) in the exposed birds. It is likely that persistence of this infection occurs due to long lasting parasitemia in avian hosts. Sporogony was abortive in mosquitoes *Culex pipiens pipiens* form *molestus*, *Culex quinquefasciatus* and *Aedes aegypti* at gametogenesis or ookinete stages. The new species is absent from juvenile birds at breeding sites in Europe, indicating that transmission occurs at African wintering grounds.

Keywords: *Plasmodium*; new species; birds; *Delichon urbicum;* molecular characterisation; vectors; persistence; transmission.

Introduction

Malaria parasites of the genus *Plasmodium* (Plasmodiidae, Haemosporida) are widespread in birds, and many species cause severe diseases in avian hosts (Zehtindjiev et al. 2008; Braga et al. 2011; Palinauskas et al. 2011). Lethal outbreaks of avian malaria have been described in wildlife (Gabaldon and Ulloa 1980; Stone et al. 1971; Atkinson et al. 2008). However, the patterns and consequences of malaria infections remain insufficiently understood in wildlife populations (Valkiūnas 2005; Glaizot et al. 2012; Marzal 2012; Garcia-Longoria et al. 2015; Marinov et al. 2015). Over 50 species of *Plasmodium* infecting birds have been named and described (Garnham 1966; Valkiūnas et al. 2008; Mantilla et al. 2013; Ilgūnas et al. 2013; Walther et al. 2014). Genetic diversity of these parasites is remarkable, indicating that many more species exist (Bensch et al. 2009; Clark et al. 2014; Perkins 2014; Outlaw and Ricklefs 2014). Knowledge about diversity of malaria parasites is crucial for better understanding evolutionary biology of *Plasmodium* parasites and epidemiology of avian malaria in wildlife.

As part of an on-going study on the diversity of avian malaria parasites and other haemosporidians (Dimitrov et al. 2014; Valkiūnas et al. 2014; Palinauskas et al. 2015), blood samples from migrating birds were collected at the Ventės Ragas Ornithological Station in Lithuania. One previously undescribed species of avian *Plasmodium* was found in a widespread Eurasian songbird, the house martin *Delichon urbicum*. This is a migratory insectivorous bird belonging to the Hirundinidae. It breeds in Europe, North Africa and temperate Asia and winters in sub-Saharan Africa and tropical Asia. The house martin tends to breed colonially and is found in both open country with low vegetation and near human habitation. It uses similar habitats both on the breeding and wintering areas (Snow and Perrins 1998). There are numerous reports of haemosporidian and other blood parasites in house martins, but *Plasmodium* spp. have only been incidentally reported in this bird species. Malaria parasites of subgenus *Novyella* have not been found in house martins and other European species of the Hirundinidae (Bennett et al. 1982; Bishop and Bennett 1992; Valkiūnas 2005).

Here, we describe a new species of malaria parasite infecting the house martin using data on the morphology of its blood stages and partial sequences of the mitochondrial and apicoplast genomes. Experiments were carried out to better understand the development of this pathogen in different avian hosts and its potential vectors. The aims of this study were (1) to provide morphological description of a new *Plasmodium* species, (2) to determine DNA sequences, which can be used for molecular characterisation and identification of this parasite, (3) to compare genetic distances and phylogenetic relationships of the new species with closely related *Plasmodium* parasites, (4) to determine experimental avian hosts and patterns of this infection development in these hosts, (5) to follow sporogonic development in experimentally infected widespread mosquito species.

Material and methods

Collection of blood samples

Fieldwork was carried out at the Ventės Ragas Ornithological Station, Lithuania in May, 2015. Twenty house martins and 29 barn swallows *Hirundo rustica* were caught with mist nets and large stationary traps. The blood was taken from birds by puncturing the brachial vein. About 30 µl of whole blood was taken in heparinized microcapillaries and stored in SET-buffer (Hellgren et al. 2004) for molecular analysis. Blood films were prepared from each bird immediately after withdrawal of the blood. They were air-dried, fixed in absolute methanol and stained with Giemsa (Valkiūnas 2005).

To find malaria infected birds and isolate parasite strains, blood films from each sampled bird were quickly examined microscopically in the field. For this purpose, one blood film was stained using 30% Giemsa solution for 15 min, air-dried using an electric fan and examined at high magnification for 3-5 min. That provided opportunities to determine malaria infected birds, collect infected blood and release the birds at the study site within one hour after their capture. One common house martin infected with undescribed *Plasmodium* sp. (parasitemia of 1.8%) was used for the new parasite strain isolation during this study. Infected blood was collected in heparinised microcapillaries and used to expose two uninfected domestic canaries *Serinus canaria* and two juvenile Eurasian siskins *Carduelis spinus.* These birds were exposed by subinoculation of about 250 µl of freshly prepared mixture, containing infected blood, 3.7% sodium citrate (anticoagulant) and 0.9% saline (4:1:5) into their pectoral muscle (Iezhova et al. 2005). Before the subinoculation experiments, all recipient birds were proved to be uninfected with haemosporidian parasites by microscopic examination of blood films and later by PCR-based testing in the laboratory (see below). The donor house martin was released after obtaining infected blood.

The observation time for all experimental birds was 172 days post exposure (dpe). Blood was taken for microscopic examination and PCR-based testing as described above once per 3 or 4 days during the first month post exposure, once per a week during the second month post exposure, and once per 1-2 weeks during reminder experiment time. Additionally, blood of one Eurasian siskin was tested 241 dpe. All birds were kept indoors in a vector-free room under controlled conditions (20 ± 1 °C, 50-60% relative humidity (RH), the natural light-dark photoperiod (L/D)) and were fed a standard diet for seed-eating bird species. Parasitemia developed in all exposed canaries and Eurasian siskins. Blood samples with intense parasitemia (>10%) were cryopreserved for future research, as described by Garnham (1966).

Histological examination of bird organs

Three exposed birds (two canaries and one Eurasian siskin) were decapitated 172 dpe. Parasitemia was 0.0001% and 0.04% in the canaries and the Eurasian siskin, respectively. Organs (brain, heart, kidneys, liver, lungs, spleen, bone marrow) and pieces of the pectoral muscles of these birds were dissected, fixed in 10% neutral formalin solution and embedded in paraffin. Histological sections of 4 µm were obtained and stained with hematoxylin-eosin (Valkiūnas 2005). Preparations were examined microscopically (see below).

Chromogenic in situ hybridization

Chromogenic in situ hybridization was performed using the same tissue samples, which were used for histological examination. We followed a previously published protocol by Dinhopl et al. (2011). In brief, 3 µm paraffin wax-embedded tissue sections were de-waxed and rehydrated. They were subjected to proteolytic treatment with proteinase K (Roche, Basel, Switzerland) 6 µg/ml in Tris-buffered saline at 37 °C for 50 min. Then, the slides were washed in distilled water and dehydrated in 96 % ethanol and 100 % isopropanol followed by air-drying. Afterwards, the slides were incubated overnight at 40 °C with hybridization mixture, 100 µl of which was composed of 50 µl formamide (50 %), 20 µl 20× standard sodium citrate (SSC), 12 µl distilled water, 10 µl dextran sulphate (50 %, w/v), 5 µl herring sperm DNA (50 mg/ml), 2 µl Denhardt's solution and 1 µl *Plasmodium* sp. probe at a concentration of 100 ng/ml.

The oligonucleotide probe (sequence: 5′-TTTAATAACTCGTTATATATATCAGTGTAGCAC-3′) was labelled with digoxigenin at the 3′ end (Eurofins MWG Operon, Ebersberg, Germany). It is specific to detect avian *Plasmodium* spp. (Dinhopl et al. 2015).

The next day, the slides were washed in decreasing concentrations of SSC (2× SSC, 1× SSC and 0.1× SSC, 10 min each at room temperature (RT)), for removal of unbound probe. Then, the slides were incubated with antidigoxigenin-AP Fab fragments (Roche) (1:200) for 1 h at RT. The hybridized probe was visualized subsequent to an additional washing step using the colour substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) (Roche). After 1 h of incubation, the colour development was stopped using TE buffer (pH 8.0) for 10 min. Finally, the slides were counterstained with haematoxylin and mounted under coverslips with Aquatex (VWR International, Vienna, Austria).

Maintenance and experimental infection of laboratory reared mosquitoes

To establish a colony of *C. p. pipiens* f. *molestus*, we used the mosquito larvae, which were obtained from Dr. Roland Kuhn. The colony was originally started from larvae collected in Hesse Region (Germany). Colonies of *C. quinquefasciatus* and *Aedes aegypti* mosquitoes were established using eggs provided by Dr. Ana Rivero (France) and Dr. Hilary Ranson (UK), respectively. All mosquito colonies have been in continuous culture for many years. We colonised these insects, as described by Žiegytė et al. (2014). Briefly, mosquitoes were kept in cages (120×45×45 cm) under standard conditions (65-70% RH and L/D photoperiod of 14/8 h). Because sporogonic development of *Plasmodium* parasites is sensitive to air temperature (Garnham 1966; Sherman 1998), two temperature conditions were used: one infected group of each species was maintained at 21±1 °C and another infected group at 26±1 °C. Adult mosquitoes were fed with 5-10% saccharose solution. Cotton wool pads moistened with this solution were provided in mosquito cages.

Two days before exposure, approximately 30 unfed females of each species were haphazardly chosen and placed inside separate experimental cages of (45×45×45cm). To increase favour of blood feeding, the experimental mosquitoes were deprived of saccharose. Eurasian siskins with gametocytemia ranging between 0.1% and 2% were placed in mosquito cages and exposed, as described by Kazlauskienė et al. (2013). Briefly, infected birds were

placed in plastic tubes (length 15 cm, diameter 5 cm) containing a rip, which was used to fix the bird legs. Both tube ends were covered with bolting silk. Only legs were exposed to mosquito bites. The birds were kept in insect cages for approximately 15-20 min once per 3-4 days. All mosquito species willingly took blood meals on bird legs. We evaluated parasitemia in all donor birds immediately after mosquito blood meals. Engorged females were taken from the experimental cages using an aspirator, placed in separate small insect cages (12×12×12 cm), maintained at the same conditions as their colonies to allow development of parasites, and dissected in intervals (see below). Experiments with all mosquito species were carried out in parallel.

Ookinete preparations (n=20) were made 1–3 dpe, oocysts preparations (n=39) - 6–14 dpe, and sporozoites preparations (n=73) - 13–25 dpe. In total, we infected and dissected 132 mosquitoes. Among them were: 37 females of *C. p. pipiens* f. *molestus* (20 individuals were maintained at 21±1 °C and 17 individuals at 26±1 °C), 46 females of *C. quinquefasciatus* (28 and18 individuals, respectively), and 49 females of *A. aegypti* (26 and 23 individuals, respectively).

Dissection of mosquitoes and making preparations of ookinetes, oocysts and sporozoites

All mosquitoes were processed individually. Before dissection, insects were lightly anesthetized by putting them into a tube closed with a cotton pad wetted in 96% ethanol for several minutes. Wings and legs of the insects were removed before dissection, which was performed under the binocular stereoscopic microscope. To eliminate contamination of samples, we either used new dissecting needle for each dissected insect or disinfected the needles in fire after each dissection.

Permanent preparations of the semi-digested midgut contents, entire midguts, and salivary glands were prepared in order to observe ookinetes, oocysts and sporozoites, respectively. All preparations were prepared according to Kazlauskienė et al. (2013). Remnants of all dissected insects were also fixed in 96% ethyl alcohol for PCR-based screening, which was used to confirm presence of corresponding lineage of parasite.

DNA extraction, PCR, sequencing and phylogenetic analysis

Total DNA was extracted from blood and insect samples using the standard ammonium-acetate protocol (Sambrook and Russell 2001) with minor modification: instead of 250 µl of fixed blood, we used 125 µl. Quantification of the DNA was performed by using nanodrop spectrophotometer (IMPLEN Nanophotometer P330). All reactions were performed in 25 µl total volumes, including 50 ng of a total genomic DNA template (2 µl), 12.5 µl of DreamTag Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 µl nuclease-free water and 1 µl of each primer. One negative control (nuclease-free water) and one positive control (an infected sample, which was positive by microscopic examination of blood films) were used per every 7 samples to control for false amplifications. No cases of false positive or negative amplifications were found.

The 479 bp length segments of parasite mitochondrial cyt *b* gene were amplified using nested PCR protocol (Bensch et al. 2000; Hellgren et al. 2004) and two pairs of primers: HaemNFI/HaemNR3, which amplify gene fragments of haemosporidians belonging to genera

Haemoproteus, Plasmodium and *Leucocytozoon*, and the primers HAEMF/HAEMR2, which are specific to *Haemoproteus* and *Plasmodium* spp. The ''Basic Local Alignment Search Tool'' (National Centre for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/BLAST) was used to determine cyt *b* lineages of detected DNA sequences. The primers ClpcF/R (outer) and ClpcF/R (nested) were used for the amplification of apicoplast gene fragments of 536 bp length (Martinsen et al. 2008).

Temperature profiles in all PCRs' were the same as in the original protocol descriptions. All amplifications were evaluated by running 2 μl of the final PCR products on a 2% agarose gels. Fragments of DNA from all positive amplifications were sequenced with corresponding primers for both strands. The obtained sequences were aligned and analysed using Bioedit program (Hall 1999).

Visualization of "double bases" in electropherograms of sequences was used to estimate presence of possible haemosporidian co-infections in wild-caught and experimentally exposed birds (Pèrez-Tris and Bensch 2005). Sequence divergence among lineages was calculated with MEGA version 5, using a Jukes-Cantor model of substitution in which all substitutions were weighted equally (Tamura et al. 2011).

For phylogenetic analysis, we downloaded haemosporidian parasite cyt *b* sequences from the MalAvi database (http://mbio-serv2.mbioekol.lu.se/Malavi/). GenBank accession numbers of sequences and codes of the parasite lineages are given in Fig. 1. The phylogenetic tree was constructed using mrBayes version 3.1 (Ronquist and Huelsenbeck 2003). The appropriate model of sequence evolution was determined by the software mrModeltest version 2.3 (Nylander 2008) to be a General Time Reversible model including variable sites and gamma-shaped rate variation across sites (GTR+I+G). Phylogenetic analyses were conducted using PAUP version 4 (Swofford 2002). Visualization of the phylogenetic tree was conducted using TreeView 1.6.6 (software available at http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

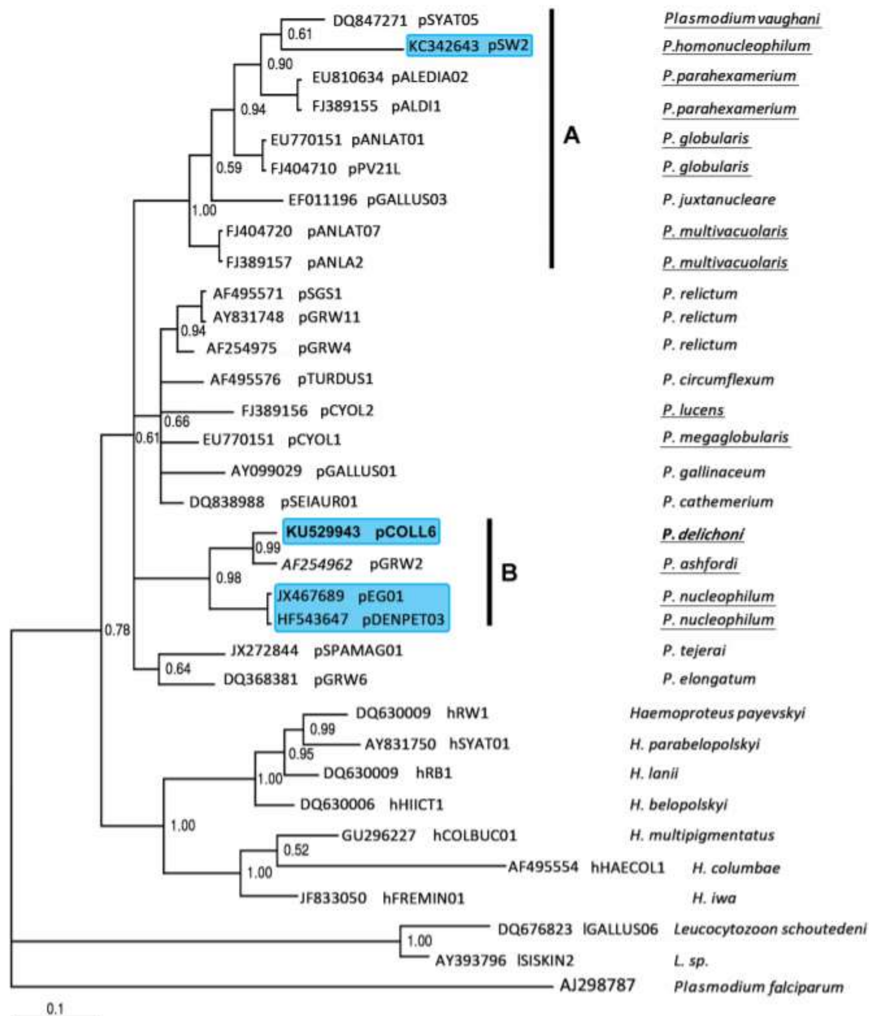

Fig. 1 Bayesian phylogeny of 33 mitochondrial cytochrome *b* gene lineages of avian haemosporidian parasites. GenBank accession numbers and codes of the lineages are given according to the MalAvi database (http://mbio-serv2.mbioekol.lu.se/Malavi/index.html). Information about *Plasmodium (Novyella) delichoni* sp. nov. (lineage pCOLL6) is given in bold, and information related to *Plasmodium* parasites with nucleophilic blood stages is provided in boxes. Names of parasites belonging to subgenus *Novyella* are underlined. Nodal support values near branches show posterior clade probabilities. *Vertical bars* indicate clades (*A, B*), in which parasite species with nucleophilic blood stages are present

Microscopic examination of blood, vector and histological preparations

Detailed microscopic analysis was carried out in the laboratory. An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides, to prepare illustrations, and to take measurements. Approximately 100-150 fields were examined in blood films at low magnification (400×), and then at least 100 fields were studied at high magnification (1000×). Intensity of parasitemia was estimated as a percentage by actual counting of the number of parasites per 1000 erythrocytes or per 10000 erythrocytes if infections were light (< 0.1%). All vector and histological preparations were first examined at low magnification (600×) and then at high magnification (1000×). The morphometric features studied (Table 1) were those defined by Valkiūnas (2005). The analyses were carried out using the 'Statistica 7' package. Student's *t*-test for independent samples was used to determine statistical significance between mean linear parameters of parasites. A *P* value of 0.05 or less was considered significant.

Table 1 Morphometry of host cells, mature gametocytes and erythrocytic meronts of *Plasmodium delichoni* (lineage pCOLL6) (n=21)

Feature	Measurements (μm) [a]
Uninfected erythrocyte	
Length	10.5-12.9 (11.8±0.7)
Width	5.4-6.9 (6.2±0.4)
Area	50.5-66.4 (58.7±4.1)
Uninfected erythrocyte nucleus	
Length	4.9-6.1 (5.6±0.4)
Width	1.8-2.4 (2.1±0.2)
Area	8.0-12.0 (9.4±1.1)
Macrogametocyte	
Infected erythrocyte	
Length	10.5-13.7 (12.3±0.8)
Width	5.1-6.8 (5.7±0.4)
Area	50.7-70.7 (58.1±5.1)
Infected erythrocyte nucleus	
Length	4.8-6.3 (5.5±0.5)
Width	1.8-2.3 (2.1±0.1)
Area	7.8-11.0 (9.5±0.9)
Gametocyte	
Length	10.0-12.7 (11.1±0.7)
Width	1.4-2.3 (1.8±0.2)
Area	16.6-27.8 (21.1±2.8)
Gametocyte nucleus [b]	
Length	-
Width	-
Area	-
Pigment granules	4.0-10.0 (7.4±1.5)
Microgametocyte	
Infected erythrocyte	
Length	11.3-14.0 (12.7±0.7)
Width	5.0-6.8 (5.8±0.4)
Area	49.7-68.7 (60.8±5.7)
Infected erythrocyte nucleus	

Length	5.0-6.3 (5.6±0.3)
Width	1.8-2.4 (2.0±0.2)
Area	8.5-11.4 (9.8±0.8)
Gametocyte	
Length	10.0-12.5 (10.9±0.7)
Width	1.2-2.5 (1.8±0.3)
Area	16.4-24.8 (19.8±2.5)
Gametocyte nucleus	
Length	1.5-3.2 (2.2±0.5)
Width	0.5-1.5 (0.9±0.3)
Area	1.0-2.6 (1.8±0.5)
Pigment granules	3.0-9.0 (6.5±1.7)
Meront	
Length	2.4-4.7 (3.8±0.6)
Width	1.3-2.7 (1.9±0.4)
Area	3.9-8.3(5.3±0.9)
No. of pigment granules [c]	-
No. of merozoites	5.0-8.0 (6.1±0.8)

[a] Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.
[b] Gametocyte nuclei are ill-defined and difficult to measure.
[c] Pigment granules often are clumped and difficult to calculate.

Ethical statement

Experimental procedures of this study were approved by the Ethical Commission of the Baltic Laboratory Animal Science Association, Lithuania; Lithuanian State Food and Veterinary Office (permit 2015-05-07, no. G2-27); Environmental Protection Agency, Vilnius (permits 2015-04-08, no. 21 and 2015-04-27, no. 25); the International Research Co-operation Agreement between the Zoological Institute of the Russian Academy of Sciences and Institute of Ecology of Nature Research Centre (25-05-2010). All efforts were made to minimize handling time and potential suffering of animals.

Results

Description of parasite

***Plasmodium (Novyella) delichoni* n. sp.**

Type host: The house martin *Delichon urbicum* (Passeriformes, Hirundinidae).
DNA sequences: Partial mitochondrial cyt *b* and apicoplast gene sequences (GenBank assession nos. KU529943 and KU530116, respectively).
Additional hosts: The barn swallow *Hirundo rustica* (Hirundinidae) and collared flycatcher (*Ficedula albicollis*) (Table 2).

Table 2 Avian hosts and countries where the pCOLL6 lineage of *Plasmodium delichoni* n. sp. has been reported

Order and family	Species	Country	Reference
Passeriformes			
Hirundinidae	*Delichon urbicum*[a]	Lithuania	This study
	D. urbicum[b]	Netherlands	Piersma et al. 2012
	Hirundo rustica[a]	Lithuania	This study
Muscicapidae	*Ficedula albicollis*[b]	Hungary	Szőllősi et al. 2009
	F. albicollis[b]	Spain	Pérez-Tris et al. 2007

[a] Reports based both on microscopic and PCR-based diagnostics.
[b] Reports based solely on PCR-based diagnostics.

Vectors: Unknown. Experimentally infected mosquitoes *C. p. pipiens* f. *molestus, C. quinquefasciatus* and *A. aegypti* were resistant. Numerous remnants of blood stages were seen in mosquito midgut contents of each species, and a few degrading ookinetes were observed in mosquitoes *A. aegypti* and *C. p. pipiens* f. *molestus* (Fig. 2). Oocysts did not develop and sporozoites were not seen in salivary glands. Sporogonic development is abortive on gametogenesis or ookinete stages in all exposed mosquito species.

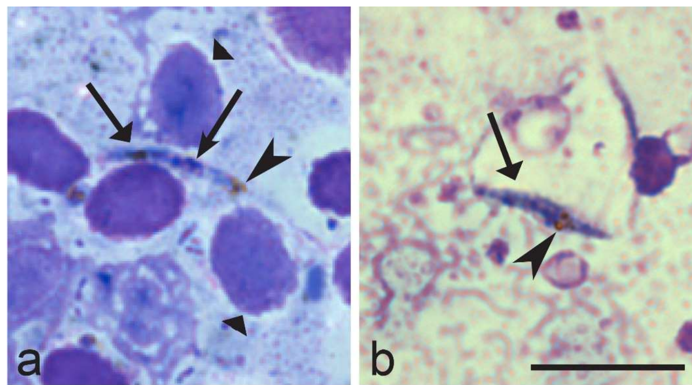

Fig. 2 Degrading ookinetes of *Plasmodium (Novyella) delichoni* sp. nov. (lineage pCOLL6) from the midgut contents of mosquitoes *Aedes aegypti* (**a**) and *Culex pipiens pipiens* form *molestus* (**b**). Long triangle arrows – ookinetes; long simple arrows – nuclei of parasites; simple arrowheads – pigment granules; triangle wide arrowheads – nuclei of semi-digested red blood cells. Giemsa-stained thin blood films. Scale bar = 10 µm

Type locality: Ventės Ragas, Šilutė District, Lithuania (55°20'28.1" N, 21°11'25.3"E).
Prevalence: As reported by PCR-based testing and microscopic examination of blood films, the overall prevalence was 12.2% in house martins and barn swallows. In all, 25.0% and 3.5% of examined house martins and barn swallows were infected, respectively.
Distribution: P. delichoni (pCOLL6) has been reported in adult house martins, barn swallows and collared flycatchers (*Ficedula albicollis*) in Europe after arrival from African wintering grounds (Table 2), but is absent from juvenile birds of these species, indicating that transmission occurs away from the European breeding grounds.
Site of infection: Mature red blood cells. No other data.

Type specimens: Hapantotype (accession numbers 48920 NS and 48921 NS, parasitemia of 1.8%, *Delichon urbicum*, Ventės Ragas, Lithuania, collected by G. Valkiūnas, 17 May 2015) was deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (the first blood passage from the type hosts *D. urbicum* to Eurasian siskin *C. spinus*, parasitemia of approximately 2%, 16 June 2015) were deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania and in the Queensland Museum, Queensland, Australia (accession nos. G466182, G466183).
Additional material: Blood films from experimentally exposed canary *S. canaria* (accession nos. 48926, 48927 NS) and Eurasian siskin *C. spinus* (accession nos. 48928-48935 NS), cryopreserved infected blood (specimen accession nos. 10/15Rcc, 11/15Rcc), and whole blood samples fixed in SET buffer (specimen accession nos. 158/15R, 287/15V, 288/15V, 291/15V, 346/15V) were deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania.
Etymology: The species name is derived from the genus name *Delichon* to which the type host of this parasite belongs.
Exoerythrocytic meronts: Phanerozoites were not found both by histological and chromogenic in situ hybridization methods in two canaries and one Eurasian siskin that were examined 172 dpe.
Parasitemia: Prepatent period varied between 15 and 19 dpe in different individual avian hosts. All blood stages were seen in the peripheral circulation during entire observation time (over 8 months).

Intense parasitemia developed in two exposed Eurasian siskins. Maximum parasitemia reached 26.4% and 70.0% in different individual Eurasian siskins 36 dpe and 46 dpe, respectively. Watery blood symptoms were readily visible during peak of parasitemia. Both exposed Eurasian siskins survived acute parasitemia, which lasted for approximately two weeks and then decreased to chronic levels. Light parasitemia (maximum 1%) developed in two exposed canaries, and it remained at low level (≤0.1%) during this study. Erythrocytic merogony is asynchronous, resulting in presence of all blood stages at any stage of parasitemia. All exposed birds survived to the end of this study.
Trophozoites (Fig. 3a): Develop in mature red blood cells. Outline is wavy or slightly amoeboid. Earliest trophozoites were seen anywhere in the cytoplasm of infected erythrocytes. As parasite develops, trophozoites attach to the host cell nuclei and usually are seen in a polar or subpolar position in relation to the nuclei (Fig. 3a); they often are difficult to visualize and can be overlooked in blood films due to small size and tight attachment to the host cell nuclei. One or two tiny pigment granules are present. Influence of parasites on infected erythrocytes is not pronounced.
Erythrocytic meronts (Figs. 3b-e, 4a-c): Develop in mature red blood cells and are closely appressed to the nuclei of infected erythrocytes from the stage of binuclear parasites to their complete maturity. Young meronts adhere to the nuclei of erythrocytes near one of their poles (Fig. 3b,c). Advanced meronts usually maintain the same position in host cells (Figs. 3d, 4a-c), but a few mature meronts were seen in sub-lateral position to the host cell nuclei (Fig. 3e). The cytoplasm is scanty, pale stained. Fully-grown meronts are of variable form, and the roundish, oval, irregular and even slightly elongate in shape parasites were seen. Nuclei usually distributed disorderly in fully-grown meronts (Figs. 3d,e, 4a-c). One refractive globule was seen

in many growing meronts (Fig. 3c), but the globules were not visible in completely mature meronts (Figs. 3e, 4a-c). The majority of meronts (over 60%) produce 6 merozoites, but meronts containing 4, 5, 8 or even 10 merozoites present. Pigment granules are of small size, dark, clumped in one or two small groups or aggregated in solid masses in mature meronts (Fig. 4b,c). Meronts usually do not influence infected erythrocytes, but largest parasites can slightly displace the nuclei of erythrocytes laterally (Fig. 3e). Segmenting meronts are numerous in the peripheral blood (Figs. 3e, 4c). Mature merozoites are approximately 1 μm in diameter; they possess a readily visible prominent nucleus, but poorly visible cytoplasm (Figs. 3e, 4a-c). Some merozoites remain attached to the host cell nuclei even after segmentation of meronts (Fig. 4c). The majority of merozoites contain a readily visible clump of chromatin (Figs. 3d, 4a-c), which is a distinctive feature of this parasite.

Macrogametocytes (Fig. 3f-m): Develop in mature red blood cells. The cytoplasm stains pale-blue, slightly granular in appearance, often contains several small vacuoles. Earliest gametocytes are elongated, slightly amoeboid in outline, usually seen in polar positions in erythrocytes (Fig. 3f). Growing gametocytes assume lateral positions to the nuclei of infected erythrocytes, and they are closely appressed to the nuclei (Fig. 3g,h). As parasite develops, gametocytes remain strictly nucleophilic (Fig. 3h-m), a characteristic feature of this species development. Growing gametocytes do not touch envelop of erythrocytes along their entire margin (Fig. 3g-k), but many fully-grown gametocytes are associated with envelope of the host cells (Fig. 3m). Gametocyte outline vary from slightly irregular (Fig. 3h,i,m) to slightly amoeboid (Fig. 3g,j). Parasite nucleus is of variable form, pale stained, resulting in its unclear boundaries, usually sub-terminal in position (Fig. 3i,l); it contains a readily distinguishable roundish nucleolus (Fig. 3k,m). Pigment granules are dark, usually grouped (Fig. 3h), roundish and oval in form, markedly variable in size, a characteristic feature of this parasite species. Small and average size (<1 μm) pigment granules predominate, but large (1 μm and bigger) elongate granules often present (Fig. 3j,k). Influence of parasites on infected erythrocytes usually is not pronounced.

Microgametocytes (Fig. 3n-p). General configuration is as for macrogametocytes with the usual haemosporidian sexual dimorphic characters. Parasite nucleus is diffuse, centrally located, often ill-defined and difficult to measure.

Specificity: Eurasian siskins and domestic canaries are susceptible and are good experimental hosts, in which long-lasting parasitemia develops. Reports of this parasite in naturally infected birds are given in Table 2.

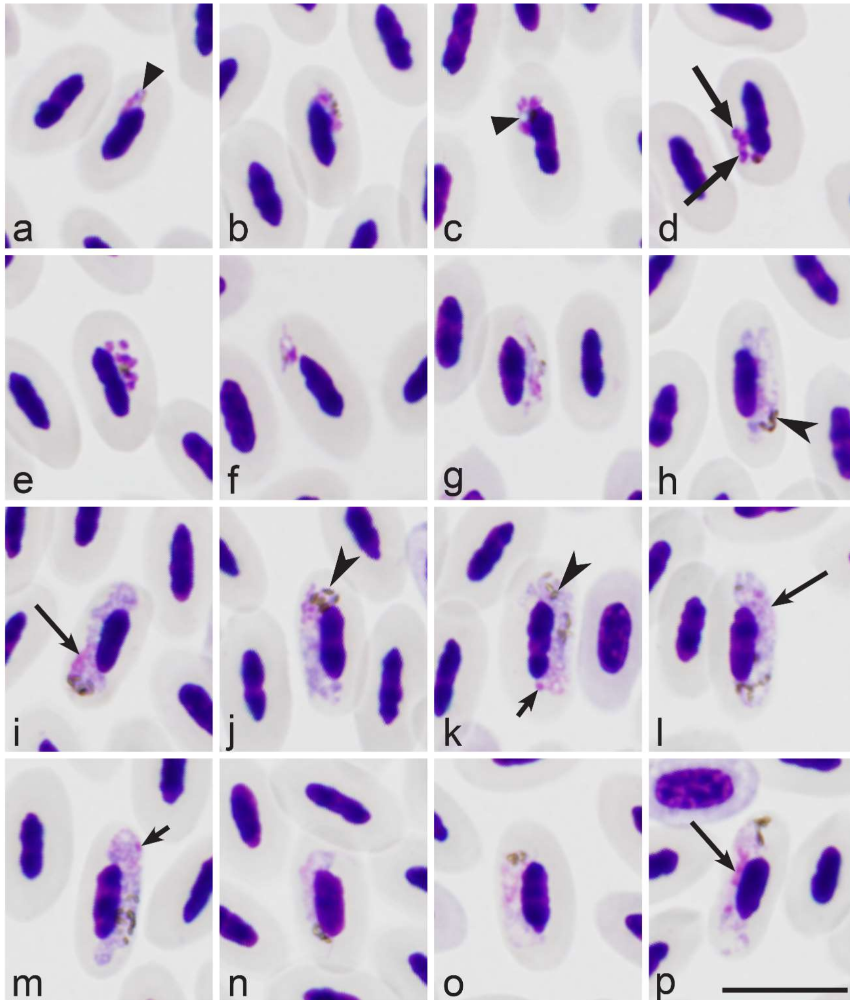

Fig. 3 *Plasmodium (Novyella) delichoni* sp. nov. (lineage pCOLL6) from the blood of house martin *Delichon urbicum:* **a** trophozoite; **b-e** erythrocytic meronts; **f** young gametocyte; **g-m** macrogametocytes; **n-p** microgametocytes. Long simple arrows – nuclei of parasites; short simple arrows – nucleoli; simple arrowheads – pigment granules; triangle arrowheads – blue, refractive globule; long triangle arrowheads – clumps of chromatin in nuclei of mature merozoites. Giemsa-stained thin blood films. Scale bar = 10 µm

Taxonomic remarks

Plasmodium delichoni sp. nov. belongs to subgenus *Novyella*, which species are characterised by presence of (1) elongate gametocytes, 2) small fully grown erythrocytic meronts, which size does not exceed or only slightly exceed that of the nuclei of infected erythrocytes, and (3) the scanty cytoplasm in erythrocytic meronts (Garnham 1966; Valkiūnas 2005). Due to strictly

nucleophilicity of blood stages (Figs. 3,4), the new species can be readily distinguished from all described *Novyella* species, except *Plasmodium nucleophilum* (Manwell 1935; Chagas et al. 2013), *Plasmodium paranucleophilum* (Manwell and Sessler 1971b) and *Plasmodium homonucleophilum* (Ilgūnas et al. 2013). *Plasmodium delichoni* can be distinguished from these parasites due to the presence of (1) large (≥1 μm in length) pigment granules in its gametocytes (Fig. 3j,k) and (2) distinct clumps of chromatin in nuclei of mature erythrocytic merozoites (Fig. 4a-c). Additionally, (1) gametocytes of *P. homonucleophilum* are not strictly nucleophilic, and the gametocytes, which do not adhere to the erythrocyte nuclei, are common (Ilgūnas et al. 2013), (2) mature erythrocytic meronts of *P. nucleophilum* often displace nuclei of infected erythrocytes (Manwell 1935), and (3) mature both gametocytes and erythrocytic meronts of *P. paranucleophilum* also displace nuclei of infected red blood cells (Manwell and Sessler 1971b). These features are not characteristics of *P. delichoni* (Figs. 3, 4).

It is important to note that main diagnostic morphological characters of blood stages observed in the type host, the house martin, maintained during the first passage of this parasite in domestic canaries and Eurasian siskins, but erythrocytic meronts producing 8 merozoites (Fig. 4c) were more often seen in domestic canaries and Eurasian siskins than in the house martin.

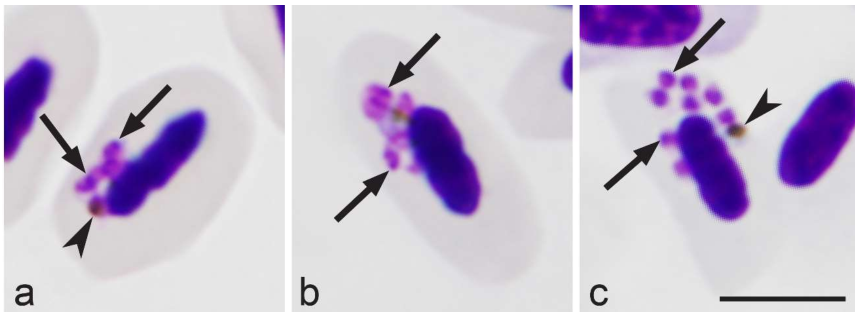

Fig. 4 Mature erythrocytic meronts of *Plasmodium (Novyella) delichoni* sp. nov. (lineage pCOLL6) from the blood of house martin *Delichon urbicum* (**a**), domestic canary *Serinus canaria* (**b**) and Eurasian siskin *Carduelis spinus* (**c**). Simple arrowheads – pigment granules; long triangle arrowheads – clumps of chromatin in nuclei of mature merozoites. Giemsa-stained thin blood films. Scale bar = 10 μm. Note that clumps of chromatin are readily visible in merozoites developed in different species of avian hosts, indicating that this is a useful taxonomic character of the new species

Phylogenetic relationships of parasites

Partial DNA sequences of cyt *b* gene are available for three species of *Plasmodium* with nucleophilic blood stages, i. e. *P. homonucleophilum*, *P. nucleophilum* and *P. delichoni.* Genetic difference in cyt *b* gene among them is large (between 5.6% and 9.8%), indicating that these species are valid. The three *Plasmodium* spp. appeared in different well-supported clades and are paraphyletic in our phylogenetic analysis (Fig. 1, clades a,b), indicating either the independent evolution of nucleophilicity of blood stages in these parasites or the insufficient sensitivity of phylogenies based on partial sequences of the cyt *b* gene in resolving the origin

of this character in avian malaria parasites. In this relation, it is important to note that 10 species of subgenus *Novyella* were used in our phylogenetic analysis, and they also are paraphyletic in the phylogenetic tree (Fig. 1). Additionally, the new species appeared to be particularly closely related to *Plasmodium ashfordi* (Fig. 1, clade b), which both gametocytes and erythrocytic meronts are non-nucleophilic. These two parasites present in the same well-supported clade (Fig. 1, clade b), and genetic distance between their cyt *b* gene sequences is 2.8%. The reported controversies between the morphological characters and the phylogeny based on partial cyt *b* gene sequences show that multigene phylogenetic analysis is needed for the better understanding evolutionary relationships among species of *Novyella* and other avian malaria parasite. However, such analysis is currently premature due to insufficient information about DNA sequences of other genes in avian *Plasmodium* spp.

Plasmodium delichoni appeared in the same clade with *P. nucleophilum* (Fig. 1, clade b), indicating possible close phylogenetic relationships between these two parasites, in which both gametocytes and erythrocytic meronts are strictly nucleophilic. The genetic distance in cyt *b* gene sequences between *P. delichoni* and *P. nucleophilum* is large (5.7%).

Discussion

This study reports a new species of avian malaria parasites, which blood stages (gametocytes, advanced trophozoites and erythrocytic meronts) are closely appressed to the nuclei of infected erythrocytes (Figs. 3, 4). This finding deserves attention because the strict nucleophilicity is not characteristic of the majority of described avian *Plasmodium* species (Hewitt 1940; Garnham 1966; Valkiūnas 2005; Mantilla et al. 2013; Walther et al. 2014). However, this feature has been reported in many other species of related haemosporidian parasites. For example, the majority of bird *Haemoproteus* species (the sister genus to *Plasmodium*) possesses more or less nucleophilic gametocytes, which are appressed to the host cell nuclei from early stages of their development in red blood cells (Desser and Bennett 1993; Valkiūnas 2005; Atkinson et al. 2008; Mehlhorn 2015). Interestingly, all described species of haemosporidians belonging to the genus *Leucocytozoon* develop in close adherence to nuclei of their host cells, which are often enlarged and/or deformed by developing both gametocytes and exoerythrocytic meronts (Desser and Bennett 1993; Forrester and Greiner 2008; Valkiūnas 2005; Lotta et al. 2015). Effects on the host cell nuclei (various modes of the nuclear displacement, atrophy, hypertrophy and even the host cell enucleation) are well expressed in many avian haemosporidian parasites, and such effects are particularly well-evident during development of *Leucocytozoon* sp. megalomeronts. The latter parasites induce remarkable increase in amounts of (1) the chromatin in nuclei of infected cells and (2) endoplasmic reticulum as wells as the number of mitochondria in the cytoplasm of the cells (Desser 1970; Wong and Desser 1978, Desser and Bennett 1993; Valkiūnas 2005; Forrester and Greiner 2008). That indicates the active participation of the host cells in the metabolism process of the parasites. The biological meaning of nucleophilicity remains insufficiently investigated in haemosporidian parasites, and its molecular mechanisms remain unknown. *Plasmodium delichoni* and domestic canary offer a convenient study system for research on mechanisms of nucleophilicity in parasitic protists because this host-parasite association is easy and relatively inexpensive to maintain in laboratory conditions.

Only sporozoites induce exoerythrocytic merogony and formation of hypnozoites in malaria parasites of mammals (Sherman 1998). In avian malaria, merozoites from erythrocytic meronts can induce development of the secondary exoerythrocytic meronts, which are known as phanerozoites (Garnham 1980; Valkiūnas 2005). Due to high and long-lasting parasitemia of *P. delichoni*, we expected to find phanerozoites in the exposed birds, but they were not seen in histological sections of internal organs in the canaries and Eurasian siskin. Chromogenic in situ hybridization using *Plasmodium* spp. specific oligonucleotide probes is a more sensitive method for detection of tissue stages, providing opportunities to visualize exoerythrocytic meronts of malaria parasites even during light intensity in organs (Dinhopl et al. 2011, 2015). Because both the histological and chromogenic in situ hybridization tools showed the same negative results, it is probable that phanerozoites do not develop in *P. delichoni*. Malaria parasites of subgenus *Novyella* have been insufficiently investigated from the point of view of exoerythrocytic merogony. Mainly blood stages have been described in species of this subgenus (Garnham 1966; Valkiūnas 2005; Atkinson et al. 2008; Mantilla et al. 2013).

Phanerozoites were reported in *P. (Novyella) nucleophilum toucani* (Manwell and Sessler 1971a; Valkiūnas 2005), *P. (Novyella) paranucleophilum* (Manwell and Sessler 1971b), *Plasmodium (Novyella) bertii* (Gabaldon and Ulloa 1981) and *Plasmodium (Novyella) vaughani* (Nelson 1966; Garnham 1966; Valkiūnas 2005). However, phanerozoites were absent from birds subinoculated with blood infected by *Plasmodium (Novyella) columbae* (Gabaldon and Ulloa 1976), *Plasmodium (Novyella) rouxi* (Garnham 1966), *Plasmodium (Novyella) hexamerium* (Manwell 1951), and *Plasmodium (Novyella) kempi* (Christensen et al. 1983); that is in accord with this study. It seems probable that phanerozoites do not develop in some *Novyella* parasites.

Phanerozoites are responsible for multiplication of parasites and also are involved in persistence of malaria infection in avian hosts (Garnham 1980; Valkiūnas 2005; Atkinson et al. 2008). Persistence of *P. delichoni* and some other *Plasmodium* spp. might occur due to long-lasting light parasitemia, as is the case in human malaria parasite *Plasmodium malaria* (Sherman 1998). Warm climates and resulting long period of transmission in sub-Saharan Africa, which is the main wintering area of European species of the Hirundinidae, might contribute to evolution of such mode of avian malaria persistence.

Plasmodium relictum and related species of avian malaria parasites of subgenus *Haemamoeba* survive cold seasons of a year, which are unfavourable periods for mosquito transmission, mainly at the stage of persisting phanerozoites (Garnham 1980; Valkiūnas 2005; Atkinson et al. 2008). However, phanerozoites might markedly damage bird organs, and they often are main reason of mortality in avian hosts (Garnham 1980; Dinhopl et al. 2015; Palinauskas et al. 2015). Because lack of phanerozoites leads to less tissue damage and milder parasite virulence, which was the case in *P. delichoni*, the persistence by means of long-lasting light parasitemia should be evolutionary advantageous due to survival of greater number of infected avian hosts, which then can serve as donors of gametocytes to infect mosquitoes. It is worth mentioning that numerous *Novyella* species have been described from African and South American birds (Valkiūnas et al. 2009; Chagas et al. 2013; Ilgūnas et al. 2013; Mantilla et al. 2013; Walther et al. 2014), in which parasites of this subgenus predominate (Valkiūnas 2005; Loiseau et al. 2012), but exoerythrocytic merogony and modes of persistence remain unknown in these infections. This study shows that mechanisms of persistence might vary in different

species of avian malaria parasites. Comparative research of tissue merogony of *Plasmodium* species transmitting in countries with warm and cold climates is needed for better understanding patterns of exoerythrocytic merogony and mechanisms of persistence of avian malaria parasites.

Numerous studies reported *Plasmodium* and *Haemoproteus* parasites in house martins and barn swallows using both microscopic examination and PCR-based testing of blood samples in Europe (Dogiel and Navtsevich 1936; Peirce 1981; Valkiūnas 2005; Marzal et al. 2008; Piesma and van der Velde 2012). Interestingly, these infections have been detected only in adult swallows after their arrival from African wintering grounds. Species of *Plasmodium* and *Haemoproteus* have not been observed in over 500 individuals of juvenile house martins and barn swallows hatched in Europe, indicating solely African transmission of these infections (Peirce 1981; Valkiūnas 2005; Marzal et al. 2008; Piesma and van der Velde 2012). The obstacles for spreading of *P. delichoni* in European breeding grounds remain unclear. This study showed lack of sporogonic development in mosquitoes, which are widespread in Europe (*C. p. pipiens*) and sub-Saharan Africa (*C. quinquefasciatus, A. aegypti*). Even gametogenesis and development of ookinetes were abortive in these mosquitoes, indicating that sexual process, ookinete formation and sporogony of *P. delichoni* require particular mosquito gut factors for successful development in vectors, as is the case in human malaria parasites (Sherman 1998). It is probable that lack of specific mosquito vectors is an important factor preventing spreading of *P. delichoni* infection in Europe. Endemic African mosquitoes should be tested for their vectorial capability. Sporogony of avian *Plasmodium* spp. completes and sporozoites develop in *Coquillettidia* mosquitoes transmitting avian malaria in Africa (Njabo et al. 2009). Species of this genus and related genera of bird-biting Culicidae should be considered in future experimental vector research in avian malaria studies. Such research is important for better understanding mechanisms preventing this disease spreading by migrating birds from tropics to the areas with temperate climates. *Plasmodium delichoni* is a convenient model parasite for such research because it develops long-lasting parasitemia in domestic canaries. These birds are easy to breed, maintain and infect in captivity, and they can be used as donors of gametocytes for experimental exposure to different mosquito species.

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References

1. Atkinson CT, Thomas NJ, Hunter DB (2008) Parasitic diseases of wild birds. Wiley-Blackwell, Oxford
2. Bennett GF, Whiteway M, Woodworth-Lynas C (1982) A host-parasite catalogue of the avian haematozoa. Mem Univ of Nfld Occ Pap Biol 5:1-243

3. Bensch S, Hellgren O, Pérez-Tris J (2009) A public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome *b* lineages. Mol Ecol Resour 9:1353-1358
4. Bensch S, Stjenman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, Torres-Pinheiro R (2000) Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc R Soc B 276:1583-1589
5. Bishop MA, Bennett GF (1992) Host-parasite catalogue of the avian haematozoa: Supplement 1, and Bibliography of the avian blood-inhabiting haematozoa: Supplement 2. Mem Univ of Nfld Occ Pap Biol 15:1-244
6. Braga ÉM, Silveira P, Belo NO, Valkiūnas G (2011) Recent advances in the study of avian malaria: An overview with an emphasis on the distribution of *Plasmodium* spp. in Brazil. Mem Inst Oswaldo Cruz 06 Suppl 1: 3-11
7. Chagas CRF, Valkiūnas G, Nery CVC, Henrique PC, Gonzalez IHL, Monteiro EF, de Oliveira Guimarães L, Romano CM, Kirchgatter K (2013) *Plasmodium (Novyella) nucleophilum* from an Egyptian Goose in Sao Paulo Zoo, Brazil: Microscopic confirmation and molecular characterization. Int J Parasitol Parasites Wildl 2:286-291
8. Christensen BM, Barnes HJ, Rowley WA (1983) Vertebrate host specificity and experimental vectors of *Plasmodium* (*Novyella*) *kempi* sp. n. from the eastern wild turkey in Iowa. J Wildl Dis 19:204-213
9. Clark NJ, Clegg SM, Lima MR (2014) A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): New insights from molecular data. Int J Parasitol 44:329-338
10. Desser SS (1970) The fine structure of *Leucocytozoon simondi*. II. Megaloschizogony. Can J Zool 48:417-421
11. Desser SS, Bennett GF (1993) The genera *Leucocytozoon*, *Haemoproteus* and *Hepatocystis*. In: Kreier JP, Baker JR (eds) Parasitic Protozoa, 2nd edn. Academic Press, New York, 4, pp 273-307
12. Dimitrov D, Zehtindjiev P, Bensch S, Ilieva M, Iezhova T, Valkiūnas G (2014) Two new species of *Haemoproteus* Kruse, 1890 (Haemosporida, Haemoproteidae) from European birds, with emphasis on DNA barcoding for detection of haemosporidians in wildlife. Syst Parasitol 87:135-151
13. Dinhopl N, Mostegl MM, Richter B, Nedorost N, Maderner A, Fragner K, Weissenböck H (2011) Application of in-situ hybridization for the detection and identification of avian malaria parasites in paraffin wax-embedded tissues from captive penguins. Avian Pathol 40:315-320
14. Dinhopl N, Nedorost N, Mostegl MM, Weissenbacher-Lang C, Weissenböck H (2015) In situ hybridization and sequence analysis reveal an association of *Plasmodium* spp. with mortalities in wild passerine birds in Austria. Parasitol Res 114:1455-1462
15. Dogiel VA, Navtsevich N (1936) The parasite fauna of the house martin. Uch zap LGU 7, Ser biol 3: 43-79 (in Russian)
16. Forrester DJ, Greiner EC (2008) Leucocytozoonosis. In: Atkinson CT, Thomas NJ, Hunter DB (eds). Parasitic diseases of wild birds. Wiley-Blackwell, Ames, pp 54-107
17. Gabaldon A, Ulloa G (1976) Revalidación y redescripción de *Plasmodium columbae* Carini, 1912. Bol Dir Malariol San Amb 16:93-106

18. Gabaldon A, Ulloa G (1980) Holoendemicity of malaria: an avian model. Trans R Soc Trop Med Hyg 74: 501-507
19. Gabaldon A, Ulloa G (1981) A new species of the subgenus *Novyella* (Haemosporina, Plasmodiidae) from *Aramides cajanea* (Gruiformes, Rallidae). In: Canning EU (ed). Parasitological topics. A presentation volume to P.C.C. Garnham, F.R.S. on the occasion of his 80th birthday, pp 100-105
20. Garcia-Longoria L, Møller AP, Balbontín J, de Lope F, Marzal A (2015) Do malaria parasites manipulate the escape behaviour of their avian hosts? An experimental study. Parasitol Res 114:4493-4501
21. Garnham PCC (1966) Malaria parasites and other Haemosporidia. Blackwell, Oxford
22. Garnham PCC (1980) Malaria in its various vertebrate hosts. In: Kreier JP (ed). Malaria. Epidemiology, chemotherapy, morphology and metabolism. Academic Press, New York, 1, pp 95-144
23. Glaizot O, Fumagalli L, Iritano K, Lalubin F, Van Rooyen J, Christe P (2012) High prevalence and lineage diversity of avian malaria in wild populations of great tits (*Parus major*) and mosquitoes (*Culex pipiens*). PLoS One 7(4):e34964
24. Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symp Ser (Oxf) 41:95-98
25. Hellgren O, Waldenström J, Bensch S (2004) A new PCR assay for simultaneous studies of *Leucocytozoon, Plasmodium,* and *Haemoproteus* from avian blood. J Parasitol 90:797-802
26. Hewitt R (1940) Bird malaria. Jonhs Hopkins Press, Baltimore
27. Iezhova TA, Valkiūnas G, Bairlein F (2005) Vertebrate host specificity of two avian malaria parasites of the subgenus *Novyella*: *Plasmodium nucleophilum* and *Plasmodium vaughani*. J Parasitol 91:472-474
28. Ilgūnas M, Palinauskas V, Iezhova TA, Valkiūnas G (2013) Molecular and morphological characterization of two avian malaria parasites (Haemosporida: Plasmodiidae), with description of *Plasmodium homonucleophilum* n. sp. Zootaxa 3666:49-61
29. Kazlauskienė R, Bernotienė R, Palinauskas V, Iezhova TA, Valkiūnas G (2013) *Plasmodium relictum* (lineages pSGS1 and pGRW11): Complete synchronous sporogony in mosquitoes *Culex pipiens pipiens*. Exp Parasitol 133:454-461
30. Loiseau C, Harrigan RJ, Robert A, Bowie RC, Thomassen HA, Smith TB, Sehgal RN (2012) Host and habitat specialization of avian malaria in Africa. Mol Ecol 21:431-441
31. Lotta IA, Gonzalez AD, Pacheco MA, Escalante AA, Valkiūnas G, Moncada LI, Matta NE (2015) *Leucocytozoon pterotenuis* sp. nov. (Haemosporida, Leucocytozoidae): Description of the morphologically unique species from the Grallariidae birds, with remarks on the distribution of *Leucocytozoon* parasites in the Neotropics. Parasitol Res 114:1031-1044
32. Mantilla J S, González A D, Valkiūnas G, Moncada LI, Matta NE (2013) Description and molecular characterization of *Plasmodium* (*Novyella*) *unalis* sp. nov. from the Great Thrush (*Turdus fuscater*) in highland of Colombia. *Parasitol Res* 112:4193-4204
33. Manwell RD (1935) How many species of avian malaria parasites are there? Am J Trop Med 15:265-283

34. Manwell RD (1951) Exoerythrocytic schizogony in *Plasmodium hexamerium.* Am J Hyg 53:244-248
35. Manwell RD, Sessler GJ (1971a) Malaria parasites of toucans. J. Protozool. 18:570-574
36. Manwell RD, Sessler GJ (1971b) *Plasmodium paranucleophilum* n. sp. from a South American tanager. J Protozool 18:629-632
37. Marinov MP, Zehtindjiev P, Dimitrov D, Ilieva M, Bobeva A, Marchetti C (2015) Haemosporidian infections and host behavioural variation: a case study on wild-caught nightingales *(Luscinia megarhynchos*). Ethol Ecol Evol, in press DOI: 10.1080/03949370.2015.1102776
38. Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol 47:261-273
39. Marzal A (2012) Recent advances in studies on avian malaria parasites. In: Okwa OO (ed) Malaria parasites. InTech, pp 135-158
40. Marzal A, Bensch S, Reviriego M, Balbontin J, De Lope F (2008) Effects of malaria double infection in birds: one plus one is not two. J Evol Biol 21:979-987
41. Mehlhorn H (2015) Encyclopedia of Parasitology, 4th eds. Springer-Verlag, Berlin, Heidelberg
42. Nelson P (1966) Exo-erythrocytic stages of a Ceylon strain of *Plasmodium vaughani* in pigeons. Trans R Soc Trop Med Hyg 60:424
43. Njabo K, Cornel AJ, Sehgal RNM, Loiseau C, Buermann W, Harrigan RJ, Pollinger J, Valkiūnas G, Smith TB (2009) *Coquillettidia* (Culicidae, Diptera) mosquitoes are natural vectors of avian malaria in Africa. Malar J 8(1):193
44. Nylander JAA (2008) MrModeltest v2.3. Program distributed by the author. Evolutionary Biology Centre, Uppsala University
45. Outlaw DC, Ricklefs RE (2014) Species limits in avian malaria parasites (Haemosporida): How to move forward in the molecular era. Parasitology 141:1223-1232
46. Palinauskas V, Valkiūnas G, Bolshakov CV, Bensch S (2011) *Plasmodium relictum* (lineage SGS1) and *Plasmodium ashfordi* (lineage GRW2): The effects of the co-infection on experimentally infected passerine birds. Exp Parasitol 127:527-533
47. Palinauskas V, Žiegytė R, Ilgūnas M, Iezhova TA, Bernotienė R, Bolshakov C, Valkiūnas G (2015) Description of the first cryptic avian malaria parasite, *Plasmodium homocircumflexum* n. sp. with experimental data on its virulence and development in avian hosts and mosquitoes. Int J Parasitol 45:51-62
48. Peirce MA (1981) Distribution and host-parasite check-list of the haematozoa of birds in Western Europe. J Nat Hist 15:419-458
49. Pérez-Tris J, Bensch S (2005) Diagnosing genetically diverse avian malarial infections using mixed-sequence analysis and TA-cloning. Parasitology 131(Pt 1):15-23
50. Perez-Tris J, Hellgren O, Križanauskienė A, Waldenström J, Secondi J, Bonneaud C, Fjeldsa J, Hasselquist D, Bensch S (2007). Within-host speciation of malaria parasites. PLoS ONE 2(2):e235
51. Perkins SL (2014) Malaria's many mates: past, present and future of the systematics of the order Haemosporida. J Parasitol 100:11-25

52. Piesma T, van der Velde M (2012) Dutch house martin *Delichon urbicum* gain blood parasite infections over their lifetime, but do not seem to suffer. J Ornithol 153:907-912
53. Ronquist F, Heulsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference
54. under mixed models. Bioinformatics 19:1572-1574
55. Sambrook J, Russell DW (2001) Molecular clonning: A laboratory manual. 3rd ed., Cold Spring Harbor, New York
56. Sherman IW (ed) (1998) Malaria: parasite biology, pathogenesis, and protection. American Society for Microbiology, Washington DC
57. Snow D, Perrins CM (eds) (1998) The birds of the Western Palearctic. Oxford University Press, Oxford
58. Stone WB, Weber BL, Parks FJ (1971) Morbidity and mortality of birds due to avian malaria. N Y Fish and Game J 18:62-63
59. Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, Massachusetts
60. Szőllősi E, Rosivall B, Hasselquist D, Török J (2009) The effect of parental quality and malaria infection on nestling performance in the collared flycatcher Ficedula albicollis. J Ornit 150:519-527
61. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731-2739
62. Valkiūnas G (2005) Avian Malaria Parasites and other Haemosporidia. CRC, Boca Raton
63. Valkiūnas G, Iezhova TA, Loiseau C, Chasar A, Smith TB, Sehgal RNM (2008) New species of haemosporidian parasites (Haemosporida) from African rainforest birds, with remarks on their classification. Parasitol Res 103:1213-1228
64. Valkiūnas G, Iezhova TA, Loiseau C, Smith TB, Sehgal RNM (2009) New malaria parasites of the subgenus *Novyella* in African rainforest birds, with remarks on their high prevalence, classification and diagnostics. Parasitol Res 104:1061-1077
65. Valkiūnas G, Palinauskas V, Ilgūnas M, Bukauskaite D, Dimitrov D, Bernotienė R, Zehtindjiev P, Ilieva M, Iezhova TA (2014) Molecular characterization of five widespread avian haemosporidian parasites (Haemosporida), with perspectives on the PCR-based detection of haemosporidians in wildlife. Parasitol Res 113:2251-2263
66. Walther EL, Valkiūnas G, González A D, Matta NE, Ricklefs RE, Cornel A, Sehgal RNM (2014) Description, molecular characterization, and patterns of distribution of a widespread New World avian malaria parasite (Haemosporida: Plasmodiidae), *Plasmodium (Novyella) homopolare* sp. nov. Parasitol Res 113: 3319-3332
67. Wong TC, Desser SS (1978) Ultrastructural observations on renal schizogony of *Leucocytozoon dubreuili* in the American robin. J Protozool 25: 302-314
68. Zehtindjiev P, Ilieva M, Westerdahl H, Hansson B, Valkiūnas G, Bensch S (2008) Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler *Acrocephalus arundinaceus*. Exp Parasitol 119:99-110
69. Žiegytė R, Bernotienė R, Bukauskaitė D, Palinauskas V, Iezhova TA, Valkiūnas G (2014) Complete sporogony of *Plasmodium relictum* (lineages pSGS1 and pGRW11)

in mosquito *Culex pipiens pipiens* form *molestus*, with implications to avian malaria epidemiology. J Parasitol 100:878-882

PAPER V

Characterization of *Plasmodium relictum*, a cosmopolitan agent of avian malaria.

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RESEARCH

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Characterization of *Plasmodium relictum*, a cosmopolitan agent of avian malaria

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Abstract

Background: Microscopic research has shown that *Plasmodium relictum* is the most common agent of avian malaria. Recent molecular studies confirmed this conclusion and identified several mtDNA lineages, suggesting the existence of significant intra-species genetic variation or cryptic speciation. Most identified lineages have a broad range of hosts and geographical distribution. Here, a rare new lineage of *P. relictum* was reported and information about biological characters of different lineages of this pathogen was reviewed, suggesting issues for future research.

Methods: The new lineage pPHCOL01 was detected in Common chiffchaff *Phylloscopus collybita,* and the parasite was passaged in domestic canaries *Serinus canaria*. Organs of infected birds were examined using histology and chromogenic in situ hybridization methods. *Culex quinquefasciatus* mosquitoes, Zebra finch *Taeniopygia guttata,* Budgerigar *Melopsittacus undulatus* and European goldfinch *Carduelis carduelis* were exposed experimentally. Both Bayesian and Maximum Likelihood analyses identified the same phylogenetic relationships among different, closely-related lineages pSGS1, pGRW4, pGRW11, pLZFUS01, pPHCOL01 of *P. relictum*. Morphology of their blood stages was compared using fixed and stained blood smears, and biological properties of these parasites were reviewed.

Results: Common canary and European goldfinch were susceptible to the parasite pPHCOL01, and had markedly variable individual prepatent periods and light transient parasitaemia. Exo-erythrocytic and sporogonic stages were not seen. The Zebra finch and Budgerigar were resistant. Neither blood stages nor vector stages of all examined *P. relictum* lineages can be distinguished morphologically.

Conclusion: Within the huge spectrum of vertebrate hosts, mosquito vectors, and ecological conditions, different lineages of *P. relictum* exhibit indistinguishable, markedly variable morphological forms. Parasites of same lineages often develop differently in different bird species. Even more, the variation of biological properties (parasitaemia dynamics, blood pathology, prepatent period) in different isolates of the same lineage might be greater than the variation in different lineages during development in the same species of birds, indicating negligible taxonomic value of such features. Available lineage information is excellent for parasite diagnostics, but is limited in predictions about relationships in certain host-parasite associations. A combination of experiments, field observations, microscopic and molecular diagnostics is essential for understanding the role of different *P. relictum* lineages in bird health.

Keywords: *Plasmodium relictum*, Birds, Morphological and molecular characterization, Review

Background

Plasmodium relictum is an invasive blood parasite, which causes malaria in many species of birds from all over the word [1–4]. Naive birds often experience severe disease and even mortality during malaria infection [5, 6], but some bird species and their populations appear to be relatively resistant and can tolerate this infection [7–11]. This parasite was the first recognized and described agent of avian malaria [12], likely due to its high prevalence in a wide range of different avian hosts and because morphological characteristics of its mature blood stages are so distinctive in blood films. Mature stages typically

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possess prominent nuclei and cytoplasm, numerous pigment granules and markedly influence the position of the nuclei of their host erythrocytes, causing lateral shifts in their position. Numerous synonymous names of this organism exist [7, 13]. These names were suggested for distinguishing the morphologically similar or even identical blood stages, which were reported in different avian hosts and/or different geographical areas [13–15]. Microscopic examination of blood films, the main avian malaria diagnostic tool used in the 20th Century, has identified *P. relictum* as the most common agent of avian malaria with reports from over 300 species of birds belonging to 11 orders from all over the world [1, 7, 16, 17]. Recent molecular studies have supported this conclusion and uncovered significant genetic diversity among different isolates of *P. relictum*, suggesting existence of intra-species genetic variation or even cryptic speciation [2, 18–22].

Partial sequences of mitochondrial cytochrome *b* gene (*cytb*) have been successfully used in distinguishing different lineages of avian malaria parasites, and they are excellent molecular markers for disease diagnostics [2, 4, 19, 23–25]. Over 100 closely related *cytb* lineages of avian *Plasmodium* were deposited in GenBank and MalAvi database (http://mbio-serv2.mbioekol.lu.se/Malavi) and many of them may belong to *P. relictum*. However, few of these molecular lineages are supported by microscopic examination of well-fixed and stained blood smears and the small genetic difference in *cytb* sequences alone cannot be considered as final proof that closely related lineages belong to the same morpho-species. For example, some morphologically distinct haemosporidian species differ in their partial *cytb* sequences just by a few nucleotide bases [26, 27]. Currently, only four lineages (pSGS1, pGRW4, pGRW11, pLZFUS01) have been linked to *P. relictum* based on morphological characters of their blood stages, and these data are helpful for distinguishing this infection in blood films [28–30].

During the past 15 years, much data have been collected about host, geographical distribution, vectors, virulence, and other biological characters of *P. relictum* based on *cytb* lineages [2, 3, 8, 19, 30–34]. This provides opportunities to examine patterns in the biology and pathology of avian *Plasmodium* infection at the level of these specific lineages. This study characterizes a new *cytb* lineage of *P. relictum* (pPHCOL01), makes comprehensive comparisons of morphological characters of blood stages of all known lineages of this parasite, and reviews their biological features to help identify some new directions for future avian malaria research.

Methods

Collection of blood and tissue samples

Fieldwork was carried out at the Ventės Ragas Ornithological Station, Lithuania between 4 and 18 May, 2017. Twenty-three Common chiffchaffs *Phylloscopus collybita* were caught with mist nets and large stationary traps. The blood was taken by puncturing the brachial vein. Three blood films were prepared immediately after withdrawal of the blood, air-dried using a battery-operated fan, fixed in absolute methanol and stained with Giemsa. About 30 µl of whole blood was taken in heparinized microcapillaries and stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) at ambient temperature while in the field and then maintained at −20 °C in the laboratory.

To detect and isolate the *Plasmodium* parasite strain, the blood films from each captured bird were quickly examined microscopically in the field, as previously described [35]. One naturally infected Common chiffchaff was detected, with parasitaemia of 0.1%. Ten blood films were prepared for microscopic examination from this bird. Additionally, blood was also collected in heparinized microcapillaries and used to expose two uninfected domestic Common canaries *Serinus canaria* forma *domestica* by sub-inoculation into their pectoral muscle of about 250 µl of a freshly prepared mixture of infected blood, 3.7% sodium citrate (anticoagulant) and 0.9% saline (4:1:5) [36].

Parasitaemia developed in both exposed canaries, and blood of these birds was passaged as described above in three additional canaries. Two Zebra finches *Taeniopygia guttate*, one Budgerigar *Melopsittacus undulatus* and two European goldfinches *Carduelis carduelis* were also exposed, as described above. Six uninfected canaries were used as controls. Blood of all control and experimental birds was tested by microscopic examination and PCR-based methods (see description below) twice before the experiment to ensure that they were uninfected with malaria parasites.

Two canaries were observed for 57 and 94 days post exposure (dpe) and then euthanized for histology and chromogenic in situ hybridization research. Two European goldfinches were observed for 127 dpe. All remaining birds were observed for 131 dpe. Post-exposure blood samples were taken for microscopic examination and PCR-based testing as described above once every 4 days during the first post-exposure month, once every week during the second month and once every 1–2 weeks during the remaining experiment time. All experimental and control birds were kept indoors in a vector-free room under natural light–dark photoperiod. They were fed a standard diet for seed-eating bird species.

Control birds were maintained in the laboratory for further research. The donor Common chiffchaff and infected experimental birds were euthanized, and pieces of organs (brain, liver, lungs, heart, kidneys, spleen) and pieces of pectoral muscles were fixed in 10% neutral

formalin, embedded in paraffin and processed using traditional histologic methods [7]. Histological sections of 4 μm were obtained, stained with haematoxylin–eosin, mounted in BioMount (BioGnost, Croatia) and examined microscopically. One smear of bone marrow was prepared from the tibia bone of each bird, air dried, fixed with absolute methanol and stained with Giemsa.

Experimental infection and investigation of *Culex quinquefasciatus* mosquitoes

Laboratory-reared *Culex quinquefasciatus* mosquitoes were maintained and exposed to canaries infected with the isolated *Plasmodium* sp., as previously described [32]. Briefly, insects were kept in cages (45 × 45 × 45 cm) under 65–70% relative humidity, 16/8 h light/dark photoperiod and 26 ± 1 °C. One experimentally infected canary was used as the donor of parasites for infecting mosquitoes. Eleven female mosquitoes took blood meals on this canary. Parasitaemia was 0.02% with few visible mature gametocytes. Preparations of midgut contents (6 insects were dissected 24–48 h post exposure), one preparation of midgut wall (one insect on 12 dpe) and 4 preparations of salivary glands (on 15, 16 and 18 dpe) were prepared and examined, as previously described [32].

Microscopic examination

Detailed microscopic analysis was carried out with various Olympus light microscopes equipped with Olympus digital cameras and imaging software. Preparations of blood stages of the lineages pSGS1, pGRW11, pGRW4, and pLZFUS01 were from collections of voucher specimens which have been deposited at P. B. Šivickis Laboratory of Parasitology, Nature Research Centre Vilnius. These were blood films from canaries whose were exposed experimentally to the parasite lineages pSGS1 (parasitaemia varied between 0.6 and 1.8%, preparation accession number 48979–48981 NS), pGRW11 (1.1–6%, 48982–48984 NS), pGRW4 (0.2–2.1%, 48985–48987 NS), and the lineage pLZFUS01 (0.5%, 48694–48696 NS) from the blood of a naturally infected Red-backed shrike *Lanius collurio* (for exposure description see [30]). Additionally, preparations with the Hawaiian isolate of *P. relictum* (pGRW4) were used. These were (1) 12 blood films from two individual canaries that were exposed experimentally by inoculation of infected blood (parasitaemia varies between 0.6 and 10%, accession nos. 48988–48999 NS) (for infection details see [5]), (2) 11 blood films from one naturally infected Apapane *Himatione sanguinea* (parasitaemia 22%, accession nos. 49000–49010 NS).

Blood films from each infected bird were examined and the observed blood stages were morphologically compared by skilled parasitologists of avian malaria parasites at the P. B. Šivickis Laboratory of Parasitology. At least 100 fields were studied at high magnification (1000×) in each preparation. Intensity of parasitaemia was estimated as a percentage by actual counting of the number of parasites per 10,000 erythrocytes. The morphometric features studied (Table 1) were those defined in [7]. The analyses were carried out using the 'Statistica 7' package as previously described [7].

In situ hybridization

Chromogenic in situ hybridization (ISH) was applied to increase detectability of tissue stages of the parasites. Organs (the same as for histological examination) from one naturally infected Common chiffchaff and two experimentally infected canaries (57 and 94 dpe, respectively) were tested using a previously described ISH protocol [37]. 3 μm paraffin-embedded tissue sections of all these organs were prepared. The sections were deparaffinized, subjected to proteolytic treatment with proteinase K (Roche, Basel, Switzerland) 6 μg/ml in Tris-buffered saline at 37 °C for 50 min. For hybridization, the slides with tissue sections were incubated overnight at 40 °C with hybridization mixture and a final probe concentration of 100 ng/ml. The used oligonucleotide probe (sequence: 5′-TTTAATAACTCGTTATATATATCAGTGTAGCAC-3′) was labelled with digoxigenin at the 3′ end (Eurofins MWG Operon, Ebersberg, Germany). This probe is specific to detect avian *Plasmodium* parasites [6, 37]. The digoxigenin-labelled hybrids were detected by incubating the slides with anti-digoxigenin-AP Fab fragments (Roche) (1:200) for 1 h at room temperature (RT). Visualization of the reaction was carried out using the colour substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) (Roche). A positive control (sections of a lung of a Eurasian blackbird *Turdus merula* naturally infected with *Plasmodium vaughani*, which was proven to be positive by previous ISH) was used to assure that the protocol worked. Preparations were examined microscopically by skilled parasitologists and pathologists; at least 50 fields of each preparation were studied at low magnification (400×), and then each preparation was examined for 10–15 min at high magnification (1000×).

Molecular and phylogenetic analysis

Total DNA was extracted from blood samples using an ammonium-acetate precipitation protocol [38]. Polymerase chain reaction using the primer set HaemNFI/NR3 and Haem/R2 was performed in order to amplify a 479 bp sequence of the parasite's *cytb* gene [18, 39]. The total volume of the reaction mix for each sample was 25 μl, which included 12.5 μl of DreamTaq Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 μl nuclease-free water, 1 μl of each primer and ~ 50 ng of a total genomic

Table 1 Morphometry of blood stages and host cells of *Plasmodium (Haemamoeba) relictum* (pPHCOL01) from the blood of Common chiffchaff *Phylloscopus collybita* (n = 21)

Feature	Measurements (μm)[a]
Uninfected erythrocyte	
Length	10.5–11.9 (11.2 ± 0.4)
Width	5.2–6.4 (5.8 ± 0.3)
Area	46.9–55.6 (51.7 ± 2.7)
Uninfected erythrocyte nucleus	
Length	5.2–6.1 (5.6 ± 0.3)
Width	1.8–2.3 (2.0 ± 0.1)
Area	8.1–10.8 (9.7 ± 0.7)
Macrogametocyte	
Infected erythtocyte	
Length	8.3–12.2 (10.6 ± 1.1)
Width	6.2–8.1 (7.0 ± 0.6)
Area	43.9–70.8 (58.3 ± 6.8)
Infected erythrocyte nucleus	
Length	4.5–6.8 (5.4 ± 0.5)
Width	2.1–3.4 (2.6 ± 0.3)
Area	9.2–15.2 (11.4 ± 1.3)
Gametocyte	
Length	5.9–8.0 (7.1 ± 0.5)
Width	3.2–4.1 (3.6 ± 0.3)
Area	15.5–22.7 (20.3 ± 1.9)
Gametocyte nucleus	
Length	2.1–3.1 (2.6 ± 0.3)
Width	1.4–2.5 (2.0 ± 0.3)
Area	2.6–5.3 (4.2 ± 0.6)
Pigment granules	8.0–17.0 (12.0 ± 2.3)
Microgametocyte	
Infected gametocyte	
Length	8.6–12.7 (10.7 ± 1.1)
Width	6.0–8.5 (7.1 ± 0.7)
Area	50.4–67.8 (59.1 ± 4.5)
Infected erythrocyte nucleus	
Length	5.0–6.3 (5.5 ± 0.4)
Width	1.8–3.3 (2.5 ± 0.4)
Area	9.4–14.8 (11.7 ± 1.4)
Gametocyte	
Length	6.1–10.0 (7.7 ± 0.9)
Width	3.5–5.4 (4.4 ± 0.5)
Area	18.9–29.4 (24.4 ± 2.7)
Gametocyte nucleus	
Length	3.0–5.0 (4.0 ± 0.5)
Width	2.4–3.8 (2.9 ± 0.4)
Area	6.4–12.8 (8.5 ± 1.7)
Pigment granules	9.0–16.0 (12.4 ± 1.8)
Meront	
Length	4.5–7.8 (5.8 ± 0.8)
Width	3.1–5.6 (4.4 ± 0.5)
Area	13.6–33.7 (19.5 ± 4.6)
Area of pigment granules	0.8–1.6 (1.2 ± 0.3)
No. of merozoites	10–22 (18.9 ± 3.8)

[a] Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation

DNA template (2 μl). Negative controls (nuclease-free water) were used after each seven samples to detect possible false amplifications, and one positive control (extracted parasite DNA from a blood sample, which was confirmed positive during previous PCR testing) was used to evaluate the success of PCR if none of the samples would have been amplified.

Temperatures for the PCR were as described in the original protocols. The success of the performed PCR was evaluated by running electrophoresis on a 2% agarose gel. Successfully amplified DNA was precipitated using 11 μl of 8 M NH 4Ac, 37 μl of 96% and 150 μl of 70% ethanol. After centrifugation, the supernatant was discarded, the samples were air-dried overnight, and then 16 μl of nuclease-free water was added on the precipitated DNA. Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, Foster City, California) were used for sequencing. Sequences were edited and aligned using BioEdit software [40]. Absence of double-base calling in sequence electropherograms was used as an indication of single infections [41]. Nucleotide BLAST (megablast algorithm) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare our amplified sequences with sequences deposited in the GenBank.

Molecular phylogenetic analysis was carried out using Bayesian and Maximum Likelihood algorithms. Sequences for the phylogenetic analysis were collected from GenBank and double-checked in MalAvi database [19]. *Plasmodium falciparum* was used as an outgroup. GenBank accession numbers and codes of the lineages are provided in the phylogenetic trees (Fig. 1). Bayesian phylogenetic tree (Fig. 1a) was constructed using MrBayes version 3.1 [42] software. The General Time Reversible Model (GTR) was used as suggested by the software MrModeltest 2.2 (https://github.com/nylander/MrModeltest2). Analysis was run for a total of 10 million generations with a sampling frequency of every 100 generations. Before the construction of the consensus tree, 25% of the initial trees were discarded as 'burn in' period. The tree was visualized using the software FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Maximum Likelihood tree (Fig. 1b) was constructed using the MEGA 7.0 [43] software; it was performed with 1,000

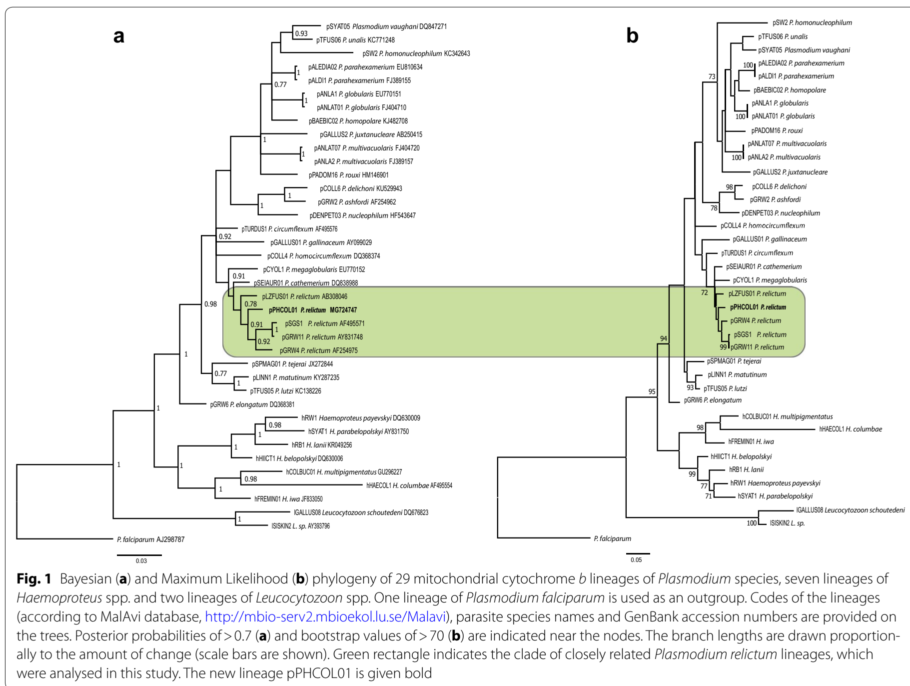


Fig. 1 Bayesian (**a**) and Maximum Likelihood (**b**) phylogeny of 29 mitochondrial cytochrome *b* lineages of *Plasmodium* species, seven lineages of *Haemoproteus* spp. and two lineages of *Leucocytozoon* spp. One lineage of *Plasmodium falciparum* is used as an outgroup. Codes of the lineages (according to MalAvi database, http://mbio-serv2.mbioekol.lu.se/Malavi), parasite species names and GenBank accession numbers are provided on the trees. Posterior probabilities of > 0.7 (**a**) and bootstrap values of > 70 (**b**) are indicated near the nodes. The branch lengths are drawn proportionally to the amount of change (scale bars are shown). Green rectangle indicates the clade of closely related *Plasmodium relictum* lineages, which were analysed in this study. The new lineage pPHCOL01 is given bold

bootstrap replications using the GTR model and the same dataset as during the Bayesian analysis.

The new sequence of lineage pPHCOL01 was deposited in GenBank (accession MG724747). Genetic differences between different lineages of *P. relictum* were calculated using the Jukes–Cantor model of substitution, as implemented in the programme MEGA 7.0 [43].

Results

Parasite lineage identification and susceptibility of experimental birds

Single infections of *P. relictum* (*cytb* lineage pPHCOL01) was identified in the donor Common chiffchaff both by microscopic examination of blood films and PCR-based amplification and sequencing. All exposed canaries were susceptible and developed a single infection with the same malaria parasite, as determined both by microscopic examination of blood films and PCR-based testing. Parasitaemia developed in one exposed European goldfinch. Two Zebra finches, one Budgerigar and one European goldfinch were resistant. All control canaries remained non-infected during this study.

Phylogenetic analysis

The reported lineage of *P. relictum* (pPHCOL01) was new. It clustered with other morphologically characterized lineages of *P. relictum* (pSGS1, pGRW4, pGRW11, pLZFUS01) in both phylogenetic analyses, supporting the close phylogenetic relationships among them (Fig. 1a, b). Genetic differences among five lineages of *P. relictum* varied between 0.2% (minimum, the lineages pSGS1 and pGRW11) and 3% (maximum, the lineages pSGS1 and pLZFUS01).

Characterization of *Plasmodium (Haemamoeba) relictum* (pPHCOL01)

See (Fig. 2, Table 1).

DNA sequence

Mitochondrial *cytb* lineage pPHCOL01 (new lineage, 479 bp, GenBank accession MG724747).

Avian hosts

Common chiffchaff *Phylloscopus collybita* is a natural host. Other natural avian hosts are unknown. Two Zebra finches and one Budgerigar that were exposed by subinoculation of infected blood were resistant. The most similar *Plasmodium* parasite lineages were reported only in sub-Saharan birds by Loiseau et al. [44] (the lineage PV40, accession HQ022817, 2 bp difference, avian host was not reported), Beadell et al. [2] (the lineage P27, accession DQ659568, 7 bp difference, the host is the Cameroon sunbird *Cyanomitra oritis)* and Lutz et al. [45] (the lineage P_AFR110, accession KM056570, 7 bp difference, the host is the Miombo tit *Parus griseiventris*).

Vectors

Remain unidentified. Sporogonic development was not observed in *Culex quinquefasciatus* mosquitoes.

Site of infection

Red blood cells; no other data.

Representative blood films

Voucher specimens (accession numbers 48965–48974 NS, *Phylloscopus collybita*, 7 May 2017, parasitaemia 0.1%, collected by D. Bukauskaitė, and 48975–48978 NS, *Serinus canaria*, 2–6 June, 2017, collected by M. Ilgūnas) were deposited in Nature Research Centre, Vilnius, Lithuania.

Prevalence

The overall prevalence was 1 of 23 (4.3%) in Common chiffchaff at the study site.

Parasitaemia and virulence

Canaries are susceptible, with long-lasting (up to 65 dpe), but light parasitaemia (< 0.01%) reported in the majority of exposed birds. One of two exposed European goldfinches developed very light (0.001%) and long-lasting (up to 127 dpe) parasitaemia. In all positive birds, parasitaemia was transient, i.e., it was not seen during all days of testing. In experimentally exposed birds, the maximum reported parasitaemia was 0.02%, and it was seen in one canary. The parasitaemia remained light or even declined into latency approximately 1–2 weeks after the first parasites seen in blood films in all positive birds, with a few parasites appearing in the circulation during entire observation time.

All blood stages (trophozoites, growing and mature meronts, growing and mature gametocytes) were reported in the peripheral circulation of naturally infected Common chiffchaff, experimentally exposed canaries and one goldfinch. This indicates asynchronous development in the blood. Mortality was not reported among exposed birds, and they appeared healthy. Clinical signs of disease were not observed during this study, and it is probable that susceptible inoculated birds can tolerate this infection.

The prepatent period varied markedly, with first parasites observed in the peripheral circulation 9, 14, 31, and 49 dpe in different canaries. Prepatent period was 11 dpe in one European goldfinch.

Morphology of blood stages of the new lineage parasites was the same in the Common chiffchaff and the experimentally exposed canaries and one goldfinch. Description of blood stages of this infection is given from preparations with parasitaemia of 0.1% in Common chiffchaff (Fig. 2).

Trophozoites

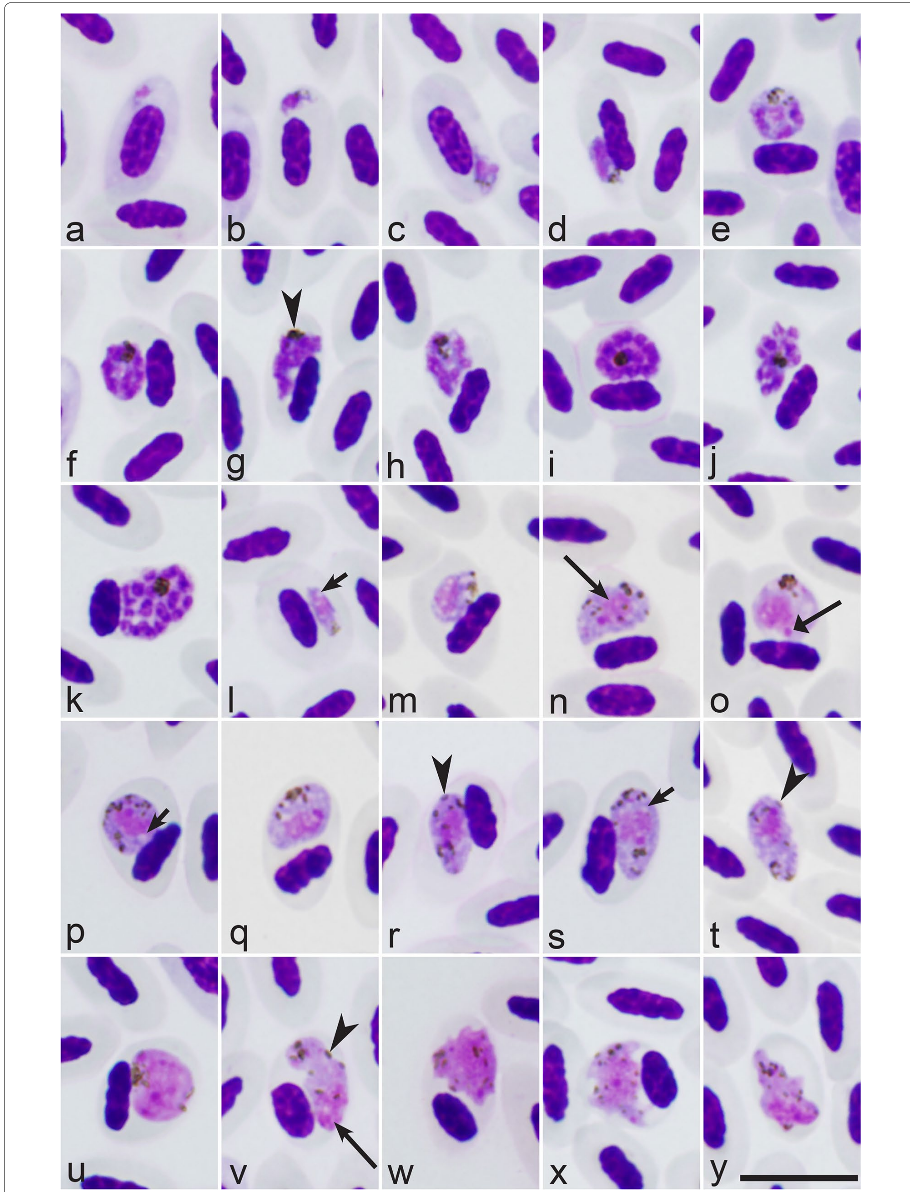
Figure 2a–d. Develop mainly in mature erythrocytes (Fig. 2b, d), but sometimes were also seen in polychromatic erythrocytes (Fig. 2a, c). Earliest trophozoites usually are of irregular form, often amoeboid in outline (Fig. 2a, b); they only slightly displace nuclei in infected erythrocytes laterally. Advanced trophozoites possess prominent nuclei and cytoplasm, but lack vacuoles (Fig. 2c, d); they were often attached to the host cell nuclei (Fig. 2d), which are slightly displaced. Pigment granules are roundish, small (< 0.5 μm), few, dark-brown, and usually grouped.

Erythrocytic meronts

Figure 2e–k. Develop in mature erythrocytes. Young growing meronts possess plentiful cytoplasm and large nuclei (Fig. 2e); size of the nuclei and amount of the cytoplasm markedly decrease as the parasites mature (compare Fig. 2e with Fig. 2f–h). Vacuoles are absent from both developing and mature meronts. Pigment granules are small (< 0.5 μm), dark-brown or black, usually grouped in young meronts (Fig. 2e), clumped and difficult to calculate in mature meronts (Fig. 2g–k). Mature meronts produce up to 22 merozoites (Table 1), which are usually arranged haphazardly (Fig. 2j, k). Growing and mature meronts markedly displace nuclei of

(See figure on next page.)

Fig. 2 *Plasmodium relictum* (lineage pPHCOL01) from the blood of Common chiffchaff *Phylloscopus collybita*. **a**–**d**—trophozoites; **e**–**k**—erythrocytic meronts; **l**–**t**—macrogametocytes; **u**–**y**—microgametocytes. Long arrows—parasite nuclei. Short arrows—vacuoles. Arrowheads—pigment granules. Triangle wide arrow—nucleolus. Giemsa-stained thin blood films. Scale bar = 10 μm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infected erythrocytes (Fig. 2e–k) and sometimes enucleate the host cells. Meronts were uncommon in peripheral circulation.

Macrogametocytes

Figure 2l–t. Predominate in peripheral circulation; they develop in mature erythrocytes. Growing and mature gametocytes are markedly variable in form, with roundish (Fig. 2o, p), oval (Fig. 2m, r–t) and various irregular shapes (Fig. 2n) present. Numerous growing and mature gametocytes adhere to the nuclei of erythrocytes (Fig. 2m, o, p, r, s). Gametocytes adhering to the erythrocyte nuclei predominate, but the gametocytes, which do not touch nuclei of erythrocytes were also seen (Fig. 2n, q). Small vacuoles were reported in the cytoplasm occasionally (Fig. 2l, p, s). Parasite nucleus is prominent, of irregular shape; nucleolus is readily visible (Fig. 2o). Pigment granules are small (< 0.5 µm) or of medium size (0.5–1 µm), black or dark-brown, mainly roundish or oval (Fig. 2n, p, q), but elongate pigment granules were seen occasionally (Fig. 2r); pigment granules are scattered in the cytoplasm (Fig. 2n, r, t) or sometimes grouped (Fig. 2o, q). Gametocytes markedly deform the infected red blood cells and displace their nuclei toward one of poles of the host cells; they often enucleate the infected cells (Fig. 2t).

Microgametocytes

Figure 2u–y. General configuration and other features are as for macrogametocytes, with usual haemosporidian sexual dimorphic characters, which are the pale staining of the cytoplasm and the diffuse large nuclei. Irregular-shape mature gametocytes are common (Fig. 2w–y).

Remarks

Examination of all blood films with the *P. relictum* lineages pSGS1, pGRW4, pGRW11, pLZFUS01 (Fig. 3) revealed the morphological identity of trophozoites, meronts and macro- and microgametocytes of these parasites in all infections that were examined. Number of merozoites in mature erythrocytic meronts of all parasite lineages and different isolates of the same lineage is markedly variable during development in the same and different species of avian hosts; it varied between 10 and 32 merozoites, but most often reported to be between 12 and 24 merozoites in all examined infections. These lineages of *P. relictum* cannot be distinguished based on this character. Additionally, the main morphological forms of blood stages reported in parasites of the new lineage pPHCOL01 (Fig. 2) were seen in blood films with single infection of all other lineage of *P. relictum* in the same and different species of avian hosts (Fig. 3). Variation in shape of each blood stage of *P. relictum* occurs, but all observed morphological forms of blood stages (Figs. 2a–y, 3a–x) were seen in parasites belonging to each examined parasite lineage. In other words, the morphological forms of all blood stages (trophozoites, growing and mature meronts, growing and mature gametocytes) in all examined *P. relictum* lineages were indistinguishable.

Interestingly, two different isolates of the lineage GRW4 (the Hawaiian strain and the strain isolated in Europe) produced indistinguishable trophozoites, meronts and gametocytes during development in canaries. Additionally, extensive microscopic examination showed that morphological and morphometric characters of blood stages of the widespread lineages pGRW4 and pSGS1 were variable during development in same and different avian hosts, and they markedly overlapped among these lineages. In other words, blood stages of the lineages pSGS1 and pGRW4 were indistinguishable from each other during their development in canary and other avian hosts (see Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3, Additional file 4: Figure S4).

Exo-erythrocytic development

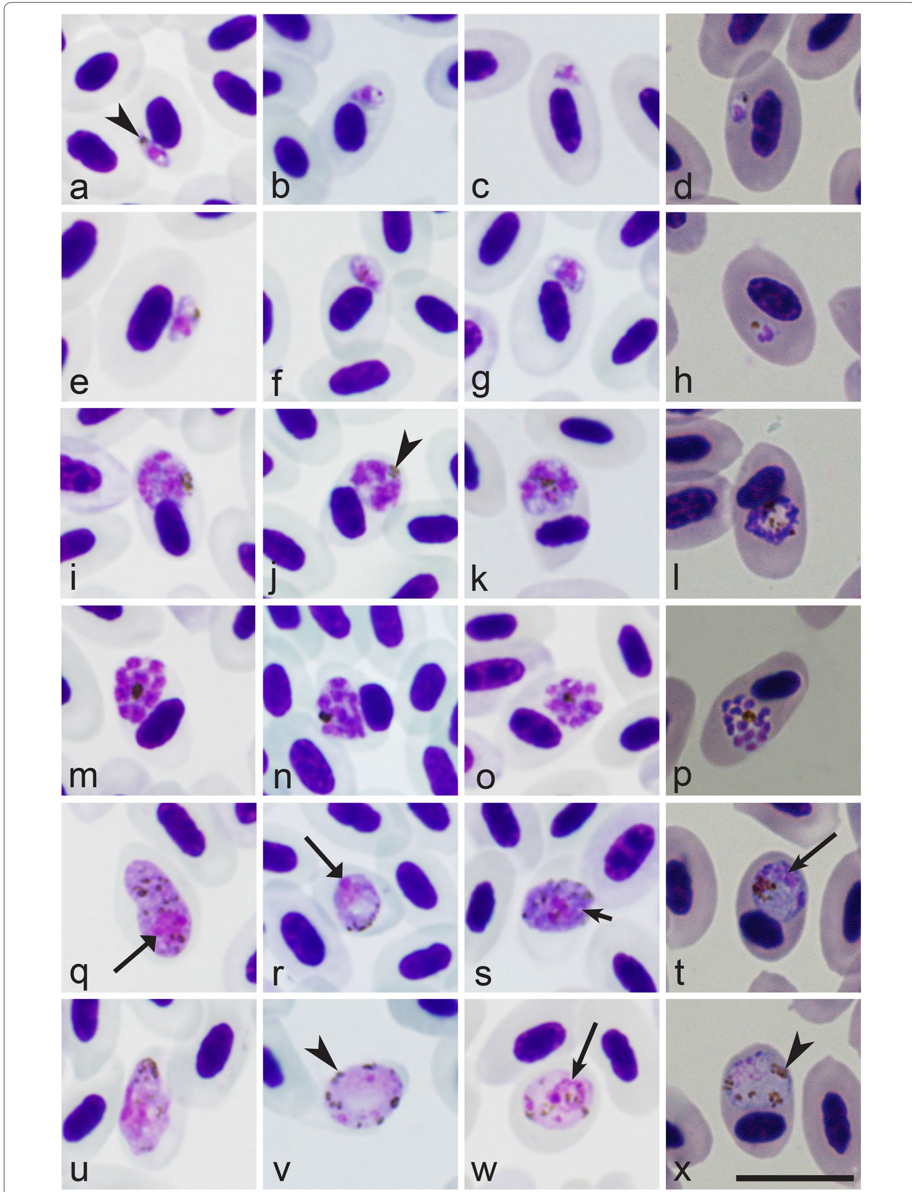
Exo-erythrocytic stages were readily visible in a positive control, assuring that the ISH protocol worked. Microscopic examination of the histological sections stained with H&E and the same organ sections treated for ISH did not reveal tissue stages of the parasite lineage pPHCOL01.

Sporogonic development

Development of ookinetes, oocysts and sporozoites was not observed in exposed *Culex quinquefasciatus* mosquitoes. Only eleven mosquitoes were exposed to pPHCOL01 lineage. Because parasitaemia was barely detectable at sub-microscopic levels in all exposed experimental birds, we were unable to repeat mosquito-infection experiments.

(See figure on next page.)

Fig. 3 *Plasmodium relictum* lineages pSGS1 (**a**, **e**, **i**, **m**, **q**, **u**), pGRW4 (**b**, **f**, **j**, **n**, **r**, **v**), pGRW11 (**c**, **g**, **k**, **o**, **s**, **w**) and pLZFUS01 (**d**, **h**, **l**, **p**, **t**, **x**). **a**–**d**—early trophozoites, **e**–**h**—advanced trophozoites, **i**–**l**—developing meronts, **m**–**p**—mature meronts, **q**–**t**—macrogametocytes, **u**–**x**—microgametocytes. All parasites are from the blood of domestic canary *Serinus canaria*, except for the pLZFUS01 lineage parasites, which are from the blood of Red-backed shrike *Lanius collurio*. Symbols are the same as in Fig. 2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Discussion

Relationship of pPHCOL01 to other lineages of *Plasmodium relictum*

This study demonstrate that the new rare lineage pPHCOL01 can be linked to *P. relictum* on both morphological and molecular grounds and provide new data about specificity and development of this infection in experimentally infected avian hosts. This is the first study to compare morphology of blood stages of different lineages of *P. relictum* using the same methodology. Parasites of all examined lineages are typical representatives of sub-genus *Haemamoeba*, whose inclusive species produce large erythrocytic meronts and gametocytes, both of which markedly influence host cell nuclear position (Figs. 2, 3). Morphological forms of blood stages of the parasite lineage pPHCOL01 found in Common chiffchaff, canary and European goldfinch were indistinguishable morphologically. Extensive comparison of blood stages of other *P. relictum* lineages gave the same results (Figs. 2, 3; Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3, Additional file 4: Figure S4). Morphological characters, which might be used for distinguishing different lineages of *P. relictum,* were not found because of marked variability of these features during each single infection in all parasite lineages. These data are in accordance with former morphological observations on blood stages of the lineages pSGS1, pGRW4 and pLZFUS01 accessed during experimental exposure of different avian hosts [28–30]. The lineages pSGS1, pGRW4, pGRW11, pLZFUS01, and pPHCOLL01 of *P. relictum* belong to the same *P. relictum* morphotype. Interestingly, the same is true for sporogonic stages of the lineages pSGS1, pGRW11 and pGRW4, which complete development in *Culex pipiens* forma *molestus* mosquitoes synchronously and produce morphologically indistinguishable ookinetes, oocysts and sporozoites at same conditions [32, 33, 46].

None of the bird species that were experimentally infected with lineage pPHCOL01 was good host for investigating dynamics of parasitaemia, and they cannot be recommended for experimental research aimed at studying blood stage infections. Canaries were susceptible, but parasitaemia was transient and light. Zebra finches and one Budgerigar were resistant. Interestingly, the available field observations indicate that the latter two avian species are likely resistant to other *P. relictum* lineages as well. Zebra finches and Budgerigars have never been reported as host of *Plasmodium* parasites by microscopic examination of blood films (this method provides opportunities to visualize blood stages), and probably might resist or tolerate many species of malaria parasites [7, 16, 17]. It is worth noting that Baron et al. [47] reported the lineage pGRW4 in New Zealand budgerigars, indicating that these birds were exposed naturally, but provided no information about whether this parasite completed its life cycle and produced erythrocytic infection in this host species. Development of this parasite might be abortive in Budgerigars, as is the case in many haemosporidian infections [48]. Thus, both Zebra finches and Budgerigars might be excellent model hosts for better understanding mechanisms of innate resistance during avian malaria.

Biological variation within *Plasmodium relictum*

Molecular techniques that amplify parasite *cytb* genes provide new opportunities to readily distinguish genetically different isolates of *P. relictum* and to identify infections caused by these parasites in avian hosts. This was impossible during the pre-molecular era of malaria research. Numerous molecular studies reported *P. relictum* in naturally infected birds [19, 23, 24, 34, 49], resulting in a solid body of information about the occurrence of these parasite lineages in various avian hosts and ecosystems all over the world (Table 2). However, comparative research on development and virulence of *P. relictum* lineages in different avian hosts and vectors has lagged behind and remains uncommon. This missing information is an obstacle to developing a better understanding of the biological properties of infections caused by different *P. relictum* lineages, limits the ability to predict disease outbreaks, and makes it more difficult to develop adequate steps for improving bird health and conservation.

Experimental research is essential for better understanding the biology of malaria parasites [5, 8, 11, 33, 50–55]. Controlled experimental studies with *P. relictum* are relatively easy to design due to availability of laboratory-friendly experimental vertebrate hosts (canaries and some species of other common birds), laboratory-colonized susceptible mosquitoes (species of the *Culex pipiens* complex) and worldwide high prevalence in many wild bird species (donors of natural infections). This makes *P. relictum* a convenient and even unique model organism to approach numerous questions about mechanisms of host-parasite interactions, including the immunological aspects during malaria infections [56–58], the ecology and evolution of host-parasite associations [25, 59–63], the host adaptations to tolerate malaria infections [10, 31, 47, 64, 65], patterns of mosquito transmission [32, 46, 53, 66–68] and many other questions.

Unfortunately, experimental information about different lineages of *P. relictum* is still limited, but available data indicate that different lineages and even different isolates of the same lineage might differ remarkably in their ability to develop in different avian hosts and in other biological properties [11]. Brief review what is

Table 2 Polymerase chain reaction-based reports of *Plasmodium relictum* lineages in avian hosts

Lineage code	Record			
	Zoogeographic region[a]	Bird order	Bird family and no. of positive bird species	Total no. of positive bird species
pSGS1	1, 2, 4, 5, 6	Anseriformes	*Anatidae* (2)[b]	115
		Charadriiformes	*Laridae* (3)	
			Recurvirostridae (1)	
			Scolopacidae (1)	
		Ciconiiformes	*Ardeidae* (2)	
		Columbiformes	*Columbidae* (1)	
		Galliformes	*Phasianidae* (4)	
		Gruiformes	*Gruidae* (1)	
		Passeriformes	*Acrocephalidae* (6)	
			Alaudidae (1)	
			Certhiidae (1)	
			Corvidae (5)	
			Emberizidae (8)	
			Estrildidae (1)	
			Fringillidae (10)	
			Furnariidae (1)	
			Hirundinidae (1)	
			Laniidae (1)	
			Motacillidae (1)	
			Muscicapidae (15)	
			Paridae (9)	
			Passeridae (7)	
			Passerellidae (1)	
			Ploceidae (4)	
			Prunellidae (1)	
			Pycnonotidae (3)	
			Scotocercidae (1)	
			Sittidae (1)	
			Sturnidae (2)	
			Sylviidae (8)	
			Thaupidae (1)	
			Troglodytidae (2)	
			Turdidae (2)	
			Tyrannidae (2)	
		Procellariiformes	*Procellariidae* (1)	
		Sphenisciformes	*Spheniscidae* (1)	
		Strigiformes	*Strigidae* (1)	
		Trochiliformes	*Trochilidae* (2)	
pGRW11	1, 2, 6	Charadriiformes	*Scolopacidae* (1)	41
		Galliformes	*Phasianidae* (2)	
		Passeriformes	*Acrocephalidae* (3)	
			Alaudidae (1)	
			Cettiidae (1)	
			Corvidae (3)	
			Emberizidae (1)	
			Fringillidae (3)	
			Hirundinidae (2)	
			Laniidae (1)	

Table 2 continued

Lineage code	Record			
	Zoogeographic region[a]	**Bird order**	**Bird family and no. of positive bird species**	**Total no. of positive bird species**
			Muscicapidae (6)	
			Paridae (4)	
			Passeridae (3)	
			Pycnonotidae (1)	
			Sylviidae (8)	
			Troglodytidae (1)	
pGRW4	1, 2, 3, 4, 5, 6, 7	Ciconiiformes	*Ardeidae* (1)	72
		Passeriformes	*Acrocephalidae* (10)	
			Bernieridae (2)	
			Cisticolidae (2)	
			Estrildidae (4)	
			Fringillidae (6)	
			Hirundinidae (3)	
			Locustellidae (2)	
			Mitidae (2)	
			Muscicapidae (10)	
			Nectariniidae (4)	
			Notiomystidae (1)	
			Paridae (2)	
			Passeridae (2)	
			Philepittidae (1)	
			Ploceidae (6)	
			Promeropidae (1)	
			Pycnonotidae (1)	
			Sylviidae (1)	
			Thraupidae (1)	
			Timaliidae (1)	
			Vangidae (1)	
			Zosteropidae (7)	
		Psittaciformes	*Psittacidae* (1)	
pLZFUS01	1, 2, 3, 5	Passeriformes	*Laniidae* (3)	6
			Parulidae (1)	
			Ploceidae (1)	
			Pycnonotidae (1)	
pPHYCOL01	1	Passeriformes	*Phylloscopidae* (1)	1

Modified from MalAvi database (http://www.iucnredlist.org/details/103843725/0)

[a] Zoogeographic regions: 1—Palaearctic, 2—Afrotropic, 3—Nearctic, 4—Neotropic, 5—Indo-Malay, 6—Australasian, 7—Oceanic (borders of the regions were considered according to http://users.tamuk.edu/kfjab02/Biology/Mammalogy/mammalogy_zoogeography.htm)

[b] Number of species is given in parenthesis

known about this biological variation is given in the following sections.

Pathology

The pathology of known lineages of *P. relictum* is highly variable in host species or incompletely known. For example, the same *P. relictum* lineage might cause severe disease in one species of avian host, but other bird species might be tolerant or even resistant [5, 8, 50, 69]. Experimental observations show that the same isolate of pSGS1 behave markedly differently in different species of birds, with the susceptibility ranging from complete resistance to light subclinical (< 0.1%) and high (> 10%) parasitaemia [8, 69]. The variation in parasitaemia dynamics and maximum intensity are often great in different individuals of the same bird species infected with pSGS1 parasite [55].

Similarly, the susceptibility of same bird species to different isolates of the same *P. relictum* lineage also might be markedly different. For example, Hawaiian isolates of pGRW4 readily infect canaries, with maximum parasitaemia ranging from light (about 0.1%) to high (up to 30% and greater) reported in birds exposed by inoculation of infected blood ([70], CTA, pers comm.). However, this bird species was either resistant or had mainly light (< 0.1%) and transient parasitaemia, which rapidly turned to chronic or even latent stages of infection after exposure to European isolates of the same parasite lineage by the same mode of infection ([11], GV, unpublished observation).

It remains unclear why different geographical isolates of the same lineage of *P. relictum* (pGRW4) behave so differently in the same species of birds. The differences between different geographic isolates of *P. relictum* lineages might be due to different clonal intra-lineage genetic diversity, which is great in Hawaiian strains of the lineage pGRW4, but remains insufficiently documented in European isolates of the same lineage [21, 31]. Marked variation in the susceptibility of same experimental bird species to different parasite lineages provide opportunities to use this host-parasite model system for comparative research aimed at a better understanding of the genetic mechanisms of tolerance and virulence during parasitic infections.

Without question, the lineages pSGS1 and pGRW4 are virulent in birds and can cause marked blood pathology and even mortality in susceptible hosts [5, 8, 11, 29, 50, 69]. The negative effects of *P. relictum* (pSGS1) on bird physiological parameters and behaviour are documented due to delicate experimental studies [54, 55]. Observations of infected, naive birds in zoos and rehabilitation centres provided evidence of the severity of disease caused by these and related parasite lineages in wild birds [71–74]. These studies are the basis of understanding the predictions and conclusions of field observations about negative influence of *P. relictum* on population decline or even extinction, particularly on oceanic islands [63, 75–78]. However, to evaluate the true virulence of a malaria parasite lineage in certain avian host species, experimental and field observations are needed, ideally in each targeting host-parasite system separately.

Even though there are numerous reports of exo-erythrocytic stages of *P. relictum* from the pre-molecular research era [1, 7, 13, 84], information about these stages and associated tissue pathology in avian hosts is still absent for parasites of all lineages of *P. relictum*. This is an obstacle to understanding of the mechanisms of persistence in birds, as well as, the association between tissue merogony and pathogenicity caused by different parasite lineages in different avian hosts. This study shows that exo-erythrocytic stages of *P. relictum* can be difficult to find during chronic infections even in experimentally infected birds with visible parasitaemia. This indicates that large multinuclear tissue stages, which are easy to see under light microscopy [6, 13], might persist for a short time and their development might be markedly dependent on the stage of infection. Application of in situ hybridization methods is promising in the investigation of tissue merogony of haemosporidians [6, 37, 78], but may not be sensitive enough to detect uninuclear hypnozoite-like intracellular stages should they occur in *P. relictum*, as is the case in human *Plasmodium vivax* infection. This suggests application of more sensitive immunofluorescent diagnostic techniques in parallel with traditional histology and in situ hybridization methods in research of exo-erythrocytic development of different lineage parasites [1, 6, 35, 37, 78].

Observation of parasites in blood films and determination of morphological characters of their blood stages remain important not only in identification of haemosporidian species [11, 27, 79], but also for distinguishing competent and abortive haemosporidian infections, which might have different consequences for the bird health. During abortive infections, the parasites might circulate within avian hosts as sporozoites or even undergo partial development within non-erythroid tissues, providing templates for PCR amplification, but the parasite would not be able to complete its life cycle due to an inability to enter red blood cells. This would result in absence of gametocytes and other blood stages in the circulation, but severe disease might occur due to damage of internal organs [48]. In the latter case, a positive PCR signal might be obtained, but parasitaemia would be absent or barely detectable due to difficulties in microscopic detection of remnants of tissue stages in the circulation [80–82]. This highlights the relevance of microscopic detection of blood stages and knowledge about morphological features of haemosporidians in pathology and epidemiological studies when used in parallel with molecular diagnostic tools.

Pre-patent period and parasitaemia

Longevity of the prepatent period cannot be used for distinguishing infections caused by different *P. relictum* lineages. Duration of the prepatent period following sporozoite-induced infection of different lineages of *P. relictum* remains largely undetermined. Prepatent periods have been observed in the Hawaiian parasite lineages pGRW4 where it was within 4 dpe in Iiwi *Drepanis coccinea* and 8 dpe in Hawaii Amakihi *Chlorodrepanis virens* [5, 50]. The prepatent period was about 5 dpe after sporozoite-induced infection of unknown lineage of *P. relictum* in canaries [83, 84].

This study demonstrated that prepatent period of infection is markedly variable in different bird species and individuals of the same species during blood-induced infection of the lineage pPHCOL01. The prepatent period is often about 1 week after the blood-induced infections of pSGS1, but varies markedly in different species of avian hosts and even individuals of the same species even after the same mode and dose of infection, and it might be as long as several weeks after infected blood-induced exposure, indicating the possibility of parasite persistence in internal organs [7, 8, 13, 69, this study].

In all investigated lineages of *P. relictum*, parasitaemia was asynchronous, with trophozoites, growing and mature meronts as well as gametocytes present in the same blood films at the same time in all species of exposed birds at any stage of parasitaemia [8, 29, 30, 33, 70, this study]. This provides opportunities to design vector research with all lineages at any stage of parasitaemia using susceptible avian hosts as donors of infections to expose mosquitoes, but all work carried out to date with different lineages has failed to demonstrate significant differences.

Host range

An interesting finding of this study is that canaries may not be suitable experimental hosts for all lineages of *P. relictum* and possibly not even isolates of the same lineage. Information about susceptibility of canaries to lineage pLZFUS01 is absent; further experimental studies are needed. This study indicates that canaries can tolerate the pPHCOL01 infection, during which light transient parasitaemia occurs and signs of illness have not been reported. Canaries are good experimental hosts for the lineages pSGS1, pGRW11 and pGRW4 due to long-lasting parasitaemia (usually, several months before latency, with infected birds maintaining infections for several years, with occurring seasonal relapses).

However, infectivity and patterns of development of different lineages and even different isolates of the same lineage might be different, sometimes significantly in canary [11, 70]. A moderate to high (> 0.1% and greater) long-lasting (several months) parasitaemia usually develops during infections with lineages pSGS1 and pGRW11 in canaries exposed by inoculation of infected blood [22, 32, 46]. The same is true for the parasite lineage pGRW4 during development in canaries, but not for all its isolates. For example, the Hawaiian and European isolates of the lineage pGRW4 develop differently in canaries. Hawaiian pGRW4 isolates develop naturally in canaries when caged birds are exposed in habitats with active natural transmission and can develop high (up to 30% and higher) long-lasting parasitaemia after sub-inoculation of infected blood, although significant individual variation is present ([70], CTA, unpublished data). Attempts to induce a long-lasting parasitaemia (several weeks or longer) and gametocytaemia exceeding 0.01% with European isolates of lineage pGRW4 were either completely unsuccessful (compete resistance was recognized in nine exposed birds) or only partially successful with extremely light transient parasitaemia (few gametocytes reported after examination of 100 microscopic fields at high magnification in four birds) ([11], GV, unpublished data). In other words, the canary is not a good host for experimental studies of erythrocytic infections with the European isolates of the lineage pGRW4, but can be used in experiments with the Hawaiian isolate. Experimental studies with other geographical isolates of *P. relictum* (pGRW4) infection have not been performed. Due to relative resistance of canaries to European isolates of lineage pGRW4, Eurasian siskin *Carduelis spinus* has been used in experiments with this parasite lineage, and this species is an excellent experimental host [33].

Hybridization and gene flow

The lineages pSGS1, pGRW4, pGRW11, pLZFUS01, and pPHCOL01 of *P. relictum* are closely related based on similarities in *cytb* sequence (Fig. 1) and cannot be distinguished by morphology (Figs. 2, 3, Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3, Additional file 4: Figure S4). Do these lineages represent distinct species of the *P. relictum* group or are they different genetic variants of the same morpho-species? Do parasites of these lineages maintain the ability to mate? Does the available information provide opportunities to approach answering these questions? This study and available experimental observations [28–30, 32, 33, 46] show that morphological data both of blood and vector stages cannot help in distinguishing parasites of the lineages pSGS1, pGRW4, pGRW11, pLZFUS01, pPHCOL01, indicating that they might belong to the same *P. relictum* morphotype, but some of them also might represent cryptic species of the *P. relictum* group.

Between-lineage hybridization experiments provide opportunities to obtain direct information about the possibility that different lineages of haemosporidian parasites can mate and exchange genetic information. Sexual processes and between-lineage hybridization of *Haemoproteus* parasites (sister genus to *Plasmodium*) can be readily induced in vitro [7]. These experiments indicate probable development of between-lineage *Haemoproteus* parasite hybrids in vitro, which can be readily distinguished morphologically on ookinete stage, but genetic information is lacking, primarily due to obstacles in accessing nuclear genetic information from single cells [85]. A recent molecular study [86] revealed that *cytb* lineages belonging to *Haemoproteus majoris* have unique alleles

in 4 investigated nuclear genes and may represent cryptic species. These lineages of *Haemoproteus majoris* are closely related and differ by only 1–6 substitutions over the 479 bp of sequenced *cytb* gene (0.2–1.3% difference). By contrast, an experimental observation in vivo [22] has demonstrated that parasites of the closely related lineages pSGS1 and pGRW11 can mate in mosquitoes *Culex pipiens* forma *molestus* and produce hybrid oocysts. Genetic differences between these lineages in the *cytb* gene are small (0.2%). According to hybridization experiments [22], the parasites of the lineages pSGS1 and pGRW11 are different variants of the same species, but information about hybridization of other lineages of *P. relictum* and other avian haemosporidian parasites is absent.

It is worth noting that partial sequences of merozoite surface protein 1 (msp1) gene were determined in 3 *P. relictum* lineages (pSGS1, pGRW11, pGRW4) in samples collected from different geographic sites using nuclear markers [21]. All three lineages were from markedly randomly sampled birds, with unclear geographical origin of infection. Four different alleles were reported in the lineage pSGS1, and three of them were shared with the lineage pGRW11, indicating possible hybridization. This is in accordance with the available experimental observations [22]. However, five different alleles were revealed in the lineage pGRW4 [21], suggesting the lack of gene flow between parasites of this lineage and the lineages pSGS1 and pGRW11. However, due to the markedly random sampling (many lineage isolates came from different species of African migrants with unclear geographical origin of infection), it is difficult to rule out that the reported genetic difference might reflect strain varieties, but not species differences. Additionally, due to common co-infections of malaria parasites in naturally infected hosts and possible selective amplification of different lineages using general primers [87], it is possible that some samples contained co-infections of different lineages. Because of this, the possibility to create between-lineage nuclear gene artefacts cannot be ruled out as well. In other words, the quality of the haemosporidian sequences should be carefully considered if samples from wildlife are used [88].

Plasmodium relictum is a unique among malaria parasites in regard to the enormous range of its avian hosts and mosquito species involved in its transmission. Therefore, direct in vivo experimental hybridization of different *P. relictum* lineages [22] would be most useful if they involved lineage isolates which are transmitted at the same site by the same mosquito species as this would make experimental studies closer to real epidemiological situations that are observed in wildlife.

Geographic distribution and prevalence

Data about vertebrate host and geographical distribution of different *P. relictum* lineages are summarized in Table 2. The lineages pLZFUS01, pPHCOL01 of *P. relictum* have been reported occasionally, mainly in birds wintering or resident in tropical countries where transmission occurs [30, this study). The parasite lineage pGRW4 has both broad host and worldwide geographical distribution, but is rare in Europe [2, 11, 21, 33]. The lineage pSGS1 and pGRW11 are also broadly distributed, but neither has been reported in several extensive studies in the mainland Americas [2, 21, 89–91]. However, Marzal et al. [3] found *P. relictum* (pSGS1) in 8 native bird species belonging to two orders in Peru, and Quillfeldt et al. [92] reported this parasite in seabirds on Falkland Islands, indicating presence of transmission, at least in South America.

The reported differences in geographical distribution of the lineages pSGS1 and pGRW11 on the one hand, and GRW4 on the other hand are difficult to explain bearing in mind the enormously broad range of their susceptible avian hosts (Table 2) and mosquito vectors, such as the globally distributed *Culex pipiens, Culex quinquefasciatus* and other mosquito species of the *Culex pipiens* complex, which are of global distribution [93–95]. It is worth noting that recent experimental studies have demonstrated complete sporogony of the pGRW4 parasites from European birds, in cosmopolitan *Culex pipiens* forma *molestus* mosquitoes at relatively low temperatures. This indicates that there are no obstacles preventing transmission of this infection in Europe during the warm period of the year [33]. The following explanations of the observed phylogeographic data are worth discussion.

First, the existence of still unclear mechanisms of geographically related limitations in transmission of the parasite lineages pSGS1 and pGRW4 cannot be ruled out. However, the observed results in the phylogeography of these parasites might also originate, at least in part, from bias in DNA amplification of different lineages during co-infections while using general primers [87]. Failure in detection of mixed infections of *Plasmodium* parasites have often been reported [41, 87, 96–98], but have not been investigated among *P. relictum* lineages. In other words, a sensitive issue is that the majority of available studies on *P. relictum* used only general primers for haemosporidian parasite DNA amplification. Such primers are selective and often do not indicate the presence of co-infection of parasites of different lineages [87]. Parasite lineage-specific primers have not been applied in phylogeographic studies of *P. relictum* lineages pSGS1 and pGRW4 and others so far. It remains unclear whether some *P. relictum* lineages are preferably amplified over

others, particularly in cases of co-infections of different lineages. Relatively simple experimental studies using the protocol by Bernotienė et al. [87] might be helpful in answering this question. Co-infections of malaria parasites are common and even predominate in some bird populations [87, 96, 97]. This information is essential for better understanding of true distribution of *P. relictum* lineages both by hosts and geographically. Application of specific primers might contribute to better understanding patterns of geographical distribution of these invasive bird infections.

Second, parasite prevalence data depend on both force of infection and the longevity of infection. If local transmission is occurring, the low prevalence of GRW4 infection in European bird populations might be a result of (1) mortality of some European birds due to this infection, as is the case with some endemic Hawaiian birds [1]; (2) resistance and ability of some bird species to tolerate the pGRW4 malaria infection [11]; or, (3) a combination of these two factors. Naive Hawaiian and New Zealand endemic birds suffer mortality from infection with *P. relictum* pGRW4 [5, 50, 75, 77, 99, 100], but introduced bird species are less susceptible and might tolerate this disease [5, 50, 70]. Little is known about the virulence of the pGRW4 infection in resident European birds and other birds worldwide [33]. Preliminary observations indicate that several European bird species (*Fringilla coelebs, Sylvia atricapilla, Passer domesticus*) can resist pGRW4 strains, which were isolated from African migrating Great read warblers *Acrocephalus arundinaceus* [11]. Further experimental studies and application of lineage specific primers might provide more certain information about distribution of these parasite lineages, their co-existence in the same avian hosts and study sites, and better understanding infections in bird health.

Vector research

The list of mosquito species, which are susceptible to *P. relictum* includes over 20 species [7, 13], however, information about vectors at parasite lineage levels is insufficient [101]. Widespread *Culex pipiens*, *Culex quinquefasciatus* and *Culex tarsalis* mosquitoes are excellent vectors for pSGS1, pGRW4, pGRW11 [22, 32, 33, 46, 52, 102–105], but data about vectors of the pLZFUS01 and pPHCOLL01 parasites are absent. It is interesting to note that mosquitoes belonging to three genera, *Aedes albopictus*, *Wyeomyia mitchellii* and *Culex quinquefasciatus*, are susceptible to the pGRW4 parasite, and the sporogony was completed in all these mosquito species, but prevalence varied significantly between species. The latter mosquito is the main vector, but other mosquito species might be involved in transmission as well [106]. However, it worth mentioning that, while sporogony was completed in a small fraction of *Wyeomyia mitchellii*, the authors [106] did express doubt in the viability of aberrant sporozoites in this mosquito species.

Culex quinquefasciatus is absent in Lithuania. This insect was used in experiments because the new *P. relictum* lineage (pPHCOL01) was isolated from a bird species wintering in Africa where *Culex quinquefasciatus* is widespread [93, 94]. Sporogony of the parasite lineage pPHCOL01 was not initiated in *Culex quinquefasciatus* probably because the donor bird has light gametocytaemia (single gametocytes were seen in donor canaries during mosquito exposure), and that might have been the main obstacle.

Numerous mosquitoes were incriminated as possible *P. relictum* vectors using microscopic methods, but mainly only oocysts were reported in the majority of the studied insects, and the development of sporozoites were accessed in a few species [101]. This questions the conclusions about true possibility and involvement of mosquitoes belonging to different genera to act as effective vectors of *P. relictum* in wildlife. More delicate studies, including the observation of sporozoites in the salivary gland are needed to reach conclusions about ability of certain mosquito species to act as vectors. It is important to note that even presence of sporozoites of *Plasmodium* parasites in salivary glands does not always guarantee that the insects can transmit infection by bite. For example, sporozoites of *Plasmodium hermani* were reported in mosquito *Wyeomyia vanduzeei*, and these sporozoites were used successfully to induce infection in turkeys by syringe inoculation, but this mosquito was unable to transmit infection by bite [107]. This example calls for more delicate vector studies for better understanding transmission of avian haemosporidians. Determination of vectors is time consuming in wildlife studies where diversity of blood-sucking dipteran insects is high. The PCR-based reports of *P. relictum* lineages in wild-caught dipteran insects markedly speed search for possible vectors by indicating significant links between insects, avian hosts and parasites [103, 104, 108–118], but cannot prove that sporozoites develop and can be transmitted by the PCR-positive insects. The observation of *Plasmodium* spp. sporozoites in salivary glands and the studies of transmission by mosquito bites remain the gold standards for determining vector competence. Combination of molecular diagnostic, experimental procedures and microscopic tools remain essential in haemosporidian vector research [33, 46, 101, 106, 119–121].

Conclusion

Plasmodium relictum is a unique species among the large group of parasites causing malaria due to its cosmopolitan distribution and exceptionally broad range

of avian hosts and mosquito vectors. These characteristics make the various *P. relictum* lineages exceptional model organisms for better understanding ecological and genetic mechanisms that make generalist pathogens so successful.

Five lineages of *P. relictum* (pSGS1, pGRW4, pGRW11, pLZFUS01, pPHCOL01) have been identified and partially characterized. Parasites of these lineages are phylogenetically closely related, and they cannot be distinguished using morphological characters of their blood or vector stages. Available data show that the same lineages develop markedly differently in different avian hosts. Remarkably, variation among biological properties (prepatent period, parasitaemia dynamics, blood pathology) between different isolates of the same lineage might be greater than the variation between different lineages during their development in the same species of avian host. This indicates the negligible value of these features for diagnosing specific parasite lineages. Currently, the lineages of *P. relictum* can be readily distinguished mainly through mtDNA sequences.

Malaria caused by *P. relictum* is of particular importance for bird health. Controlled laboratory experimental studies show that the lineages pSGS1 and pGRW4 are virulent in birds and can cause marked blood pathology and even mortality in susceptible hosts. However, the exo-erythrocytic stages and tissue pathology caused by them in avian hosts is unknown for parasites of all lineages of *P. relictum*. This is a prominent obstacle for development of the effective prevention and treatment options for birds.

Certainly, more research is needed on biology of *P. relictum* lineages. The existence of still unclear geographically related limitations in transmission of the most prevalent lineages pSGS1 and pGRW4 has been often suspected in explanation of the restricted distribution of these parasites globally. However, methodological issues in the diagnosis of these parasite lineages remain and limit our ability to study co-infections in broadly distributed lineages of *P. relictum*. The information about frequency of co-infection occurrence in lineages of *P. relictum* is inadequate. Mainly general primers have been applied in PCR-based detection and phylogeographic studies of *P. relictum*, and this method is insufficiently sensitive in determining haemosporidian co-infections. It is predicted that available information about both host and geographical distribution of these lineages might be significantly updated if more sensitive diagnostic tools are applied for distinguishing co-infections of these and other *P. relictum* lineages.

Although closely related lineages of *P. relictum* can hybridize, within-species diversity may also indicate the presence of possible cryptic speciation in the *P. relictum* group. Speciation processes have been insufficiently addressed in experimental parasitology studies mainly because of difficulties in accessing and measuring mate-recognition signals in parasites. By focusing on the extracellular sexual process of oogamy, which can be readily visualized both in vivo and in vitro, and the development of oocysts possessing numerous copies of nuclear genes, experimental hybridization can be readily accessed using haemosporidian parasite lineages [22, 33]. Methodologies of between-lineage hybridization of avian *Plasmodium* parasites as well as sister *Haemoproteus* species have been developed [22, 85]. It is important to gain more information about true range of cryptic speciation in pathogens, particularly due to increasingly frequent outbreaks of zoonotic infections, which appear after host switching, leading to the emergence of new diseases [1, 6, 34, 75]. Such studies would also provide directions on how to approach future taxonomic reconstructions on species levels in the genus *Plasmodium* and other haemosporidians. Phylogenetic analysis based on partial *cytb* sequences placed different lineages of *P. relictum* in a tight cluster. Importantly, parasites of these lineages often occur in sympatry in many cases thus, are convenient model organisms to answer questions about the range of cryptic speciation in wildlife malaria and other related haemosporidian parasites.

Additional files

Additional file 1: Figure S1. Mature erythrocytic meronts of the lineage pGRW4 of *Plasmodium relictum* in Hawaiian (a–t) and European (u–x) isolates during development in naturally infected Apapane *Himatione sanguinea* (a–h) and experimentally infected domestic canary *Serinus canaria* (i–x). Note that the size and shape of mature meronts, number of nuclei in them, influence of the meronts on host cells are markedly variable and overlap in both isolates. Meronts of both isolates cannot be distinguished by morphological characters and patterns of their influence on host cells during their development in the same and different avian hosts. Furthermore, meronts of the lineages pGRW4 cannot be distinguished from meronts of the lineage pSGS1 (see Additional file 2: Figure S2). Arrowheads—pigment granules. Giemsa-stained thin blood films. Scale bar = 10 µm.

Additional file 2: Figure S2. Mature erythrocytic meronts of the lineage pSGS1 of *Plasmodium relictum* in European isolate during development in experimentally infected Eurasian siskin *Carduelis spinus* (a–h) and domestic canary *Serinus canaria* (i–t). Note that size and shape of mature meronts, number of nuclei in them, influence of meronts on host cells are markedly variable. Meronts of this parasite lineage cannot be distinguished by morphological characters and patterns of their influence on host cells during their development in different avian hosts. Furthermore, meronts of the lineages pSGS1 cannot be distinguished from meronts of the lineage pGRW4 (see Additional file 1: Figure S1). Arrowheads—pigment granules. Giemsa-stained thin blood films. Scale bar = 10 µm.

Additional file 3: Figure S3. Mature macrogametocytes (a–o) and microgametocytes (p–x) of the lineage pGRW4 of *Plasmodium relictum* in Hawaiian (a–j, p–v) and European (k–o, w, x) isolates during development in naturally infected Apapane *Himatione sanguinea* (a–f, p–s) and experimentally infected domestic canary *Serinus canaria* (g–j, k–o, t–x). Note that

size and shape of mature gametocytes, number and position of pigment granules, morphology of parasite nuclei and influence of gametocytes on host cells are markedly variable and overlap in both isolates. Gametocytes of both isolates cannot be distinguished by morphological characters and patterns of their influence on host cells during their development in the same and different avian hosts. Furthermore, mature gametocytes of the lineage pGRW4 cannot be distinguished from mature gametocytes of the lineage pSGS1 (see Additional file 4: Figure S4). Long arrows—parasite nuclei. Short arrow—vacuole. Arrowheads—pigment granules. Triangle wide arrow—nucleolus. Giemsa-stained thin blood films. Scale bar = 10 µm.

Additional file 4: Figure S4. Mature macrogametocytes (a–k) and microgametocytes (l–t) of the lineage pSGS1 of the European isolate of *Plasmodium relictum* during development in experimentally infected Eurasian siskin *Carduelis spinus* (a–g, l–n) and domestic canary *Serinus canaria* (h–k, o–t). Note that size and shape of mature gametocytes, number and position of pigment granules, morphology of parasite nuclei and influence of gametocytes on host cells are markedly variable and overlap during development in different avian hosts. Mature gametocytes of the lineage pSGS1 cannot be distinguished from mature gametocytes of the lineage pGRW4 (see Additional file 3: Figure S3). Long arrows—parasite nuclei. Short arrow—vacuole. Arrowheads—pigment granules. Triangle wide arrows—nucleoli. Giemsa-stained thin blood films. Scale bar = 10 µm.

Authors' contributions
GV designed this study and wrote the manuscript. GV, MI, DB, TAI did field and experimental work. TAI and GV analyzed morphological data. DB carried out vector research. MI, KF, HW did histological and in situ hybridization investigations. CA provided data on the Hawaiian lineage pGRW4. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

Ethics approval and consent to participate
Experimental procedures were performed by licensed researchers and were approved by the Ethical Commission of the Baltic Laboratory Animal Science Association, Lithuania; Lithuanian State Food and Veterinary Office (2015-05-07, no. G2-27); Environmental Protection Agency, Vilnius (2017-04-26, no. 23); and the International Research Cooperation Agreement between the Zoological Institute of the Russian Academy of Sciences and Institute of Ecology of Nature Research Centre (2015-09-04).

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References

1. Atkinson CT, Thomas NJ, Hunter DB. Parasitic diseases of wild birds. Oxford: Wiley-Blackwell; 2008.
2. Beadell JS, Ishtiaq F, Covas R, Melo M, Waren BH, Atkinson CT, et al. Global phylogeographic limits of Hawaii's avian malaria. Proc R Soc Lond B Biol Sci. 2006;273:2935–44.
3. Marzal A, García-Longoria L, Cárdenas Callirgos JM, Sehgal RN. Invasive avian malaria as an emerging parasitic disease in native birds of Peru. Biol Invasions. 2014;17:39–45.
4. Drovetski SV, Aghayan SA, Mata VA, Lopes RJ, Mode NA, Harvey JA, et al. Does the niche breadth or trade-off hypothesis explain the abundance—occupancy relationship in avian Haemosporidia? Mol Ecol. 2014;23:3322–9.
5. Atkinson CT, Woods KL, Dusek RJ, Sileo LS, Iko WM. Wildlife disease and conservation in Hawaii: pathogenicity of avian malaria (*Plasmodium relictum*) in experimentally infected iiwi (*Vestiaria coccinea*). Parasitology. 1995;111(Suppl):S59–69.
6. Ilgūnas M, Bukauskaitė D, Palinauskas V, Iezhova TA, Dinhopl N, Nedorost N, et al. Mortality and pathology in birds due to *Plasmodium* (*Giovannolaia*) *homocircumflexum* infection, with emphasis on the exoerythrocytic development of avian malaria parasites. Malar J. 2016;15:256.
7. Valkiūnas G. Avian malaria parasites and other Haemosporidia. Boca Raton: CRC; 2005.
8. Palinauskas V, Valkiūnas G, Bolshakov CV, Bensch S. *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. Exp Parasitol. 2008;120:372–80.
9. Loiseau C, Zoorob R, Robert A, Chastel O, Julliard R, Sorci G. *Plasmodium relictum* infection and MHC diversity in the house sparrow (*Passer domesticus*). Proc Biol Sci. 2011;278:1264–72.
10. Larcombe S, Bichet C, Cornet S, Faivre B, Sorci G. Food availability and competition do not modulate the costs of *Plasmodium* infection in dominant male canaries. Exp Parasitol. 2013;135:708–14.
11. Dimitrov D, Palinauskas V, Iezhova TA, Bernotienė R, Ilgūnas M, Bukauskaitė D, et al. *Plasmodium* spp.: an experimental study on vertebrate host susceptibility to avian malaria. Exp Parasitol. 2015;148:1–16.
12. Grassi B, Feletti R. Malariaparasiten in den Vögeln. Centralbl Bakteriol Parasitenkd. 1891;9:403–9, 429–33, 461–7.
13. Garnham PCC. Malaria parasites and other Haemosporidia. Oxford: Black-well; 1966.
14. Johnston HT, Cleland JB. Notes on some parasitic Protozoa. Proc Linn Soc NSW. 1909;34:501–13.
15. Brumpt E. Paludisme aviaire: *Plasmodium paddae* n. sp. du calfat (*Padda oryzivora*). Utilisation de ce parasite pour les recherches chimiothérapiques du paludisme. C R Hebd Séances Acad Sci. 1935;200:967–70.
16. Bennett GF, Whiteway M, Woodworth-Lynas C. A host-parasite catalogue of the avian haematozoa. Mem Univ Nfld Occ Pap Biol. 1982;5:1–243.
17. Bishop MA, Bennett GF. Host-parasite catalogue of the avian haematozoa: supplement 1, and bibliography of the avian blood-inhabiting haematozoa. Mem Univ Nfld Occ Pap Biol. 1992;2:1–244.
18. Bensch S, Stjernman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, et al. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc Biol Sci. 2000;276:1583–9.
19. Bensch S, Hellgren O, Pérez-Tris J. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol Ecol Resour. 2009;9:1353–8.
20. Marzal A, Ricklefs RE, Valkiūnas G, Albayrak T, Arriero E, Bonneaud C, et al. Diversity, loss and gain of malaria parasites in a globally invasive bird. PLoS ONE. 2011;6:e21905.
21. Hellgren O, Atkinson CT, Bensch S, Albayrak T, Dimitrov D, Ewen JG, et al. Global phylogeography of the avian malaria pathogen *Plasmodium relictum* based on MSP1 allelic diversity. Ecography. 2015;38:842–50.
22. Palinauskas V, Bernotienė R, Žiegytė R, Bensch S, Valkiūnas G. Experimental evidence for hybridization of closely related lineages in *Plasmodium relictum*. Mol Biochem Parasitol. 2017;217:1–6.
23. Clark NJ, Clegg SM, Lima MR. A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): New insights from molecular data. Int J Parasitol. 2014;44:329–38.

24. Perkins SL. Malaria's many mates: past, present and future of the systematics of the order *Haemosporida*. J Parasitol. 2014;100:11–25.
25. Outlaw DC, Ricklefs RE. Species limits in avian malaria parasites (Haemosporida): how to move forward in the molecular era. Parasitol. 2014;141:1223–32.
26. Hellgren O, Križanauskienė A, Valkiūnas G, Bensch S. Diversity and phylogeny of mitochondrial cytochrome *b* lineages from six morphospecies of avian *Haemoproteus* (Haemosporida: Haemoproteidae). J Parasitol. 2007;93:889–96.
27. Mantilla JS, González AD, Valkiūnas G, Moncada LI, Matta NE. Description and molecular characterization of *Plasmodium* (*Novyella*) *unalis* sp. nov. from the Great Thrush (*Turdus fuscater*) in highland of Colombia. Parasitol Res. 2013;112:4193–204.
28. Palinauskas V, Kosarev V, Shapoval A, Bensch S, Valkiūnas G. Comparison of mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites of the subgenera *Haemamoeba* and *Giovannolaia* (Haemosporida: Plasmodiidae). Zootaxa. 2007;1626:39–50.
29. Valkiūnas G, Zehtindjiev P, Hellgren O, Ilieva M, Iezhova TA, Bensch S. Linkage between mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites, with a description of *Plasmodium* (*Novyella*) *ashfordi* sp. nov. Parasitol Res. 2007;100:1311–22.
30. Ilgūnas M, Palinauskas V, Iezhova TA, Valkiūnas G. Molecular and morphological characterization of two avian malaria parasites (Haemosporida: Plasmodiidae), with description of *Plasmodium homonucleophilum* n. sp. Zootaxa. 2013;3666:49–61.
31. Jarvi SI, Farias ME, Lapointe DA, Belcaid M, Atkinson CT. Next-generation sequencing reveals cryptic mtDNA diversity of *Plasmodium relictum* in the Hawaiian Islands. Parasitology. 2013;140:1741–50.
32. Kazlauskienė R, Bernotienė R, Palinauskas V, Iezhova TA, Valkiūnas G. *Plasmodium relictum* (lineages pSGS1 and pGRW11): complete synchronous sporogony in mosquitoes *Culex pipiens pipiens*. Exp Parasitol. 2013;133:454–61.
33. Valkiūnas G, Žiegytė R, Palinauskas V, Bernotienė R, Bukauskaitė D, Ilgūnas M, et al. Complete sporogony of *Plasmodium relictum* (lineage pGRW4) in mosquitoes *Culex pipiens pipiens*, with implications on avian malaria epidemiology. Parasitol Res. 2015;144:3075–85.
34. Ricklefs RE, Medeiros M, Ellis VA, Svensson-Coelho M, Blake JG, Loiselle BA, et al. Avian migration and the distribution of malaria parasites in New World passerine birds. J Biogeogr. 2017;44:1113–23.
35. Valkiūnas G, Ilgūnas M, Bukauskaitė D, Žiegytė R, Bernotienė R, Jusys V, et al. *Plasmodium delichoni* n. sp.: description, molecular characterisation and remarks on the exoerythrocytic merogony, persistence, vectors and transmission. Parasitol Res. 2016;115:2625–36.
36. Iezhova TA, Valkiūnas G, Bairlein F. Vertebrate host specificity of two avian malaria parasites of the subgenus *Novyella*: *Plasmodium nucleophilum* and *Plasmodium vaughani*. J Parasitol. 2005;91:472–4.
37. Dinhopl N, Mostegl MM, Richter B, Nedorost N, Maderner A, Fragner K, et al. Application of in situ hybridization for the detection and identification of avian malaria parasites in paraffin wax-embedded tissues from captive penguins. Avian Pathol. 2011;40:315–20.
38. Sambrook J, Fritsch EF, Mamiatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
39. Hellgren O, Waldenström J, Bensch S. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium* and *Haemoproteus* from avian blood. J Parasitol. 2004;90:797–802.
40. Hall TA. BioEdit: a user—friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series. 1999;41:95–8.
41. Pérez-Tris J, Bensch S. Diagnosing genetically diverse avian malarial infections using mixed-sequencing analysis and TA ecloning. Parasitology. 2005;131:15–23.
42. Ronquist F, Heulsenbeck JP. MrBayes 3: Bayesian phylogeneticinference under mixed models. Bioinformatics. 2003;19:1572–4.
43. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870–4.
44. Loiseau C, Harrigan RJ, Robert A, Bowie RC, Thomassen HA, Smith TB, et al. Host and habitat specialization of avian malaria in Africa. Mol Ecol. 2012;21:431–41.
45. Lutz HL, Hochachka WM, Engel JI, Bell JA, Tkach VV, Bates JM, et al. Parasite prevalence corresponds to host life history in a diverse assemblage of afrotropical birds and haemosporidian parasites. PLoS ONE. 2015;10:e0121254.
46. Žiegytė R, Bernotienė R, Bukauskaitė D, Palinauskas V, Iezhova TA, Valkiūnas G. Complete sporogony of *Plasmodium relictum* (lineages pSGS1 and pGRW11) in mosquito *Culex pipiens pipiens* form *molestus*, with implications to avian malaria epidemiology. J Parasitol. 2014;100:878–82.
47. Baron HR, Howe L, Varsani A, Doneley RJ. Disease screening of three breeding populations of adult exhibition budgerigars (*Melopsittacus undulatus*) in New Zealand reveals a high prevalence of a novel polyomavirus and avian malaria infection. Avian Dis. 2014;58:111–7.
48. Valkiūnas G, Iezhova TA. Exo-erythrocytic development of avian malaria and related haemosporidian parasites. Malar J. 2017;16:101.
49. Sehgal RN. Manifold habitat effects on the prevalence and diversity of avian blood parasites. Int J Parasitol Parasites Wildl. 2015;4:421–30.
50. Atkinson CT, Dusek RJ, Woods KL, Iko WM. Pathogenicity of avian malaria in experimentally-infected Hawaii. Amakihi J Wildl Dis. 2000;36:197–204.
51. Zehtindjiev P, Ilieva M, Westerdahl H, Hansson B, Valkiūnas G, Bensch S. Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler *Acrocephalus arundinaceus*. Exp Parasitol. 2008;119:99–110.
52. LaPointe DA, Goff ML, Atkinson CT. Thermal constraints to the sporogonic development and altitudinal distribution of avian malaria *Plasmodium relictum* in Hawai'i. J Parasitol. 2010;96:318–24.
53. Cornet S, Nicot A, Rivero A, Gandon S. Evolution of plastic transmission strategies in avian malaria. PLoS Pathog. 2014;10:e1004308.
54. Garcia-Longoria L, Møller AP, Balbontín J, de Lope F, Marzal A. Do malaria parasites manipulate the escape behaviour of their avian hosts? An experimental study. Parasitol Res. 2015;114:4493–501.
55. Mukhin A, Palinauskas V, Platonova E, Kobylkov D, Vakoliuk I, Valkiūnas G. The strategy to survive primary malaria infection: an experimental study on behavioural changes in parasitized birds. PLoS ONE. 2016;11:e0159216.
56. Grim KC, McCutchan T, Li J, Sullivan M, Graczyk TK, McConkey G, et al. Preliminary results of an anticircumsporozoite DNA vaccine trial for protection against avian malaria in captive African black-footed penguins (*Spheniscus demersus*). J Zoo Widl Med. 2004;35:154–61.
57. Sepil I, Lachish S, Hinks AE, Sheldon BC. Mhc supertypes confer both qualitative and quantitative resistance to avian malaria infections in a wild bird population. Proc Biol Sci. 2013;280:20130134.
58. Ellis VA, Cornet S, Merrill L, Kunkel MR, Tsunekage T, Ricklefs RE. Host immune responses to experimental infection of *Plasmodium relictum* (lineage SGS1) in domestic canaries (*Serinus canaria*). Parasitol Res. 2015;114:3627–36.
59. Spencer KA, Buchanan KL, Leitner S, Goldsmith AR, Catchpole CK. Parasites affect song complexity and neural development in a songbird. Proc Biol Sci. 2005;272:2037–43.
60. Yohannes E, Palinauskas V, Valkiūnas G, Lee RW, Bolshakov CV, Bensch S. Does avian malariae infection affects feather stable isotope signatures? Oecologia. 2011;167:937–42.
61. Garroway CJ, Radersma R, Sepil I, Santure AW, De Cauwer I, Slate J, et al. Fine-scale genetic structure in a wild bird population: the role of limited dispersal and environmentally based selection as causal factors. Evolution. 2013;67:3488–500.
62. Outlaw RK, Counterman B, Outlaw DC. Differential patterns of molecular evolution among Haemosporidian parasite groups. Parasitology. 2015;142:612–22.
63. Soares L, Marra P, Gray L, Ricklefs RE. The malaria parasite *Plasmodium relictum* in the endemic avifauna of eastern Cuba. Conserv Biol. 2017;31:1477–82.
64. Foster JT, Woodworth BL, Eggert LE, Hart PJ, Palmer D, Duffy DC, et al. Genetic structure and evolved malaria resistance in Hawaiian honeycreepers. Mol Ecol. 2007;16:4738–46.
65. Atkinson CT, Saili KS, Utzurrum RB, Jarvi SI. Experimental evidence for evolved tolerance to avian malaria in a wild population of low elevation Hawai'i 'Amakihi *(Hemignathus virens*). EcoHealth. 2013;10:366–75.
66. Vézilier J, Nicot A, Gandon S, Rivero A. *Plasmodium* infection brings forward mosquito oviposition. Biol Lett. 2015;11:20140840.
67. Pigeault R, Nicot A, Gandon S, Rivero A. Mosquito age and avian malaria infection. Malar J. 2015;30:383.

68. Medeiros MC, Ricklefs RE, Brawn JD, Hamer GL. *Plasmodium* prevalence across avian host species is positively associated with exposure to mosquito vectors. Parasitology. 2015;142:1612–20.
69. Palinauskas V, Valkiūnas G, Križanauskienė A, Bensch S, Bolshakov CV. *Plasmodium relictum* (lineage P-SGS1): further observation of effects on experimentally infected passeriform birds, with remarks on treatment with Malarone™. Exp Parasitol. 2009;123:134–9.
70. van Riper IIIC. The impact of introduced vectors and avian malaria on insular passeriform bird population in Hawaii. Bull Soc Vector Ecol. 1991;16:59–83.
71. Braga EM, Silveira P, Belo NO, Valkiūnas G. Recent advances in the study of avian malariae: an overview with an emphasis on the distribution of *Plasmodium* spp. in Brazil. Mem Inst Oswaldo Cruz. 2011;106(Suppl 1):3–11.
72. Bueno MG, Lopez RP, de Menezes RM, de J Costa-Nascimento M, Lima GF, Araújo RA, et al. Identification of *Plasmodium relictum* causing mortality in penguins (*Spheniscus magellanicus*) from São Paulo Zoo, Brazil. Vet Parasitology. 2010;173:123–7.
73. Vanstreels RE, da Silva-Filho RP, Kolesnikovas CK, Bhering RC, Ruoppolo V, Epiphanio S, et al. Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. Vet Res. 2015;46:30.
74. Inumaru M, Murata K, Sato Y. Prevalence of avian haemosporidia among injured wild birds in Tokyo and environs, Japan. Int J Parasitol Parasites Wildl. 2017;6:299–309.
75. Howe L, Castro IC, Schoener ER, Hunter S, Barraclough RK, Alley MR. Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. Parasitol Res. 2012;110:913–23.
76. Atkinson CT, Utzurrum RB, Lapointe DA, Camp RJ, Crampton LH, Foster JT, et al. Changing climate and the altitudinal range of avian malaria in the Hawaiian Islands—an ongoing conservation crisis on the island of Kaua'i. Glob Chang Biol. 2014;20:2426–36.
77. Sijbranda DC, Hunter S, Howe L, Lenting B, Argilla L, Gartrell BD. Cases of mortality in little penguins (*Eudyptula minor*) in New Zealand associated with avian malaria. N Z Vet J. 2017;65:332–7.
78. Dinhopl N, Nedorost N, Mostegl MM, Weissenbacher-Lang C, Weissenböck H. *In situ* hybridization and sequence analysis reveal an association of *Plasmodium* spp. with mortalities in wild passerine birds in Austria. Parasitol Res. 2015;114:1455–62.
79. Valkiūnas G, Ilgūnas M, Bukauskaitė D, Palinauskas V, Bernotienė R, Iezhova TA. Molecular characterization and distribution of *Plasmodium matutinum*, a common avian malaria parasite. Parasitology. 2017;144:1726–35.
80. Olias P, Wegelin M, Zenker W, Freter S, Gruber AD, Klopfleisch R. Avian malaria deaths in parrots, Europe. Emerg Infect Dis. 2011;17:950–2.
81. Moens MAJ, Valkiūnas G, Paca A, Bonaccorso E, Aguirre N, Pérez-Tris J. Parasite specialization in a unique habitat: hummingbirds as reservoirs of generalist blood parasites of Andean birds. J Anim Ecol. 2016;85:1234–45.
82. Palinauskas V, Žiegytė R, Iezhova TA, Ilgūnas M, Bernotienė R, Valkiūnas G. Description, molecular characterisation, diagnostics and life cycle of *Plasmodium elongatum* (lineage pERIRUB01), the virulent avian malaria parasite. Int J Parasitol. 2016;46:697–707.
83. Corradetti A, Garnham PCC, Neri L, Scanga M, Cavallini C. A redescription of *Plasmodium* (*Haemamoeba*) *relictum* (Grassi and Feletti, 1891). Parassitologia. 1970;12:1–10.
84. Garnham PCC. Malaria in its various vertebrate hosts. In: Kreier JP, editor. Malaria. Part 1. Epidemiology, chemotherapy, morphology and metabolism. New York: Academic Press; 1980. p. 95–144.
85. Valkiūnas G, Palinauskas V, Križanauskienė A, Bernotienė R, Kazlauskienė R, Iezhova TA. Further observations on in vitro hybridization of hemosporidian parasites: patterns of ookinete development in *Haemoproteus* spp. J Parasitol. 2013;9:124–36.
86. Nilsson E, Taubert H, Hellgren O, Huang X, Palinauskas V, Markovets MY, et al. Multiple cryptic species of sympatric generalists within the avian blood parasite *Haemoproteus majoris*. J Evol Biol. 2016;29:1812–26.
87. Bernotienė R, Palinauskas V, Iezhova TA, Murauskaitė D, Valkiūnas G. Avian haemosporidian parasites (Haemosporida): a comparative analysis of different polymerase chain reaction assays in detection of mixed infections. Exp Parasitol. 2016;163:31–7.
88. Pacheco MA, Matta NE, Valkiunas G, Parker PG, Mello B, Stanley CE Jr, et al. Mode and rate of evolution of haemosporidian mitochondrial genomes: timing the radiation of avian parasites. Mol Biol Evol. 2017;msx285.
89. Durrant KL, Beadell JS, Ishtiaq F, Graves GR, Olson SL, Gering E, et al. Avian malaria in South America: a comparison of temperate and tropical zones. Ornithol Monogr. 2006;60:98–111.
90. Merino S, Moreno J, Vasquez RA, Martinez J, Sánchez-Monsálvez I, Estades CF, et al. Haematozoa in forest birds from southern Chile: latitudinal gradients in prevalence and parasite lineage richness. Austral Ecol. 2008;33:329–40.
91. Lacorte GA, Félix GMF, Pinheiro RRB, Chaves AV, Almeido-Neto G, Neves FS, et al. Exploring the diversity and distribution of neotropical avian malaria parasites—a molecular survey from Southeast Brazil. PLoS ONE. 2013;8:e57770.
92. Quillfeldt P, Martínez J, Hennicke J, Ludynia K, Gladbach A, Masello JF, et al. Hemosporidian blood parasites in seabirds—a comparative genetic study of species from Antarctic to tropical habitats. Naturwissenschaften. 2010;97:809–17.
93. Vinogradova EB. Mosquitoes *Culex pipiens pipiens*: taxonomy, distribution, ecology, physiology, genetics and control. Sofia: PenSoft; 2000.
94. Farajollahi A, Fonseca DM, Kramer LD, Marm KA. "Bird biting" mosquitoes and human disease: a review of the role of *Culex pipiens* complex mosquitoes in epidemiology. Infect Genet Evol. 2011;11:1577–85.
95. Samy AM, Elaagip AH, Kenawy MA, Ayres CF, Peterson AT, Soliman DE. Climate change influences on the global potential distribution of the mosquito *Culex quinquefasciatus*, vector of West Nile virus and lymphatic filariasis. PLoS ONE. 2016;11:e0163863.
96. Valkiūnas G, Bensch S, Iezhova TA, Križanauskienė A, Hellgren O, Bolshakov CV. Nested cytochrome *b* polymerase chain reaction diagnostics underestimate mixed infections of avian blood haemosporidian parasites: microscopy is still essential. J Parasitol. 2006;92:418–22.
97. Martínez J, La Martínez-De Puente J, Herrero J, Del Cerro S, Lobato E, Rivero-De Aguilar J, et al. A restriction site to differentiate *Plasmodium* and *Haemoproteus* infections in birds: on the inefficiency of general primers for detection of mixed infections. Parasitology. 2009;136:713–22.
98. Zehtindjiev P, Križanauskienė A, Bensch S, Palinauskas V, Asghar M, Dimitrov D, et al. A new morphologically distinct avian malaria parasite that fails detection by established PCR-based protocols for amplification of the cytochrome b gene. J Parasitol. 2012;98:657–65.
99. Massey JG, Graczyk TK, Cranfield MR. Characteristics of naturally acquired *Plasmodium relictum capistranoae* infections in naive Hawaiian crows (*Corvus hawaiiensis*) in Hawaii. J Parasitol. 1996;82:182–5.
100. Schoener ER, Banda M, Howe L, Castro IC, Alley MR. Avian malaria in New Zealand. N Z Vet J. 2014;62:189–98.
101. Santiago-Alarcon D, Palinauskas V, Schaefer HM. Diptera vectors of avian Haemosporidian parasites: untangling parasite life cycles and their taxonomy. Biol Rev Camb Philos Soc. 2012;87:928–64.
102. Work TM, Washino RK, Van Riper C 3rd. Comparative susceptibility of *Culex tarsalis*, *Anopheles franciscanus*, and *Culiseta inornata* (Diptera: Culicidae) to *Plasmodium relictum* (Haemosporidia: Plasmodiiae). J Med Entomol. 1990;27:68–71.
103. Kim KS, Tsuda Y, Sasaki T, Kobayashi M, Hirota Y. Mosquito blood–meal analysis for avian malaria study in wild bird communities: laboratory verification and application to *Culex sasai* (Diptera: Culicidae) collected in Tokyo, Japan. Parasitol Res. 2009;105:1351–7.
104. Kim KS, Tsuda Y. Avian *Plasmodium* lineages found in spot surveys of mosquitoes from 2007 to 2010 at Sakata wetland, Japan: do dominant lineages persist for multiple years? Mol Ecol. 2012;21:5374–85.
105. Cornet S, Nicot A, Rivero A, Gandon S. Both infected and uninfected mosquitoes are attracted toward malaria infected birds. Malar J. 2013;12:179.
106. LaPointe DA, Goff ML, Atkinson CT. Comparative susceptibility of introduced forest-dwelling mosquitoes in Hawai'i to avian malaria, *Plasmodium relictum*. J Parasitol. 2005;91:843–9.
107. Nayar JK, Young MD, Forrester DJ. *Wyeomyia vanduzeei*, an experimental host for wild turkey malaria *Plasmodium hermani*. J Parasitol. 1980;66:166–7.
108. Carlson JS, Martínez-Gómez JE, Cornel A, Loiseau C, Sehgal RN. Implications of *Plasmodium* parasite infected mosquitoes on an insular avifauna: the case of Socorro Island, México. J Vector Ecol. 2011;36:213–320.

109. Ejiri H, Sato Y, Kim KS, Tsuda Y, Murata K, Saito K, et al. Blood meal identification and prevalence of avian malaria parasite in mosquitoes collected at Kushiro Wetland, a subarctic zone of Japan. J Med Entomol. 2011;48:904–8.
110. Ejiri H, Sato Y, Sawai R, Sasaki E, Matsumoto R, Ueda M, et al. Prevalence of avian malaria parasite in mosquitoes collected at a zoological garden in Japan. Parasitol Res. 2009;105:629–33.
111. Fabian MM, Toma H, Arakawa T, Sato Y. Malaria parasite developmental analyses by the nested polymerase chain reaction method: an implication for the evaluation of mosquito infection rates in epidemiological studies. Southeast Asian J Trop Med Public Health. 2004;35:820–7.
112. Glaizot O, Fumagalli L, Iritano K, Lalubin F, Van Rooyen J, Christe P. High prevalence and lineage diversity of avian malaria in wild populations of great tits (*Parus major*) and mosquitoes (*Culex pipiens*). PLoS ONE. 2012;7:e34964.
113. Ishtiaq F, Guillaumot L, Clegg SM, Phillimore AB, Black RA, Owens IPF, et al. Avian haematozoan parasites and their associations with mosquitoes across Southwest Pacific Islands. Mol Ecol. 2008;17:4545–55.
114. Kim KS, Tsuda Y, Yamada A. Bloodmeal identification and detection of avian malaria parasite from mosquitoes (Diptera: Culicidae) inhabiting coastal areas of Tokyo Bay, Japan. J Med Entomol. 2009;46:1230–4.
115. Kimura M, Darbro JM, Harrington LC. Avian malaria parasites share congeneric mosquito vectors. J Parasitol. 2010;96:144–51.
116. la Martínez-de Puente J, Martínez J, Rivero-de Aguilar J, Herrero J, Merino S. On the specificity of avian blood parasites: revealing specific and generalist relationships between haemosporidians and biting midges. Mol Ecol. 2011;20:3275–87.
117. Bernotienė R, Valkiūnas G. PCR detection of malaria parasites and related haemosporidians: the sensitive methodology in determining bird-biting insects. Malar J. 2016;15:283.
118. Ionică AM, Zittra C, Wimmer V, Leitner N, Votýpka J, Modrý D, et al. Mosquitoes in the Danube Delta: searching for vectors of filarioid helminths and avian malaria. Parasit Vectors. 2017;10:324.
119. Njabo K, Cornel AJ, Sehgal RNM, Loiseau C, Buermann W, Harrigan RJ, et al. *Coquillettidia* (Culicidae, Diptera) mosquitoes are natural vectors of avian malaria in Africa. Malar J. 2009;8:193.
120. Njabo KY, Cornel AJ, Bonneaud C, Toffelmier E, Sehgal RN, Valkiūnas G, et al. Nonspecific patterns of vector, host and avian malaria parasites associations in a central African rainforest. Mol Ecol. 2011;20:1049–61.
121. Valkiūnas G, Kazlauskienė R, Bernotienė R, Palinauskas V, Iezhova TA. Abortive long-lasting sporogony of two *Haemoproteus* species (Haemosporida, Haemoproteidae) in the mosquito *Ochlerotatus cantans*, with perspectives on haemosporidian vector research. Parasitol Res. 2013;112:2159–69.

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PAPER VI

Patterns of *Plasmodium homocircumflexum* virulence in experimentally infected passerine birds.

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Patterns of *Plasmodium homocircumflexum* virulence in experimentally infected passerine birds

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Abstract

Background: Avian malaria parasites (genus *Plasmodium*) are cosmopolitan and some species cause severe pathologies or even mortality in birds, yet their virulence remains fragmentally investigated. Understanding mechanisms and patterns of virulence during avian *Plasmodium* infections is crucial as these pathogens can severely affect bird populations in the wild and cause mortality in captive individuals. The goal of this study was to investigate the pathologies caused by the recently discovered malaria parasite *Plasmodium homocircumflexum* (lineage pCOLL4) in four species of European passeriform birds.

Methods: One cryopreserved *P. homocircumflexum* strain was multiplied and used for experimental infections. House sparrows (*Passer domesticus*), common chaffinches (*Fringilla coelebs*), common crossbills (*Loxia curvirostra*) and common starlings (*Sturnus vulgaris*) were exposed by subinoculation of infected blood. Experimental and control groups (8 individuals in each) were observed for over 1 month. Parasitaemia, haematocrit value and body mass were monitored. At the end of the experiment, samples of internal organs were collected and examined using histological and chromogenic in situ hybridization methods.

Results: All exposed birds were susceptible, with similar average prepatent period and maximum parasitaemia, yet virulence was different in different bird species. Mortality due to malaria was reported in chaffinches, house sparrows and crossbills (7, 5 and 3 individuals died respectively), but not in starlings. Exoerythrocytic meronts (phanerozoites) were observed in the brain of all dead experimental birds. Blockage of blood vessels in the brain led to cerebral ischaemia, invariably causing brain damage, which is likely the main reason of mortality. Phanerozoites were observed in parenchymal organs, heart and muscles of all infected individuals, except starlings.

Conclusion: This study shows that *P. homocircumflexum* is generalist and the same lineage caused similar parasitaemia-related pathologies in different host species. Additionally, the mode of exo-erythrocytic development is different in different birds, resulting in different mortality rates. This should be taken into consideration in studies addressing pathology during avian malaria infections.

Keywords: Avian malaria, *Plasmodium*, Birds, Phanerozoites, Pathology

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Background

With the exception of Antarctica, agents of avian malaria (Plasmodiidae, Haemosporida) have been reported all over the world [1, 2]. In all, 55 species of these pathogens have been recognized [3], and many new agents of avian malaria were discovered recently [4–9]. Species of *Plasmodium* have complex life cycles, which remain incompletely investigated for the majority of these pathogens [10]. These parasites can cause severe health disorders in domestic, wild and captive birds, sometimes even leading to lethal malaria [10–14].

There are two main causes of pathology during avian malaria infections: blood pathology [12, 15] and organ damage due to phanerozoites—tissue meronts developing during secondary exo-erythrocytic merogony [11, 12, 16, 17]. During avian malaria, merozoites from erythrocytic meronts can induce exo-erythrocytic merogony (development of phanerozoites), and that is not the case during malaria in mammals. If blood pathology caused by erythrocytic stages of avian malaria parasites has been relatively well studied [1, 12], the damage caused by tissue stages and patterns of their occurrence remain insufficiently understood. Because phanerozoites develop in various non-specialized reticuloendothelial cells (macrophages, endothelial cells of capillaries), they can occur and cause damage of various organs in susceptible vertebrate hosts [10–12, 18, 19]. Due to the secondary exo-erythrocytic merogony, avian malaria may be a more virulent disease than human malaria. Additionally, the cause of virulence in avian malaria is more difficult to predict than during malaria in mammals due to unclear patterns of phanerozoite occurrence.

Recently, the issue of virulence of avian malaria pathogens has attracted much attention. However, majority of the investigations focused mainly on parasitaemia during *Plasmodium* infections [20–25]. These studies provided valuable information about blood-related pathological changes but are limited to truly evaluate mechanisms of the virulence during avian malaria due to the lack of information about pathology caused in organs. Knowledge about patterns of exo-erythrocytic development of malaria and other haemosporidian parasites remains insufficient. Most studies dealing with exo-erythrocytic development were carried out between 1930s and 1960s [10]—before molecular diagnostic techniques were introduced in avian malaria research. Application of molecular diagnostic methods showed that the diversity of parasites (both inter- and intra-species) is far greater than previously believed. Thus, it becomes even more difficult to address exo-erythrocytic development of particular parasites and their lineages, calling for the application of modern diagnostic tools and experimental research in avian malaria studies.

Phanerozoite stage is difficult to access in wild-caught naturally infected avian hosts, in which the longevity of malaria infection usually is unknown. Additionally, the exo-erythrocytic meronts also are sometimes difficult to visualize using the traditional histological approaches, particularly during light malaria infections. Molecular diagnostic tools have been developed and might aid with the detection and identification of phanerozoites [26, 27]. Several recent studies reported detection of exo-erythrocytic stages of avian malaria parasites, but these were mainly case reports [19, 28–30]. Patterns of the exo-erythrocytic development of avian *Plasmodium* species are still insufficiently understood. Experimental work would be helpful to develop this knowledge, which is crucial to answer questions related to bird health, treatment, ecology and possible threats to biodiversity.

Malaria parasite *Plasmodium homocircumflexum* (lineage pCOLL4) was recently discovered and described in Europe [8, 31]. A pilot study was conducted with the aim to investigate the effects of this infection on three individual birds belonging to three species [17]. The obtained data suggested that Eurasian siskins (*Carduelis spinus*), common crossbills (*Loxia curvirostra*) and common starlings (*Sturnus vulgaris*) were readily susceptible to *P. homocircumflexum* infections. Moreover, this parasite developed phanerozoites in these birds and was lethal in all tested bird individuals. This called for more a detailed investigation of pathology caused by *P. homocircumflexum* in avian hosts.

The goal of this study was to experimentally investigate (1) the dynamics of parasitaemia and the parasitaemia related health parameters (haematocrit value and body mass) during *P. homocircumflexum* (lineage pCOLL4) infection in four common European bird species (common crossbill, common starling, house sparrow *Passer domesticus* and common chaffinch *Fringilla coelebs*) and (2) the development of secondary exo-erythrocytic meronts and pathologies caused in these birds. Parasitaemia, haematocrit level and bird body mass were monitored at consistent time intervals, and birds were screened using histological and chromogenic *in situ* hybridization methods for detection of the exo-erythrocytic stages.

Methods

Study site

This study was carried out at the Biological Station of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea (55°05′ N, 20°44′ E) during the months of May–August, 2015 and 2016. Juvenile wild birds (<7 months old) were used. They were caught using funnel traps and mists nets and identified to species and age according to [32]. Prior to the experiments, all birds were screened for haemosporidian

Table 1 Susceptibility of passerine birds to *Plasmodium homocircumflexum* (lineage pCOLL4) infection after experimental exposure

Bird species, group and infection dose	No. exposed (no. infected)	No. died	Prepatent period (days)	Maximum parasitemia[a]	Minimum hematocrit value[a]	Maximum weight, g[a]	No. of individuals with phanerozoites located in						
							Brain	Heart	Liver	Lung	Spleen	Kidney	Muscle
Loxia curvirostra													
Experimental (3×10^6)[b]	8 (8)	7	4–8	3.3–23.3 (10)	12.5–33.3 (23.8)	37.4–45.4 (42.5)	7	8	8	8	8	8	8
Control	8 (0)	0	–	–	27.6–50 (43.7)	39.2–48.8 (43.9)	0	0	0	0	0	0	0
Sturnus vulgaris													
Experimental (9.5×10^5)[b]	8 (8)	3	4–12	0.1–30 (13)	18.6–33.3 (28.15)	65.5–91.1 (80.2)	0	0	0	0	0	0	0
Control	8 (0)	1	–	–	31.4–37 (34.2)	71.8–83.8 (77.4)	0	0	0	0	0	0	0
Passer domesticus													
Experimental (8×10^5)[b]	8 (8)	5	5	0.1–41.4 (9.4)	11.4–40 (28.1)	24.5–30.2 (28.4)	4	4	8	8	7	7	4
Control	8 (0)	2	–	–	28.6–45.5 (37.5)	26.8–31 (28.75)	0	0	0	0	0	0	0
Fringilla coelebs													
Experimental (5×10^5)[b]	8 (8)	3	5	0.1–40.4 (11.5)	21–42 (31)	20.7–23.7 (22.)	2	2	4	7	4	4	2
Control	8 (0)	1	–	–	31–47.8 (40.3)	20.6–24.9 (23.1)	0	0	0	0	0	0	0

[a] Variation of individual parameters, followed in parentheses by the average for entire group

[b] Dose (total number of mature meronts) inoculated in each individual bird. It was difficult to standardize dose of infection in all experimental groups, mainly due to difficulty to standardize and calculate certain number of mature erythrocytic meronts in each inoculum, particularly in different year experiments. That is why comparison of quantitative data between bird species should be done with caution

parasites using microscopic examination of blood films, and only non-infected birds were selected. The non-infected status of all birds prior the experiments was confirmed by polymerase chain reaction (PCR)-based testing in the laboratory as described below. Recipient birds were infected by subinoculation of *P. homocircumflexum* (lineage pCOLL4) infected blood and maintained until their death or the end of the experiment (between 40 and 64 days post exposure (DPE) in different experiments), at which point they were euthanized. The study aimed to maintain experiments until mortality was recognized in experimental groups, and that happened at different intervals in different bird species (see the "Results" section).

Experimental design

All experimental bird species were unavailable in 1 year thus experiments were performed in two successive years using the same clone of malaria parasite. In 2015, juvenile house sparrows (*Passer domesticus*), common chaffinches (*Fringilla coelebs*) were available for experimental research. These bird species are abundant in Europe and were relatively easy to obtain in necessary numbers that year. Juvenile common crossbills (*Loxia curvirostra*) and common starlings (*Sturnus vulgaris*) were available for this experiment in 2016; these species were selected based on a pilot study [17], which showed that *P. homocircumflexum* develops phanerozoites in these birds. The common crossbills, common chaffinches and house sparrows were kept indoors in a vector-free room. The common starlings were kept outside in cages covered with a fine-mesh bolting silk preventing access of blood-sucking insects to birds. All birds were maintained at a natural light–dark photoperiod.

A strain of *P. homocircumflexum* (lineage pCOLL4, GenBank accession no. KC884250), originally isolated from a naturally infected red-backed shrike was used to infect the experimental birds. This strain was obtained from the biobank of the P. B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania [8]. Because of (1) the small size of donor birds and the resulting limited amount of blood, which could be withdrawal without damaging the bird and (2) the different number of mature meronts in infected blood of different donor birds during exposure, all experimental bird groups were exposed to different doses of infections (Table 1). That is why quantitative data of experiments with different bird species (level of parasitaemia, haematocrit value, body mass) should be used with caution for comparison between bird species. These data reflect parasite development within certain bird species after infection with certain dose of infection.

In 2015 and 2016, one sample of this isolate was thawed according to [11] and used to multiply the parasite by passage in live birds. In 2015, 5 Eurasian siskins (*Carduelis spinus*) were exposed for multiplication of the same strain. First, the infected blood was obtained from 2 siskins, mixed and used to infect 8 experimental house sparrows ($\sim 8 \times 10^5$ mature erythrocytic meronts were inoculated in each recipient). Second, the infected blood from another 3 siskins was collected, mixed and used to infect 8 experimental common chaffinches ($\sim 5 \times 10^5$ mature meronts were inoculated in each recipient). In 2016, blood from one siskin was used to infect 8 experimental common starlings ($\sim 9.5 \times 10^5$ mature meronts were inoculated in each recipient). When parasitaemia developed in the starlings, blood from 3 individuals was mixed and used to infect 8 experimental common crossbills ($\sim 3 \times 10^6$ mature meronts were inoculated into the recipient). Due to unavailability of siskins in 2016, crossbills were used to multiply the strain. Both siskins and crossbills are closely related members of the Fringillidae. Because the parasite strain was the same, it was predicted that the use of different parasite donors would not influence susceptibility of recipient avian hosts or development of phanerozoites.

In all experiments, 100 µl of infected donor blood was mixed with 25 µl sodium citrate and 125 µl 0.9% saline solution per recipient as described by [33]. The mixture was sub-inoculated into the pectoral muscles of the experimental birds. In all, 8 birds of each species were inoculated with *P. homocircumflexum* (lineage pCOLL4). Blood from uninfected common crossbills was inoculated into 8 non-infected birds of each species with the aim to standardize the stress level in experiment and control groups at the start of the experiment. These birds were maintained as control groups at the same conditions as the corresponding species of experimental groups.

The crossbills, house sparrows, chaffinches and starlings, were observed and sampled for 40, 48, 57 and 64 days, respectively. Differences in the period of sampling between different bird species are due to different mortality rates, which were reported within each bird species during this study. Blood from crossbills and starlings was taken for microscopic examination and PCR-based testing every 4 days, and it was obtained from sparrows and chaffinches, starting on 5 DPE, every 3 days during the experiment. Approximately 50 µl of blood was collected in heparinized microcapillaries after puncturing the brachial vein with a sterile needle. Three drops of blood were used to make three blood films, which were air-dried, fixed in absolute methanol, stained with Giemsa and examined microscopically as described by [34]. Approximately 35 µl of the blood was fixed in non-lysis SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA,

pH 8.0) for molecular analysis; these samples were stored at − 4 °C in the field and maintained at − 20 °C in the laboratory.

After the birds' death or euthanasia at the end of the experiment, brain, heart, kidney, liver, lungs, spleen, and a piece of the pectoral muscle of the experimental birds were dissected and fixed in 10% neutral formalin. In the laboratory the collected tissues were embedded in paraffin blocks. Histological sections of 4 µm were prepared, stained with haematoxylin–eosin (H&E) [12] and examined microscopically. Additionally, one thin smear of bone marrow was prepared on a glass slide from each bird. These preparations were processed and examined as the blood films.

Morphological analysis

An Olympus BX51 light microscope equipped with Olympus DP12 digital camera and imaging software Olympus DP-SOFT was used to examine slides and to prepare illustrations. Each blood slide was examined for 15–20 min at medium magnification (400×), and then at least 100 fields were studied at high magnification (1000×). Intensity of parasitaemia was calculated as a percentage by actual counting of the number of parasites per 1000 erythrocytes or per 10,000 erythrocytes if infections were light [35]. Histological preparations were examined at low magnification (200×) for 10–15 min., followed by examination at medium magnification (400×) for 10–15 min and then at high magnification (1000×) for another 20–30 min.

Statistical analyses

Statistical analyses were carried out using the 'R' package [36]. Normality of data distribution was evaluated using Shapiro–Wilk test. Wilcoxon test was applied for data which were not distributed according to normal distribution in order to evaluate the differences between the means. Fisher's exact test was used to evaluate if there was a statistically significant difference between mortality in control and experimental group separately in each bird species. Because of different doses of infection in different bird species (Table 1, see also the "Experimental design" section above), mortality rates were not compared between bird species.

Molecular analysis

Total deoxynucleic acid (DNA) was extracted from collected blood samples using an ammonium-acetate protocol [37] with one modification, 125 µl of fixed blood was used instead of 250 µl. A nested-PCR protocol [38] was applied for the molecular analysis. Primer pair HaemFNI/HaemNR3 was used for the first PCR according condition described by [38]. This primer pair amplifies a partial sequence of the mitochondrial cytochrome b (*cytb*) gene of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* species. Reaction mix for the first PCR consisted of 12.5 µl of Dreamtaq Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 µl of nuclease-free water, 1 µl of each primer and 2 µl of template DNA. For the second PCR, the primer pair HaemF/HaemR2 was used according to the conditions described by [39]. The later primer pair amplifies a 479 bp fragment of *cytb* gene. The reaction mix for the second PCR was as for the first one (only this time using different primers) and instead of genomic DNA, 2 µl of the first PCR product for the second PCR was used. PCR success was evaluated by performing electrophoresis on a 2% agarose gel. 2 µl of the second PCR was used for this evaluation. One negative control (nuclease-free water) and one positive control (a *Plasmodium* sample, which was positive in previous testing) were used to determine possible false amplifications. No case of false amplification was found. Positive PCR products were sequenced from the 5′ end using the HAEMF primer [39]. Dye terminator cycle sequencing (Big Dye) was used. Samples were loaded onto an ABI PRISM TM 3100 capillary sequencing robot (Applied Biosystems, USA). Sequences of parasites were edited and examined using the BioEdit program [40]. The 'Basic Local Alignment Search Tool' using the megablast algorithm were applied to identify the *cytb* lineages of detected DNA sequences. Identified sequences were double checked using the 'Basic Local Alignment Search Tool' in MalAvi database [41].

In situ hybridization

Chromogenic in situ hybridization (ISH) was performed on tissue sections that appeared to be non-infected during microscopic examination of H&E preparation. The procedure was carried out according to [26]. In short, paraffin embedded tissue sections of 3 µm thickness were treated in proteinase K (Roche, Basel, Switzerland) 6 µg/ml and Tris-buffered saline solution at 37 °C for about 50 min. Hybridization was carried out overnight at 40 °C with hybridization mixture placed on the histological sections. Concentration of the probe used during the incubation was 100 ng/ml. The probe labelled with digoxigenin at the 3′ end (Eurofins MWG Operon, Ebersberg, Germany) is aimed at 18S ribosomal ribonucleic acid (rRNA) strand and is specific to avian *Plasmodium* spp. [26]. The hybrids were detected by incubating slides with antidigoxigenin-AP Fab fragments (Roche) (1:200) for 1 h at room temperature followed by visualization reaction using the colour substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) (Roche). Probe specificity has been extensively tested previously [26, 27]. Conducted pilot study [17] showed that *P. homocircumflexum* (lineage pCOLL4)

phanerozoites were readily detectable using this method. Tissues from a deceased wild Blackbird *Turdus merula* free of avian malaria parasites were used as a negative control and an irrelevant oligonucleotide probe (designed for *Leishmania* spp.) was applied on the experimental samples to detect any false hybridizations, and tissues of *Plasmodium elongatum* (lineage pERIRUB01) infected canary were used as a positive control.

Results

Both microscopic and PCR-based analyses showed that all birds used in this work were uninfected with haemosporidian parasites prior to experiments. Control birds remained uninfected during this study, indicating that all infection in experimental groups were induced exclusively during experiments. Parasitaemia developed in all experimentally infected birds (Table 1), with mature erythrocytic meronts and gametocytes (Fig. 1) present in all infected individuals. That indicates susceptibility of all exposed bird species. Morphologically indistinguishable blood stages developed in all exposed bird species. Both microscopic examination and PCR-based testing revealed presence of a single infection, pCOLL4 lineage of *P. homocircumflexum* in all experimental birds.

Data on common crossbills' susceptibility to *P. homocircumflexum* (prepatent period, maximum parasitaemia, mortality rate, development of phanerozoites) are provided in Table 1 and Fig. 2a. Average parasitaemia reached the peak of 7.8% on 12 DPE (Fig. 2a). After the peak, parasitaemia remained and fluctuated in exposed individuals, but average parasitaemia did not reach the peak levels again. Experimental infection had a significant negative effect on the average haematocrit value of common crossbills ($p < 0.05$) (Fig. 2a). Average haematocrit value of the experimental group decreased more than twofold compared to the control group. The decrease of haematocrit value coincided with high parasitaemia. After the initial decrease, average haematocrit value maintained low and did not reach the initial value. There were no significant average body mass changes in the exposed common crossbills in comparison to controls ($p = 0.19$) (Fig. 2a). Seven of 8 infected common crossbills died between 25 and 40 DPE (Fig. 2a; Table 1), but all control crossbills survived. Mortality

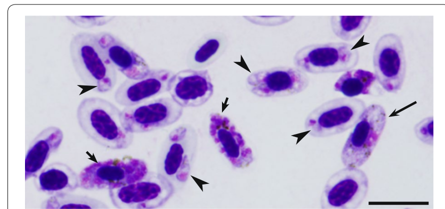

Fig. 1 High parasitaemia of *Plasmodium (Giovannolaia) homocircumflexum* (cytochrome b lineage pCOLL4) in an experimentally infected European siskin *Carduelis spinus*. Numerous developing young parasites (simple arrowheads), two mature meronts (short arrows) and one mature macrogametocyte (long arrow) are shown, indicating complete life cycle in avian hosts. Giemsa stained blood film. Scale bar = 10 µm

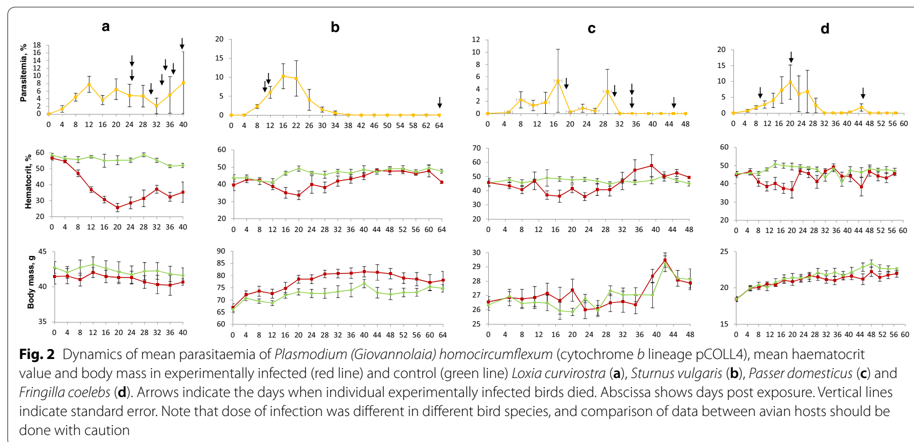

Fig. 2 Dynamics of mean parasitaemia of *Plasmodium (Giovannolaia) homocircumflexum* (cytochrome *b* lineage pCOLL4), mean haematocrit value and body mass in experimentally infected (red line) and control (green line) *Loxia curvirostra* (**a**), *Sturnus vulgaris* (**b**), *Passer domesticus* (**c**) and *Fringilla coelebs* (**d**). Arrows indicate the days when individual experimentally infected birds died. Abscissa shows days post exposure. Vertical lines indicate standard error. Note that dose of infection was different in different bird species, and comparison of data between avian hosts should be done with caution

rate in experimental group was significantly higher than in control group ($p < 0.001$). Phanerozoites were seen in histological sections in all examined organs of the dead common crossbills (Table 1), and they were numerous in brain. Phanerozoites were also observed in the same organs of one crossbill that survived during the experiment, but they were absent in the brain of this individual bird, indicating that the brain pathology is an essential reason of mortality.

Data on common starlings' susceptibility to *P. homocircumflexum* (prepatent period, maximum parasitaemia, mortality rate, development of phanerozoites) are provided in Table 1 and Fig. 2b. Average parasitaemia reached the peak of 10.2% on 16 DPE (Fig. 2b). After the peak, parasitaemia fluctuated in exposed individuals, but average parasitaemia did not reach the peak level again. Average body mass of the experimental group statistically significantly increased comparing to the control group ($p < 0.05$) (Fig. 2b). Three of 8 common starlings died during the experiment (8 DPE, 9 DPE and 64 DPE) (Fig. 2b). One common starling died in the control group on the 40 DPE. There was no significant difference in mortality in control and experimental groups ($p = 0.57$). No phanerozoites were observed in any common starlings—neither dead, nor the survived individuals during histological examination of organs (Table 1), and this result was confirmed by the negative in situ hybridization tests.

Data on house sparrows' susceptibility to *P. homocircumflexum* (prepatent period, maximum parasitaemia, mortality rate, development of phanerozoites) are provided in Table 1 and Fig. 2c. Average parasitaemia reached the peak of 5.7% on 17 DPE (Fig. 2c). After the peak, parasitaemia fluctuated markedly, but average parasitaemia did not reach the peak levels again. Average body mass of exposed house sparrows changed significantly ($p = 0.04$) (Fig. 2c). Until 20 DPE, the average body mass of the experimental group increased in comparison to controls ($p = 0.042$). This coincided with the increased parasitaemia. After 20 DPI, the average body mass of the experimental group decreased. It is worth nothing that the decrease in average body mass of the control group was also detected after 20 DPI (Fig. 1). After 34 DPE, the average body mass of both experimental and control group increased sharply. Five of 8 infected sparrows died (Fig. 2c; Table 1). One bird died 18 DPE, others died between 31 and 45 DPE. There was no significant difference in mortality in control and experimental groups ($p = 0.31$). Phanerozoites were seen in the lungs, liver, spleen and kidney of the experimentally infected house sparrow that died on 18 DPE. In the infected sparrows that died between 31 DPE and 45 DPE, phanerozoites were seen in all examined tissues, including the brain (Fig. 3a, c, e, g, i, k, m). In the house sparrows that survived the experiment, few phanerozoites were located in the lungs, liver and kidney, but were absent in brain. It is important to note that three house sparrows died during light chronic parasitaemia between 36 DPE and 45 DPE, showing that decrease of parasitaemia is not always indication of improved health during avian malaria. On 20 DPE and 43 DPE, two house sparrows from the control group died; malaria parasite were not reported in them.

Data on common chaffinches' susceptibility to *P. homocircumflexum* (prepatent period, maximum parasitaemia, mortality rate, development of phanerozoites) are provided in Table 1 and Fig. 2d. Average parasitaemia reached the peak of 9.7% on 20 DPE (Fig. 2d). After the peak, parasitaemia fluctuated in exposed individuals, but average parasitaemia did not reach the peak levels again. The decrease in the average haematocrit value were noted on 29 DPE and 44 DPE, which again coincided with increases in average parasitaemia (Fig. 2d). Average body mass of experimental common chaffinches was significantly lower ($p = 0.001$) than of control group (Fig. 2d). Three infected common chaffinches died on 10 DPE, 21 DPE and 46 DPE. Phanerozoites were observed in all examined tissues of the chaffinches which died on 21 DPE and 46 DPE (Fig. 3b, d, f, h, j, l, n), but no phanerozoites were found in the chaffinch that died on 10 DPE. There was no significant difference in mortality in control and experimental groups ($p = 0.57$). In chaffinches that survived the experiment, phanerozoites were located in the parenchymal organs (lung, liver, kidney and spleen), and they were absent in brain (Table 1). One control chaffinch died on the 11 DPE; malaria parasites were not reported in this individual.

Phanerozoites observed in the brain were more or less elongate (Fig. 3a, b), and they followed the shape of the brain capillaries. Large phanerozoites filling the entire diameter of the capillaries were common, and they blocked the blood flow (Fig. 3a, b). Merozoites were readily visible in mature phanerozoites (Fig. 3a). Phanerozoites appeared to be roundish in most cases in other organs, such as lung (Fig. 3c, d), liver (Fig. 3e, f), spleen (Fig. 3g) and kidney (Fig. 3i), but phanerozoites of irregular shape were also seen (Fig. 3h, j). Phanerozoites were of oval shape in the heart (Fig. 3k, l), and the ones in the pectoral muscle were mostly slender and elongate (Fig. 3m, n). Inflammatory response to the phanerozoites appeared to be mild or was not seen.

In all experiments, the decrease of the average haematocrit value coincided with increased parasitaemia of *P. homocircumflexum* (Fig. 2). This was a general pattern with minor variations in different species of avian hosts independently of dose of infection. The same lineage of malaria parasite influenced body mass of birds, but

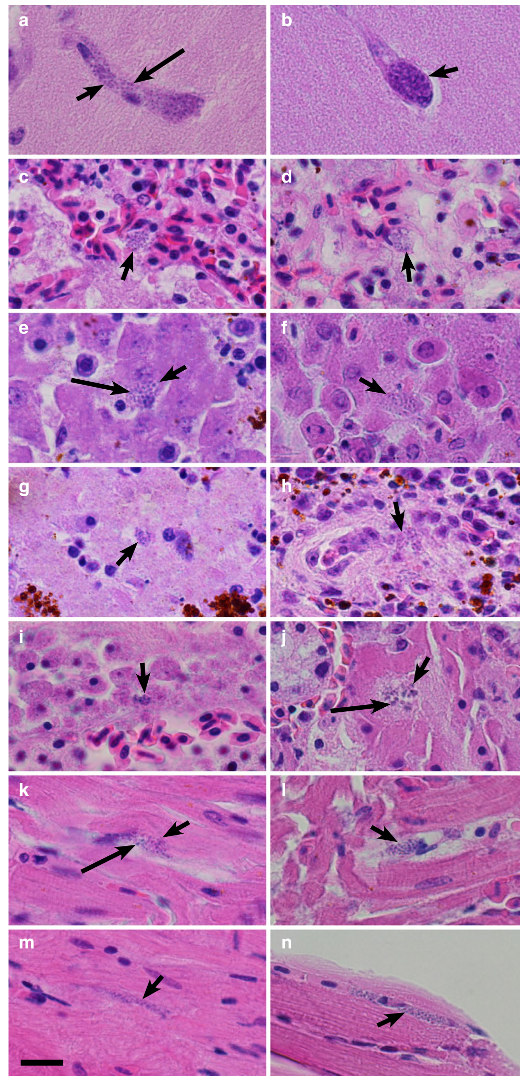

Fig. 3 Phanerozoites of *Plasmodium (Giovannolaia) homocircumflexum* (cytochrome *b* lineage pCOLL4) in histological sections of brain (**a**, **b**), lung (**c**, **d**), liver (**e**, **f**), spleen (**g**, **h**), kidney (**i**, **j**), heart (**k**, **l**) and pectoral muscle (**m**, **n**) of experimentally infected *Passer domesticus* (**a**, **c**, **e**, **g**, **i**, **k**, **m**) and *Fringilla coelebs* (**b**, **d**, **f**, **h**, **j**, **l**, **n**). Morphologically similar phanerozoites were reported in all infected experimental birds, but they were absent in starlings. Short arrows: phanerozoites, long arrows: merozoites. Haematoxylin–eosin stained preparations. Scale bar = 20 µm

markedly differently in different avian host species, ranging from the decrease of body mass in exposed crossbills and chaffinches to the increase in common starlings and house sparrows.

Development of phanerozoites occurred in all exposed avian hosts, except starlings (Table 1). Presence of phanerozoites was associated with brain damage; this stage likely lead to mortality of infected individuals in all experimental groups since the phanerozoites were seen in brain of all dead experimental birds. Starlings were resistant in regard of development of phanerozoites in spite of being inoculated with the second highest dose of infection. In all susceptible experimental birds, the spleen and liver were markedly enlarged, of black colour in comparison to controls.

The dose of infection did not influence the susceptibility of experimental birds, the minimum prepatent period nor the average maximum parasitaemia (Table 1). These parameters were similar in all experimental groups irrespectively on the dose of infection. Development of phanerozoites also was not strictly related to dose of infection because the dose was similar in experimentally exposed house sparrows and common starlings, but phanerozoites developed only in the former species (Table 1). The highest mortality was reported in common crossbills, which were injected with largest dose of parasites.

Discussion

This study was designed with the aim to describe the pathologies caused by *P. homocircumflexum* (lineage pCOLL4) in different species of avian hosts. Due to complicated methodology of strain multiplication and calculation of the number of mature meronts in inoculated blood during different experiments, it was impossible to standardize and calculate the dose of infection in each experiment precisely. This shortcoming prevents detailed comparison quantitative data between different avian hosts. However, the following key findings are innovative, are not related to dose of infection and should be discussed. First, *P. homocircumflexum* (lineage pCOLL4) developed high parasitaemia in all exposed wild passerine birds, indicating broad specificity and potentially big invasive ability in regard to vertebrate host range. Second, this parasite is virulent, with high maximum parasitaemia reported in all exposed birds. Third, general pattern of parasitaemia dynamics and haematocrit value changes were similar in all exposed birds. Fourth, phanerozoites developed in all bird species, except starlings, resulting in different mortality rates. It is also interesting to note that this infection influenced body mass of birds, but markedly differently in different host species; however, the reported differences might be related to different dose of infection and should be treated with caution.

This study shows that *P. homocircumflexum* (lineage pCOLL4) is able to infect and develop parasitaemia in distantly related passeriform bird species belonging to different families. Both the susceptibility and minimum prepatent period as well as maximum average parasitaemia were similar in different bird species without relation to the inoculated dose of mature erythrocytic meronts. All individual birds belonging to the Fringillidae, Passeridae and Sturnidae families were susceptible to this infection, indicating that *P. homocircumflexum* is truly a generalist parasite. This observation is in accordance with PCR-based records of this parasite lineage in wild birds. In all, *P. homocircumflexum* was reported in birds belonging to 14 species ([8, 17, 31, 42], present study). However, only in 7 bird species (*Lanius collurio*, *Serinus canaria*, *Carduleis spinus*, *Loxia curvirostra*, *Sturnus vulgaris*, *Passer domesticus* and *Fringilla coelebs*) mature gametocytes were observed (Fig. 1), indicating completion of life cycle in these avian hosts and the potential ability of the parasite to infect vectors ([8, 17], present study). In other published reports, the lineage pCOLL4 or synonymous lineages were detected only by PCR-based analysis [31, 42], and it was unclear if this infection completes or aborts development in the reported PCR-positive individuals. Abortive haemosporidian infections seem to be common in wildlife but are dead ends of haemosporidian parasite transmission [43]. The pathogen's ability to infect the broad range of vertebrate hosts and produce infective stages (gametocytes) is an important point to consider in regard to epidemiology of this infection. Vectors species of *P. homocircumflexum* (pCOLL4) remain unknown. Running hypothesis is that migrating European birds are naturally infected in Africa, but transmission might be interrupted due to lack of susceptible mosquito species in Europe [8]. Because common European birds are readily susceptible, get sick and often die (Table 1), further research is essential for better understanding true infection prevalence in wildlife populations and mechanisms preventing transmission of this parasite at breeding grounds of European birds. It is worth noting that experimentally infected birds in the present study are sedentary or short-distance migrants.

Further studies in these bird local populations as well as in the blood of the juveniles of long-distance migrants (for example, the red-backed shrike from which the strain was originally isolated) in Europe are needed to prove or reject the running hypothesis. It is possible that the available data about low prevalence of *P. homocircumflexum* (pCOLL4) might indicate high mortality of susceptible birds in the wild. The lack of suitable vector might also be an important limiting factor at present. However, it is difficult to predict how the epidemiological situation would develop due to climate change and spread of new

mosquito species in Europe [44, 45]. That calls for epidemiological research of *P. homocircumflexum* (pCOLL4).

This study supplements the results of [20] experiments, in which 5 species of passeriform birds (common crossbills, common chaffinches (*Fringilla coelebs*), common starlings, house sparrows and Eurasian siskins) were exposed to *Plasmodium relictum* (lineage pSGS1) infection, but showed different dynamics of parasitaemia and parasitaemia related haematocrit and body mass changes. Interestingly, the susceptibility of these bird species to *P. relictum* was markedly different [20], but it was the same during infection of *P. homocircumflexum* in this study, in which all individuals of all bird species developed parasitaemia. Additionally, the common starlings were more resistant to *P. relictum* in comparison to other bird species in both experiments. However, in this study, all common starlings were susceptible to *P. homocircumflexum* infection and developed parasitaemia, but this bird species was completely resistant to *P. relictum* with no parasite reported in blood [20].

In this study, the partial resistance of starlings to *P. homocircumflexum* was manifested not in parasitaemia, but in absence of phanerozoites in all exposed birds (Table 1). In other words, the available experimental observations indicate that the development of the same pathogen might be different in different species of avian hosts not only on the parasitaemia stage, but also on the exo-erythrocytic stage. The mechanisms responsible for the observed differences in parasite development in different species of birds remain unclear, and they might be due to the species-related innate resistance, which varies between different bird species [46–48]. However, immunity issues remain insufficiently investigated, and belong to the weakest understood points of avian malaria infections [12]. It is unclear how the avian immune system combats infections and how various biological factors (stress, co-infections, previous diseases, nutrition) influence immunity [49, 50]. Further experimental studies are needed for better understanding how immunological factors affect the success of parasitic infections. The host-parasite models developed in this study provide theoretical backgrounds and experimental opportunities in sampling materials for addressing comparative immunological research.

Avian malaria is a disease that causes blood pathology due to direct destruction of erythrocytes [11, 12] or damage of stem cells in bone marrow, leading to interruption of erythropoiesis [8, 11, 51]. During this study, parasites were not reported in stem cells of bone marrow, but high parasitaemia developed in the majority of birds (Table 1), indicating the anemia due to direct destruction of red blood cells by developing parasites and their removal in spleen and liver, which were enlarged, of black color and overfilled by infected erythrocytes and pigment granules in all dissected sick birds. The haematocrit values of the exposed birds decreased significantly during high parasitaemia in all tested bird species. This finding agrees with reports of former studies with different *Plasmodium* species in different species of birds [20, 52–54]. Interestingly, in the case of house sparrows, the decrease of haematocrit value was overcompensated and even exceeded that of the control group of the same species in the end of experiment (Fig. 2c). The greatest effect on haematocrit value was reported in infected common crossbills (Fig. 2a), and this suggests not only direct destruction of erythrocytes by parasites, but also active removal of infected erythrocytes by cells of the reticuloendothelial system in the spleen and liver. In the cases of common starlings and common chaffinches, the changes of haematocrit value were strictly positively correlated with the increase of parasitaemia, and then this parameter returned to normal levels when parasitaemia decreased. These results agree with results from previous studies measuring the haematocrit value in experimentally infected birds [20, 55].

Unexpectedly, infected common starlings and house sparrows showed a significant increase in body mass (Fig. 2b, c). This could be related to the availability of food: all birds were fed *ad libitum* and sick birds might eat more than controls. Our preliminary visual observations on control and infected birds support this hypothesis however, the food consumption was not measured during the study. It is probable that the same result hardly would be achieved in nature where the food supply is limited due to competition and the threat of predators. It is interesting to note that the infected common starlings were particularly active to the offered food during this study, and they were observed starting to eat even while the feeder's hand was still holding the feeder in the cage. In other words, they were not afraid of people during feeding. Similar behaviour observation has been reported in previous study [20] where the exposed Eurasian siskins were not scared by people entering the room and continued consuming food. It might be that some species of birds increase food consumption as a compensatory mechanism during loss of energy during malaria. However, it seems this is not the case in all bird species because chaffinches along with crossbills were not seen to increase the food consumption and their body mass did not increase in comparison to controls (Fig. 2a, d), which might be an indication of a possible existence of species-related mechanisms responsible for food consumption [56]. This study shows that increased body mass is not always an indication of good health during avian malaria.

This is the first study, which reports clear differences in exo-erythrocytic development of the same parasite lineage in different avian hosts. In other words, it was documented that not only susceptibility and parasitaemia dynamics, but also exo-erythrocytic development of the same pathogen might be different in different species of avian hosts. Phanerozoites developed in 3 species of birds infected with different doses of parasite, but they did not appear in common starlings (Table 1). It seems that the phanerozoites of *P. homocircumflexum* (pCOLL4) start to develop around 20 DPE in experimentally infected birds because phanerozoites were not seen in the chaffinch that died on 10 DPE but were observed in all tissues of the chaffinch that died 21 DPE. There might be individual variation in timing of phanerozoite formation, but phanerozoites likely appeared in the tissues between the 10 DPE and 21 DPE. In other words, some period of parasite adaptation to the avian host is needed before merozoites acquire ability to inhabit reticuloendothelial cells in organs. In dead house sparrows, phanerozoites were seen in lungs, liver, spleen and kidney on the 18 DPE, and they were present in all organs, including the brain 31 DPE. Common crossbills started dying 25 DPE during the experiment, and phanerozoites were seen in all of the examined tissues. These results second and expand on the results by [8, 17] who reported phanerozoites of *P. homocircumflexum* in all examined organs of birds which died 19 DPE and 38 DPE. Interestingly, phanerozoites were observed in the brain of all birds that died starting from 21 DPE until the end of the experiment, except for the starling, in which no phanerozoites developed. Phanerozoites in the brain were not observed in any of the birds that survived the experiment. This suggests that phanerozoites in the brain most likely cause the death in all *P. homocircumflexum* malaria cases, as was determined by prior works in some other malaria parasites [12].

Several birds from control groups died during this study, and this partly complicates the understanding of the experimental results. Two of the control house sparrows, one control common chaffinch and one control common starling died during this long-lasting experiment (Table 1), indicating that maintaining of wild birds in captivity and experimental manipulations are stressful for them. This raises the question whether the all deaths observed in the experimental groups were truly related to the effects of malaria or were, they—at least in part—caused by other unknown factors. The experiment with common crossbills was most successful, and it could help answering this question. Mainly, 7 of 8 infected common crossbills died during the experiment ($p < 0.001$) and phanerozoites were seen in every examined organ, including the brain. One of 8 crossbills with parasitaemia of approximately 8% survived the experiment; it was euthanized and phanerozoites were seen in all examined organs except for the brain. Also, none of the control group crossbills died. This allows the assumption, that most likely phanerozoites in the brain, along with damage to parenchymal organs were the cause of death of the experimental birds. It seems probable that even though several control group birds died in the cases of house sparrows, common starlings and common chaffinches (behaviour of these birds was particularly stressful in captivity), it is likely that phanerozoites of *P. homonucleophilum* developing in the brain are essential reason of mortality during this study.

It is important to note that in the case of common starlings, results of virulence differed from those obtained during our former pilot study [17] during which only one juvenile common starling was exposed to *P. homocircumflexum* infection. Development of the parasitaemia followed a similar pattern in both experiments. Mainly, after similar prepatent period, the parasitaemia developed to reach a peak and then decreased, eventually turning into a chronic stage. On 36 DPE of the pilot study the common starling suddenly died. Phanerozoites were located in the examined organs (brain, heart, liver, lung, kidney, spleen and a piece of the pectoral muscle) and positive ISH result confirmed that these exo-erythrocytic meronts were correctly identified as *Plasmodium* phanerozoites. That was not the case in this study. Two starlings died very early in the experiment (8 DPE and 9 DPE). Histological and ISH examination did not reveal any developed exo-erythrocytic stages. The low parasitaemia and absence of parasites in tissues suggest that these two individuals died of factors other than malaria. Even though parasitaemia developed in surviving birds in this study, as was also indicated by the pilot study—it reached a peak and turned into chronic stage, but exposed birds survived longer than expected according to the pilot study. Only one common starling infected with *P. homocircumflexum* died on 64 DPE. After the end of the experiment, all infected starlings were examined histologically. Contrary to the pilot study, no phanerozoites were detected in neither the surviving starlings, nor the one that died during the experiment. The negative histological results were confirmed by ISH tests performed in all examined organs of all experimental starlings. This raises a question for the reason of this difference is in these two experiments. Previous studies have suggested that common starlings can resist *P. relictum* (lineage pSGS1) infection [12, 20]. Since the starling in the pilot study was caught from the wild, it is impossible to know if it had not contracted some immune system suppressing disease prior to the experiment in the

pilot study. This result also suggests that there might be marked individual variation in susceptibility of birds to support exo-erythrocytic development, which initiation mechanism remains unclear. The intriguing question remains, why common starlings are able to resist malaria *P. relictum* and *P. homocircumflexum* infections. This avian host and the parasites might be used as model organisms to study molecular mechanisms of innate resistance during avian malaria.

Conclusion

Contrary to the common belief, bird malaria parasites cause not only severe blood pathology, but also inhabit and damage various internal organs including brain, heart, liver, lungs, kidneys and spleen. Knowledge about the development of tissue stages remain insufficient during avian malaria, especially in wildlife. This study shows that wild birds belonging to three families (house sparrows, chaffinches, common crossbills and common starlings) are highly susceptible to *P. homocircumflexum* infection, which is broad generalist malaria parasite. Mortality due to malaria was reported in three of the four exposed bird species, and the brain damage due to cerebral ischemia caused by phanerozoites was associated with mortality in the majority of exposed birds. This finding contributes to better understanding the pathology during avian malaria infections and indicate possible directions for development of treatment, which must address not only blood stages, but also tissue stages of the parasite, which damage organs all over the body of birds.

Abbreviations

BCIP: 5-bromo-4-chloro-3-indolyl phosphate; *cytb*: mitochondrial cytochrome *b*; DNA: deoxynucleic acid; DPE: days post exposure; H&E: haematoxylin–eosin; ISH: chromogenic in situ hybridization; NBT: 4-nitro blue tetrazolium chloride; PCR: polymerase chain reaction; rRNA: ribosomal ribonucleic acid.

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Authors' contributions

Conceptualization: MI, VP, GV; Formal analysis: DB; Funding acquisition: GV; Investigation: MI, DB, VP, TI, KF, HW, EP; Resources: HW, GV; Supervision: GV; Visualization: MI, TI; Writing—original draft preparation: MI; Writing—review and editing: DB, VP, TJ, KF, HW, EP, GV. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The experiments described in this study comply with the current laws of Lithuania and Russia. All experimental procedures were according to the All Union State standard (ГОСТ № P53434-2009 "Principles of good laboratory practice") of Russian Federation. All experimental procedures were approved by the Biological Station Rybachy of the Zoological Institute, Russian Academy of sciences and are in accordance to the International Research Co-operation Agreement between the Biological Station Rybachy and Nature Research Centre (Vilnius, Lithuania) (№ 25-05-2010). Work with birds was also approved by the Forest and Nature Protection Agency of Kaliningrad Region, Russia (№ 18, 5-05-2016). None of the experimental birds suffered apparent injury during experiments. All efforts were made to minimize handling time and potential suffering of birds.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Atkinson CT, Thomas NJ, Hunter DB. Parasitic diseases of wild birds. Oxford: Wiley-Blackwell Press; 2008.
2. Marzal A. Recent advances in studies on avian malaria parasites. In: Okwa OO, editor. Malaria parasites. InTech; 2012. p. 135–58.
3. Valkiūnas G, Iezhova T. Keys to avian malaria parasites. Malar J. 2018;17:212.
4. Chagas CRF, Valkiūnas G, Nery CVC, Henrique PC, Gonzalez IHL, Monteiro EF, et al. *Plasmodium* (Novyella) *nucleophilum* from an Egyptian Goose in São Paulo Zoo, Brazil: microscopic confirmation and molecular characterization. Int J Parasitol Parasites Wildl. 2013;2:286–91.
5. Mantilla JS, González AD, Valkiūnas G, Moncada LI, Matta NE. Description and molecular characterization of *Plasmodium* (Novyella) *unalis* sp. nov. from the Great Thrush (Turdusfuscater) in highland of Colombia. Parasitol Res. 2013;112:4193–204.
6. Ilgūnas M, Palinauskas V, Iezhova T, Valkiūnas G. Molecular and morphological characterization of two avian malaria parasites (Haemosporida: Plasmodiidae), with description of *Plasmodium homonucleophilum* n. sp. Zootaxa. 2013;3666:49–61.
7. Walther EL, Valkiūnas G, González AD, Matta NE, Ricklefs RE, Cornel A, et al. Description, molecular characterization, and patterns of distribution of a widespread New World avian malaria parasite (Haemosporida: Plasmodiidae), *Plasmodium* (Novyella) *homopolare* sp. nov. Parasitol Res. 2014;113:3319–32.
8. Palinauskas V, Žiegytė R, Ilgūnas M, Iezhova TA, Bernotienė A, Bolshakov C, et al. Description of the first cryptic avian malaria parasite, *Plasmodium homocircumflexum* n. sp. with experimental data on its virulence and development in avian hosts and mosquitoes. Int J Parasitol. 2015;45:51–62.
9. Valkiūnas G, Ilgūnas M, Bukauskaitė D, Palinauskas V, Bernotienė R, Iezhova T. Molecular characterization and distribution of *Plasmodium matutinum*, a common avian malaria parasite. Parasitology. 2017;144:1726–35.
10. Valkiūnas G, Iezhova T. Exo-erythrocytic development of avian malaria and related haemosporidian parasites. Malar J. 2017;16:101.

11. Garnham PCC. Malaria parasites and other haemosporidia. Hoboken: Blackwell Scientific Publications Press; 1966.
12. Valkiūnas G. Avian malaria parasites and other Haemosporidia. Boca Raton: CRC Press; 2005.
13. Braga EM, Silveira P, Belo NO, Valkiūnas G. Recent advances in the study of avian malariae: an overview with an emphasis on the distribution of *Plasmodium* spp. in Brazil. Mem Inst Oswaldo Cruz. 2011;106(Suppl 1):3–11.
14. Grilo ML, Vanstreels RET, Wallace R, Garcia-Parraga D, Braga EM, Chitty J, et al. Malaria in penguins—current perceptions. Avian Pathol. 2016;45:393–407.
15. Hayworth AM, Van Riper C, Weathers WW. Effects of *Plasmodium relictum* on the metabolic rate and body temperature in Canaries (*Serinus canarius*). J Parasitol. 1987;73:850–3.
16. Vanstreels RE, da Silva-Filho RP, Kolesnikovas CK, Bhering RC, Ruoppolo V, Epiphanio S, et al. Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. Vet Res. 2015;46:30.
17. Ilgūnas M, Bukauskaitė D, Palinauskas V, Iezhova TA, Dinhopl N, Nedorost N, et al. Mortality and pathology in birds due to *Plasmodium* (Giovannolaia) *homocircumflexum* infection, with emphasis on the exoerythrocytic development of avian malaria parasites. Malar J. 2016;15:256.
18. Fix AS, Waterhouse C, Greiner EC, Stoskopf MK. *Plasmodium relictum* as a cause of avian malaria in wild-caught magellanic penguins (*Spheniscus magellanicus*). J Wildl Dis. 1988;24:610–9.
19. Vanstreels RET, Kolesnikovas CKM, Sandri S, Silveira P, Belo NO, Ferreira FC, et al. Outbreak of avian malaria associated to multiple species of *Plasmodium* in Magellanic penguins undergoing rehabilitation in southern Brazil. PLoS ONE. 2014;9:e94994.
20. Palinauskas V, Valkiūnas G, Bolshakov VC, Bensch S. *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. Exp Parasitol. 2008;120:372–80.
21. Palinauskas V, Valkiūnas G, Križanauskienė A, Bensch S, Bolshakov CV. *Plasmodium relictum* (lineage P-SGS1): further observation of effects on experimentally infected passeriform birds, with remarks on treatment with Malarone™. Exp Parasitol. 2009;123:134–9.
22. Cellier-Holzem E, Esparza-Salas R, Garnier S, Sorci G. Effect of repeated exposure to *Plasmodium relictum* (lineage SGS1) on infection dynamics in domestic canaries. Int J Parasitol. 2010;40:1447–53.
23. Bichet C, Cornet S, Larcombe S, Sorci G. Experimental inhibition of nitric oxide increases *Plasmodium relictum* (lineage SGS1) parasitemia. Exp Parasitol. 2012;132:417–23.
24. Cornet S, Bichet C, Larcombe S, Faivre B, Sorci G. Impact of host nutritional status on infection dynamics and parasite virulence in a bird-malaria system. J Anim Ecol. 2014;83:256–65.
25. Ellis VA, Cornet S, Merrill L, Kunkel MR, Tsunekage T, Ricklefs RE. Host immune responses to experimental infection of *Plasmodium relictum* (lineage SGS1) in domestic canaries (*Serinus canaria*). Parasitol Res. 2015;114:3627–36.
26. Dinhopl N, Mostegl MM, Richter B, Nedorost N, Maderner A, Fragner K, et al. Application of in situ hybridization for the detection and identification of avian malaria parasites in paraffin wax-embedded tissues from captive penguins. Avian Pathol. 2011;40:315–20.
27. Dinhopl N, Nedorost N, Mostegl MM, Weissenbacher-Lang C, Weissenböck H. In situ hybridization and sequence analysis reveal an association of *Plasmodium* spp. with mortalities in wild passerine birds in Austria. Parasitol Res. 2015;114:1455–62.
28. Howe L, Castro IC, Schoener ER, Hunter S, Barraclough RK, Alley MR. Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. Parasitol Res. 2012;110:913–23.
29. Martinsen ES, Sidor IF, Flint S, Cooley J, Pokras MA. Documentation of malaria parasite (*Plasmodium* spp.) infection and associated mortality in a Common Loon (*Gavia immer*). J Wildl Dis. 2017;53:859–63.
30. Verwey JK, Peters A, Monks D, Raidal SR. Spillover of avian haemosporidian parasites (Haemosporidia: *Plasmodium*) and death of captive psittacine species. Aust Vet J. 2018;96:93–7.
31. Pérez-Tris J, Hellgren O, Križanauskienė A, Waldenström J, Secondi J, Bonneaud C, et al. Within-host speciation of malaria parasites. PLoS ONE. 2007;2:e235.
32. Vinogradova NV, Dolnik VR, Efremov VD, Payevsky VA. Guide for identification of sex and age of passerine birds of the fauna of the USSR. Handbook. Moscow: Nauka Press; 1976 **(in Russian)**.
33. Iezhova TA, Valkiūnas G, Bairlein F. Vertebrate host specificity of two avian malaria parasites of the subgenus Novyella: *Plasmodium nucleophilum* and *Plasmodium vaughani*. J Parasitol. 2005;91:472–4.
34. Valkiūnas G, Iezhova TA, Križanauskienė A, Palinauskas V, Sehgal RNM, Bensch S. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. J Parasitol. 2008;94:1395–401.
35. Godfrey RD, Fedynich AM, Pence DB. Quantification of hematozoa in blood smears. J Wildl Dis. 1987;23:558–65.
36. R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2013. http://www.R-project.org/. Accessed 13 Dec 2018.
37. Sambrook J, Fritsch EF, Mamiatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
38. Hellgren O, Waldenström J, Bensch S. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol. 2004;90:797–802.
39. Bensch S, Stjernman M, Hasselquist D, Ostman O, Hansson B, Westerdahl H, et al. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc Biol Sci. 2000;267:1583–9.
40. Hall A. BioEdit: a user-friendly biological sequence alignment editor and analysis program of Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–8.
41. Bensch S, Hellgren O, Perez-Tris J. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome *b* lineages. Mol Ecol Resour. 2009;19:1353–8.
42. Durrant KL, Beadell JS, Ishtiaq F, Graves GR, Olson SL, Gering E, et al. Avian hematozoa in South America: a comparison of temperate and tropical zones. Ornithol Monogr. 2006;60:98–111.
43. Moens MAJ, Valkiūnas G, Paca A, Bonaccorso E, Aguirre N, Pérez-Tris J. Parasite specialization in a unique habitat: hummingbirds as reservoirs of generalist blood parasites of Andean birds. J Anim Ecol. 2016;85:1234–45.
44. Rochlin I, Ninivaggi DV, Hutchinson ML, Farajollahi A. Climate change and range expansion of the Asian Tiger mosquito (Aedes albopictus) in Northeastern USA: implications for public health practitioners. PLoS ONE. 2013;8:e60874.
45. Samy AM, Elaagip AH, Kenawy MA, Ayres CFJ, Peterson AT, Soliman DE. Climate change influences on the global potential distribution of the mosquito *Culex quinquefasciatus*, vector of West Nile Virus and lymphatic filariasis. PLoS ONE. 2016;11:e0163863.
46. Matson KD, Ricklefs RE, Klasing KC. A hemolysis–hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. Dev Comp Immunol. 2005;29:275–86.
47. Matson KD, Cohen AA, Klasing KC, Ricklefs RE, Scheuerlein A. No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl. Proc R Soc B. 2006;273:815–22.
48. Mendes L, Piersma T, Hasselquist D, Matson KD, Ricklefs RE. Variation in the innate and acquired arms of the immune system among five shorebird species. J Exp Biol. 2006;209:284–91.
49. Råberg L, Grahn M, Hasselquist D, Svensson E. On the adaptive significance of stress-induced immunosuppression. Proc R Soc B. 1998;265:1637–41.
50. Kogut MH. Impact of nutrition on the innate immune response to infection in poultry. J Appl Poult Res. 2009;18:111–24.
51. Corradetti A, Neri I, Cavallini C. Non identiti di *Plasmodium paddae* e di *Plasmodium praecoz* (*relictm*). Parassitologia. 1968;10:145–9.
52. Permin A, Juhl J. The development of *Plasmodium gallinaceum* infections in chickens following single infections with three different dose levels. Vet Parasitol. 2002;105:1–10.
53. Paulman A, McAllister MM. *Plasmodium gallinaceum*: clinical progression, recovery, and resistance to disease in chickens infected via mosquito bite. Am J Trop Med Hyg. 2005;73:1104–7.
54. Williams RB. Avian malaria: clinical and chemical pathology of *Plasmodium gallinaceum* in the domesticated fowl *Gallus gallus*. Avian Pathol. 2005;34:29–47.
55. Palinauskas V, Žiegytė R, Šengaut J, Bernotienė R. Different paths—the same virulence: experimental study on avian single and co-infections

with *Plasmodium relictum* and *Plasmodium elongatum*. Int J Parasitol. 2018;48:1089–96.
56. Atkinson CT, Lease JK, Drake BM, Shema NP. Pathogenicity, serological responses, and diagnosis of experimental and natural malarial infections in native Hawaiian thrushes. Condor. 2001;103:209–18.

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PAPER VII

The life cycle of the avian haemosporidian parasite *Haemoproteus majoris*, with emphasis on the exo-erythrocytic and sporogonic development.

Ilgūnas, M., Chagas, C.R.F, Bukauskaitė, D., Bernotienė, R., Iezhova, T., Valkiūnas, G.

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RESEARCH

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The life-cycle of the avian haemosporidian parasite *Haemoproteus majoris*, with emphasis on the exoerythrocytic and sporogonic development

Mikas Ilgūnas*, Carolina Romeiro Fernandes Chagas, Dovilė Bukauskaitė, Rasa Bernotienė, Tatjana Iezhova and Gediminas Valkiūnas

Abstract

Background: *Haemoproteus* parasites (Haemosporida, Haemoproteidae) are cosmopolitan in birds and recent molecular studies indicate enormous genetic diversity of these pathogens, which cause diseases in non-adapted avian hosts. However, life-cycles remain unknown for the majority of *Haemoproteus* species. Information on their exoerythrocytic development is particularly fragmental and controversial. This study aimed to gain new knowledge on life-cycle of the widespread blood parasite *Haemoproteus majoris*.

Methods: *Turdus pilaris* and *Parus major* naturally infected with lineages hPHYBOR04 and hPARUS1 of *H. majoris*, respectively, were wild-caught and the parasites were identified using microscopic examination of gametocytes and PCR-based testing. Bayesian phylogeny was used to determine relationships between *H. majoris* lineages. Exoerythrocytic stages (megalomeronts) were reported using histological examination and laser microdissection was applied to isolate single megalomeronts for genetic analysis. *Culicoides impunctatus* biting midges were experimentally exposed in order to follow sporogonic development of the lineage hPHYBOR04.

Results: Gametocytes of the lineage hPHYBOR04 are indistinguishable from those of the widespread lineage hPARUS1 of *H. majoris*, indicating that both of these lineages belong to the *H. majoris* group. Phylogenetic analysis supported this conclusion. Sporogony of the lineage hPHYBOR04 was completed in *C. impunctatus* biting midges. Morphologically similar megalomeronts were reported in internal organs of both avian hosts. These were big roundish bodies (up to 360 μm in diameter) surrounded by a thick capsule-like wall and containing irregularly shaped cytomeres, in which numerous merozoites developed. DNA sequences obtained from single isolated megalomeronts confirmed the identification of *H. majoris*.

Conclusions: Phylogenetic analysis identified a group of closely related *H. majoris* lineages, two of which are characterized not only by morphologically identical blood stages, but also complete sporogonic development in *C. impunctatus* and development of morphologically similar megalomeronts. It is probable that other lineages belonging to the same group would bear the same characters and phylogenies based on partial *cytb* gene could be used to predict life-cycle features in avian haemoproteids including vector identity and patterns of exoerythrocytic merogony. This study reports morphologically unique megalomeronts in naturally infected birds and calls for research on exoerythrocytic development of haemoproteids to better understand pathologies caused in avian hosts.

Keywords: *Haemoproteus*, Molecular characterization, Exoerythrocytic development, Megalomeronts, Sporogony

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Background

Blood parasites belonging to the genus *Haemoproteus* Kruse, 1890 (Haemosporida: Haemoproteidae) have been reported in birds all over the world, except for Antarctica. These are some of the most extensively studied pathogens of birds [1]. Two subgenera have been distinguished in this genus: *Haemoproteus* comprising species transmitted by louse flies (Hippoboscidae) and *Parahaemoproteus* comprising species transmitted by biting midges (Ceratopogonidae) [2]. Over 150 species belonging to this genus were described using morphological characters of their blood stages (gametocytes) [2–4] and available molecular data suggest that the number might be even greater [5–7].

Despite the cosmopolitan distribution and great species diversity, information about complete life-cycles of the vast majority of *Haemoproteus* parasites is lacking. This is particularly true for exoerythrocytic and sporogonic development of these pathogens [8, 9]. Numerous recent studies have addressed the taxonomy, genetic diversity, ecology, evolutionary biology and genetics of avian haemosporidians [1, 6, 7, 10–13]. Such studies were designed mainly by analysing the blood stages (gametocytes), which are present in the circulation and are relatively easy to sample. This provided opportunities to gain new knowledge on the molecular biology and ecology of these parasites, particularly their molecular diagnostics, however contributing scarce information about exoerythrocytic development in avian hosts and sporogonic development in vectors. Knowledge about these parts of the haemosporidian life-cycles is essential for better understanding the epidemiology of haemoproteosis and pathologies caused by these parasites but remains markedly fragmentary [14, 15]. Early studies usually considered *Haemoproteus* parasites as relatively benign in the vertebrate hosts [16]. However, the application of molecular diagnostic methods has challenged this opinion due to the discovery of numerous well-documented cases of severe haemoproteosis in non-adapted avian hosts [17–23]. Severe pathologies and even mortality have been reported, particularly when *Haemoproteus* infection was established in non-adapted ("wrong") avian hosts. In such cases, exoerythrocytic development can be initiated but occurs incompletely and finally is aborted, yet it leads to severe disease [24]. Interestingly, sporogonic stages (ookinetes) of avian *Haemoproteus* parasites can markedly damage the midguts of blood-sucking insects (both vectors and non-vectors) and even kill them after blood meals with heavy gametocytaemia, but this issue and its biological significance remains insufficiently understood in wildlife [25].

Studies addressing the transmission and sporogonic development of avian haemoproteids remain uncommon [9, 14, 26–29] and vector species are unknown for the great majority of *Haemoproteus* species and their lineages [10, 30, 31]. Biting midges of the genus *Culicoides* have been successfully used in the experimental sporogony research of avian haemoproteids [9, 14]. These blood-sucking dipterans are abundant in temperate climate zones in Europe and wild-caught insects can be used for experimental exposure [14, 28]. Additionally, protocols have been developed to maintain *Culicoides nubeculosus* and some other species of biting midges in captivity, providing opportunities for experimental sporogony research [29, 32, 33]. Molecular detection of *Haemoproteus* lineages in wild-caught insects remains important because it provides useful information about links between parasite lineages and blood-sucking insects in the wild [34–37], but is insufficient to prove if the PCR-positive insects are competent vectors that can support complete sporogony and development of the infective sporozoites. Experimental observations and other methods providing opportunities to access sporozoites in insects remain essential in vector research [38].

This study aimed to contribute new knowledge about the genetic diversity, exoerythrocytic development and sporogony of *Haemoproteus majoris*, a widespread blood parasite of passeriform birds. One fieldfare *Turdus pilaris* naturally infected with the lineage hPHYBOR04 of *Haemoproteus majoris* was sampled and the parasites were identified to species level using the morphology of blood stages and partial cytochrome *b* (*cytb*) gene sequence. Exoerythrocytic development of this parasite is reported and sporogony followed in experimentally exposed *Culicoides impunctatus* biting midges. Because morphologically unique exoerythrocytic stages (megalomeronts) were detected, one great tit *Parus major* naturally infected with a closely related lineage hPARUS1 of *H. majoris* was also sampled and examined histologically for the presence of megalomeronts. The main goals of this study were: (i) to identify the gametocytes of the hPHYBOR04 lineage to the species level; (ii) to determine closely related lineages of this parasite; (iii) to investigate the exoerythrocytic development of the hPHYBOR04 lineage and to test a hypothesis that its closely related lineage hPARUS1 develop similar megalomeronts; (iv) to investigate the sporogonic development of the lineage hPHYBOR04 in experimentally exposed *C. impunctatus*.

Methods

Study site and selection of *Haemoproteus* species infected birds

Birds were sampled at the Ventės ragas ornithological station (55°20′38.93″N, 21°11′34.05″E) in May of 2018 and vector competence experiments were carried out in the Labanoras Forest (55°12′25.77″N, 25°55′26.47″E)

in June of the same year in Lithuania. Birds were caught using mist nets, zig-zag traps and funnel type traps, which were available at the ornithological station. Blood was drawn from each individual bird by puncturing the brachial vein. Thin blood films were prepared on glass slides, fixed with absolute methanol, stained with Giemsa and examined under a microscope as described by Valkiūnas et al. [39]. In parallel, about 30 μl of blood from each individual bird was also collected and fixed in SET buffer [40] for polymerase chain reaction (PCR)-based testing. SET buffer-fixed samples were stored at −4 °C in the field and at −20 °C once back to the laboratory. One fieldfare *Turdus pilaris* and one great tit *Parus major* were found naturally infected with single *Haemoproteus* infections. These birds were selected for examination of blood stages, vector research and histological investigation. The status of single infection in experimental birds was determined by microscopic examination of blood films in the field and later confirmed by observations of electropherograms of DNA sequences in the laboratory (double-base calling was not reported).

Design of sporogony research

Wild *Culicoides impunctatus* biting midges were exposed to *H. majoris* infection by allowing them to take blood meals on the selected fieldfare, as described and illustrated by Valkiūnas [2] and Žiegytė et al. [30]. Briefly, the infected fieldfare was held in hands protected by rubber gloves and biting midges were allowed to feed naturally between 22:00 and 23:00 h. This bird was exposed to bites at a site with high density of biting midges for approximately 30 min. The procedure was repeated 3 times in 3 successive days. *Culicoides impunctatus* willingly took bird blood meal on the parasite donor bird and numerous feeding insects were observed on the bird's head. When approximately 20 females began taking blood meals on the bird's head, the head with the feeding insects was carefully placed into an unzipped insect cage (approximately 12 × 12 × 12 cm) made of fine-mesh bolting silk. The engorged females flew off bird's head after finishing the blood meal. The cage with engorged biting midges was then closed using a zipper. Males and non-fed females were removed from the cages. The cages with the engorged biting midges were transported to the laboratory where they were kept at 23 ± 1 °C, 70 ± 5% relative humidity and light:dark photoperiod of 17:7 h. The experimentally exposed biting midges were fed by placing one pad of cotton moistened with 10% saccharose solution on top of the cage. A total of 38 females were collected after blood meal on the fieldfare with intensity of mature gametocytes parasitaemia of 0.03%. The insects were dissected at set intervals of time for detection of ookinetes (5 insects), oocysts (6 insects) and sporozoites (27 insects), as described by Valkiūnas [2] and Žiegytė et al. [30]. Ninety-six non-fed females of *C. impunctatus* were collected at the study site and fixed in 96% ethanol. They were used to evaluate the possibility of natural infections in biting midges at the study site.

Sporogonic stage samples

The experimentally exposed biting midges were dissected and preparations of ookinetes, oocysts and sporozoites were made at set time intervals. Briefly, the insects were anesthetized by placing them in a tube covered with a cotton-pad moistened with 96% ethanol. For visualizing the ookinetes, midguts of the blood-fed *C. impunctatus* females were extracted and gently crushed on objective glass slides 12 h post-exposure (hpe); thin preparations were made, fixed and stained the same way as blood films.

For oocyst observation, temporary preparations were made between 3–6 days post-exposure (dpe). Midguts were gently dissected and placed on a glass slide. Then, a drop of 2% mercurochrome solution was placed on each midgut, which was covered with a coverslip and examined under a microscope, as described by Žiegytė et al. [30].

To visualize sporozoites, the salivary glands were isolated from the biting midge females 6–9 dpe and gently crushed on glass slides to prepare small thin smears. The smears were fixed with absolute methanol and stained with 4% Giemsa solution for 1 h.

After each insect dissection, residual parts of their bodies were fixed in 96% ethanol and used for PCR-based analysis to confirm insect species identification and the presence of the corresponding parasite lineage in vectors. Dissection needles were disinfected in fire to prevent contamination after each dissection.

Histological samples

At the end of the study, the naturally infected experimental fieldfare and one great tit infected with *H. majoris* (lineages hPHYBOR04 and hPARUS1, respectively) were euthanized. Brain, heart, kidneys, liver, lungs, spleen and a piece of the pectoral muscle were collected from each bird, fixed in 10% neutral formalin and embedded in paraffin blocks. Histological sections of 4 μm were prepared, stained with haematoxylin-eosin (H&E) and examined microscopically [2]. Additionally, histological sections of 4 μm were also prepared on paraffin membrane slides (MMI-MembraneSlide, Molecular Machines and Industries, Zurich, Switzerland) for laser microdissection studies.

Parasite morphological analysis

Blood stages

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera and AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) imaging software was used to examine blood slides, prepare illustrations and take measurements of gametocytes. The blood films were examined for 15–20 min at medium magnification (400×) and then at least 100 fields were studied at high magnification (1000×). Intensity of parasitaemia was calculated as a percentage by actual counting of the number of parasites per 1000 erythrocytes or per 10,000 erythrocytes if the infections were light [41].

Exoerythrocytic stages

An Olympus BX51 light microscope (Olympus) equipped with an Olympus DP12 digital camera and Olympus DP-SOFT imaging software was used to examine H&E stained histological sections. First, each histological preparation was examined at medium magnification (400×). If present, tissue stages of haemosporidian parasites can be readily visible. If exoerythrocytic meronts were found, they were examined and illustrated under set of different magnifications (100, 200, 400 and 1000×) for better visualization of parasite location and structure.

Sporogonic stages

An Olympus BX43 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus SZX2-FOF digital camera and QCapture Pro 6.0, Image Pro Plus (Teledyne Imaging, Surrey, Canada) imaging software was used to examine ookinete, oocyst and sporozoite preparations. All preparations were examined under high (1000×) magnification. Images of parasites were collected and used for measurement using the program QCapture Pro 6.0 (Teledyne Imaging, Surrey, Canada).

Molecular analysis

DNA extraction and PCR from blood samples

Total DNA was extracted from blood samples fixed in SET buffer using the standard ammonium-acetate protocol [42]. Partial mitochondrial cytochrome *b* (*cytb*) sequences were amplified using a nested-PCR protocol [40, 43]. The total volume of the PCR mix was 25 µl and it consisted of 12.5 µl of Dreamtaq Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania), 8.5 µl of nuclease-free water, 1 µl of each primer and 2 µl of template DNA. The primer pair HaemNFI/HaemNR3 was applied for the initial PCR according to the protocol described by Hellgren et al. [40]. The primer pair HAEMF/HAEMR2 was applied for the second reaction according to the protocol by Bensch et al. [43]. Two microlitres of the first PCR product was used for the second PCR instead of genomic DNA. Nuclease-free water (negative control) and a *Haemoproteus* sample, which was positive in previous testing (positive control), were used to determine possible false amplifications. No case of false amplification was found.

Laser microdissection, DNA extraction and PCR using single megalomeronts

To confirm the identity of megalomeronts observed in the H&E stained histological section, laser microdissection was applied to isolate single megalomeronts. An Olympus IX71 light microscope (Olympus, Tokyo, Japan) equipped with Olympus/MMI CellCut Plus laser system and PTP function software (Predefined Target Position, Molecular Machines and Industries, Zurich, Switzerland) was used to cut single megalomeronts from non-stained histological sections. Adjustments of the contrast allowed for easy identification of these structures on paraffin membrane preparations (Fig. 1). Dissected megalomeronts were removed from the membrane using the adhesive silicone caps of the MMI IsolationCaps (Molecular Machines and Industries, Zurich, Switzerland) test tubes.

DNA from the dissected megalomeronts was isolated using the Chelex (Bio-Rad, Hercules, California, USA) DNA extraction protocol according to Palinauskas et al. [44]. Briefly, 0.2 g of Chelex was suspended in 1 ml of nuclease-free water and incubated in a 56 °C water bath for 1h. Next, the suspension was allowed to cool to room temperature. Then, 25 µl was placed on the wall of each tube, vortexing the suspension between each pipetting. After that, 0.7 µl of Proteinase K (10 mg/ml) was added to each Chelex drop. The tubes were then closed and flipped upside down for the Chelex/Proteinase K mixture to cover the adhesive cap of each tube. Tubes (still upside down) were incubated in a 56 °C water bath for 1h followed by 12 min incubation at 95 °C to inactivate the Proteinase K. The tubes were briefly spun down, the adhesive caps were replaced with regular caps and the tubes were centrifuged at 13.400× *g* for 12 min. The supernatant was used for PCR immediately. The total volume of the PCR mix was 25 µl and consisted of 12.5 µl of Dreamtaq Master Mix (Thermo Fisher Scientific), 8.5 µl of nuclease-free water, 1 µl of each primer and 2 µl of template DNA.

The primer pair HQF/HQR, which amplifies a short (194 bp) fragment of the mitochondrial *cytb* gene, was used for the PCR according to the conditions described by Ciloglu et al. [45]. The main goal of the amplification of the short sequences was to prove that the observed megalomeronts truly belong to *Haemoproteus* species. Due to the fixation of the histological samples in formalin and DNA extraction using the Chelex protocol, the relatively long haemosporidian parasite barcoding

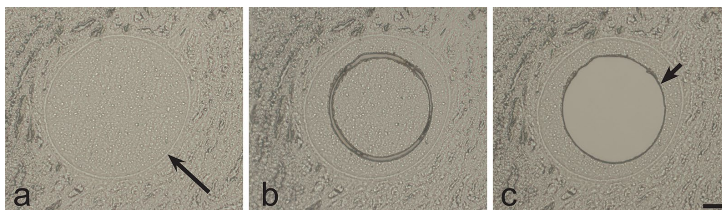


Fig. 1 Laser microdissection of single megalomeronts of *Haemoproteus majoris* (lineage hPARUS1) from kidneys of the great tit *Parus major*. **a** Single intact megalomeront on a membrane slide before dissection. **b** Same single intact megalomeront on a membrane slide after dissection. **c** Same single megalomeront on a membrane slide after the removal of its dissected part. Note that only the central portion of the megalomeront was dissected for genetic analysis as was evident due to the intact capsule-like wall covering the parasite. Long simple arrow: capsule-like wall of megalomeront; short simple arrow: the hole in the membrane left after the dissection of the megalomeront. Unstained histological sections. *Scale-bar*: 20 µm

sequence (479 bp) of *cytb* could not be amplified. However, shorter fragments were amplified and used for the parasite genus identification. The primer pair HQF/HQR readily amplifies *Haemoproteus* spp. sequences [45] and was selected for the identification of megalomenronts to the generic level. Nuclease-free water (negative control) was used to determine possible false amplifications. No case of false amplification was found.

DNA extraction from vectors and PCR

DNA extraction from vectors and the applied PCR protocols were identical to those used for blood samples with one exception. Because vector samples for molecular analysis were stored in 96% ethanol, the fixed insects were transferred into SET buffer. This was achieved by placing the insect remains into an empty 1.5 ml tube, air-drying the remaining ethanol and pouring 250 µl SET buffer onto the dry insect.

Because the *C. impunctatus* used in the experiments were wild-caught, the prevalence of possible natural *Haemoproteus* spp. infection was determined in free-living biting midges that were sampled at the study site. These insects were tested by PCR-based methods. DNA was extracted from 24 pools of biting midges, each containing 4 flies. Additionally, remains of all experimental biting midges were collected after the dissections during sporogony research; they were fixed in 96% ethanol and tested by PCR amplification in order to confirm the identity of the parasite lineages in the experimentally exposed flies and confirm the species identification of biting midges. For the latter test, the insect specific primers LCO149 and HCO2198 were applied to amplify a fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene [46].

DNA sequencing and phylogenetic analysis

The success of the performed PCRs was evaluated by running electrophoresis on a 2% agarose gel. Two microlitres of the PCR products were used to test the success of amplification for each performed reaction. Successfully amplified PCR products were sequenced from both 5'- and 3'-ends using dye terminator cycle sequencing (Big Dye). Sequencing was carried out using an ABI PRISM TM 3100 capillary sequencing robot (Applied Biosystems, Foster City, California, USA).

Sequences were edited and examined using the BioEdit software [47]. The 'Basic Local Alignment Search Tool' (megablast algorithm) was used to identify the amplified *cytb* sequences and *cox*1 sequences of insects in the NCBI GenBank [48] and the 'Basic Local Alignment Search Tool' of the MalAvi database was used to double-check the identification [5]. A Bayesian phylogenetic tree was constructed using partial mitochondrial *cytb* sequences (479 bp). A total of 41 *Haemoproteus* spp. and 11 *Plasmodium* spp. lineages were used to construct the tree. All known *H. majoris* lineages were used; these were obtained from the MalAvi database (http://130.235.244.92/Malavi/). Additionally, several lineages that are most similar to the known lineages of this parasite were selected using the National Center for Biotechnology Information (NCBI) BLAST algorithm. *Plasmodium* lineages were included in the analysis with the aim to show that these and *Haemoproteus* spp. sequences do not position randomly in the tree but form separate clades. One *Leucocytozoon* sp. lineage was used as the outgroup.

The phylogenetic tree was computed using the MrBayes version 3.1 software [49]. Best-fit model of evolution (GTR) was selected by the software MrModeltest 3.7 [50]. The analysis was run for a total of 10 million generations and sample frequency was set to every 100th generation. Before the construction of the consensus tree, 25% of the initial trees were discarded as 'burn in'. The constructed phylogenetic tree was visualized in FigTree v1.4.3 [51]. Genetic distances between lineages were calculated using the Jukes-Cantor model of substitution, as implemented in the program MEGA 7.0 [52].

Statistical analysis

Statistical analysis of mean lineal parameters of parasites was carried out using 'R' version 3.4.3 and packages *Rcmdr* and *RcmdrMisc* [53]

Results

Parasite lineages and phylogenetic analysis

Single infection of the lineage hPARUS1 of *H. majoris* and the unidentified to the species level lineage hPHYBOR04 were found in the great tit and fieldfare, respectively, during PCR-based screening. This finding was in accordance with blood film microscopic examination, which allowed detecting the presence of single *Haemoproteus* infections of morphologically distinct gametocytes in these birds as well. Phylogenetic analysis grouped these parasite lineages in one well-supported clade (Fig. 2, Clade A) together with other lineages of *H. majoris* (hWW2, hPHSIB1 and hCCF5), suggesting a close phylogenetic relationship among them. Genetic differences among the morphologically identified *cytb* lineages of *H. majoris* varied between 0.2% (hPHYBOR04-hCWT4 and hCWT4-hWW2) and 1.3% (hPHYBOR04-hCCF5).

Characterization of *Haemoproteus* (*Parahaemoproteus*) *majoris* (lineage hPHYBOR04)

To date, parasites of the lineage hPHYBOR04 have not been identified to the species level and their gametocytes have not been described. A description of the parasite is given below.

Haemoproteus (Parahaemoproteus) majoris (lineage hPHYBOR04)

Avian hosts: According to this study, MalAvi database and the GenBank data, this lineage has been recorded in fieldfare *Turdus pilaris* (Lithuania; this study), Arctic warbler *Phylloscopus borealis* (USA, S. Galen & S. Perkins unpublished data, GenBank: MG726191) and Gray-cheeked thrush *Catharus minimus* (USA, S. Galen & S. Perkins unpublished data, MalAvi database).

Vector: *Culicoides impunctatus* (see description of the sporogonic stages below).

Site of infection: Gametocytes develop in mature erythrocytes; megalomeronts were seen in kidneys (see description below).

Representative blood films: Voucher specimens (accessions 49045-49057 NS, intensity of parasitaemia 0.1–1.0%, *T. pilaris*, sampled at Ventes ragas, Lithuania, collected by M. Ilgūnas, from 17 May to 11 June 2018) were deposited at the Nature Research Centre, Vilnius, Lithuania and the Queensland Museum, Queensland, Australia (accession number G466217).

Representative histological sections: Histological preparations of kidneys of *T. pilaris* are deposited at Nature Research Centre, Vilnius, Lithuania (accession number 49154 NS) and the Queensland Museum, Queensland, Australia (accession number G466218).

Representative vector preparations: Preparations of ookinetes from midguts and sporozoites from salivary glands of *Culicoides impunctatus* are deposited at Nature Research Centre, Vilnius, Lithuania (accession numbers 49152 and 49153 NS, respectively).

Representative DNA sequences: Mitochondrial *cytb* lineage hPHYBOR04 (479 bp, GenBank: MN219405).

Description

Young gametocytes

Earliest forms present free in cytoplasm, located anywhere in infected erythrocytes, but more frequently in polar or subpolar positions to erythrocyte nuclei (Fig. 3a-c). Gametocytes, reaching size of nuclei of erythrocytes in length, closely appressed to nuclei of infected erythrocytes (Fig. 3d) present, and this contact seen during gametocyte growth and maturation (Fig. 3e, f). Advanced gametocytes extended longitudinally along nuclei of erythrocytes and adhered to envelop of erythrocytes (Fig. 3e). Gametocyte nuclei prominent (Fig. 3c–e). Pigment granules small (< 0.5 mm), often grouped (Fig. 3e). Volutin granules visible and aggregated close to periphery in advanced gametocytes (Fig. 3e). Outlines varying from even (Fig. 3b, d) to irregular (Fig. 3a, e) and ameboid (Fig. 3b) present.

Macrogametocytes

Developing in mature erythrocytes. Cytoplasm blue, slightly heterogeneous in appearance, possessing fine

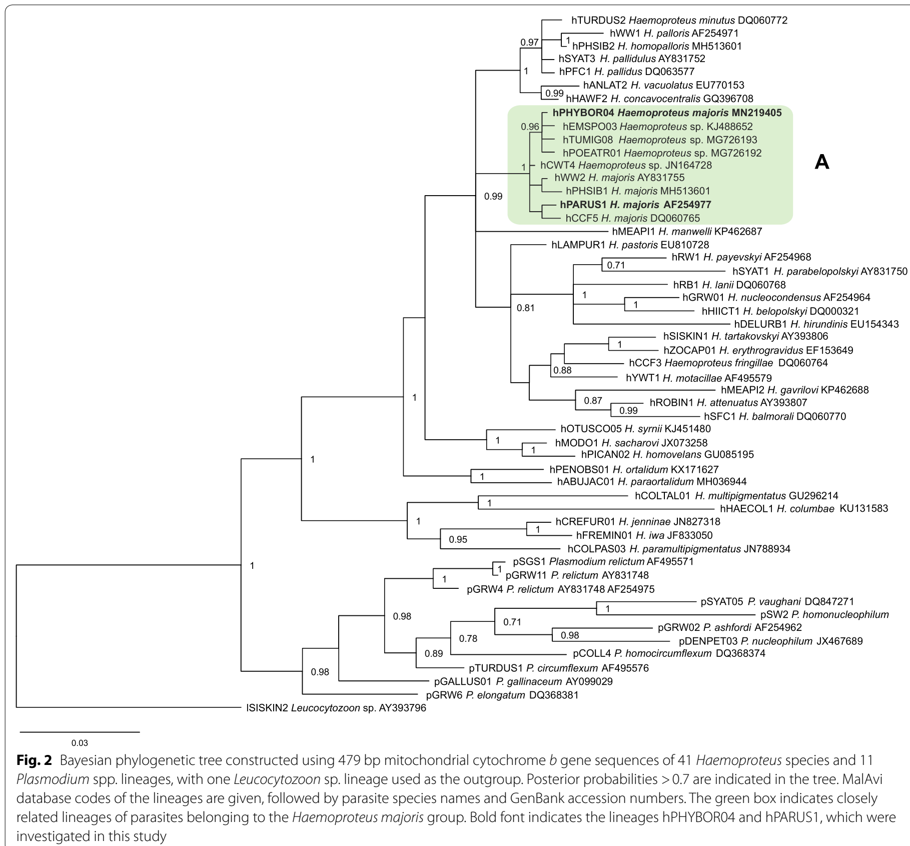


Fig. 2 Bayesian phylogenetic tree constructed using 479 bp mitochondrial cytochrome *b* gene sequences of 41 *Haemoproteus* species and 11 *Plasmodium* spp. lineages, with one *Leucocytozoon* sp. lineage used as the outgroup. Posterior probabilities > 0.7 are indicated in the tree. MalAvi database codes of the lineages are given, followed by parasite species names and GenBank accession numbers. The green box indicates closely related lineages of parasites belonging to the *Haemoproteus majoris* group. Bold font indicates the lineages hPHYBOR04 and hPARUS1, which were investigated in this study

homogenously dispersed volutin (Fig. 3l). Gametocytes growing along and closely adhering to nuclei of infected erythrocytes (Fig. 3f–h), slightly displacing nuclei laterally, enclosing them with ends, but not encircling completely (Fig. 3f–l) present. Advanced gametocytes closely appressed both to nuclei and envelope of erythrocytes and finally fill up poles of erythrocytes (Fig. 3h–l). Central part of growing gametocyte frequently constricted (Fig. 3f–h), giving dumbbell-like appearance to parasite (Fig. 3g, h); tips of dumbbell-shaped gametocytes adhering to envelope of erythrocytes (Fig. 3f–h). Dumbbell-shaped growing macrogametocytes common; after parasite maturation, dumbbell-shaped gametocytes not present anymore. Parasite nucleus variable in form, frequently roundish or oval (Fig. 3h, i), usually subterminal (Fig. 3h, i, k), but occasionally seen in central (Fig. 3j) or terminal (Fig. 3h, l) positions. Nucleolus not seen. Pigment granules of medium size (0.5–1 mm), roundish, oval and sometimes slightly elongate in form, usually more or less randomly scattered throughout cytoplasm (Fig. 3h–l). Outline of macrogametocytes usually even or slightly wavy, but slightly ameboid forms seen occasionally. Nuclei of infected erythrocytes slightly displaced laterally (Fig. 3h–l).

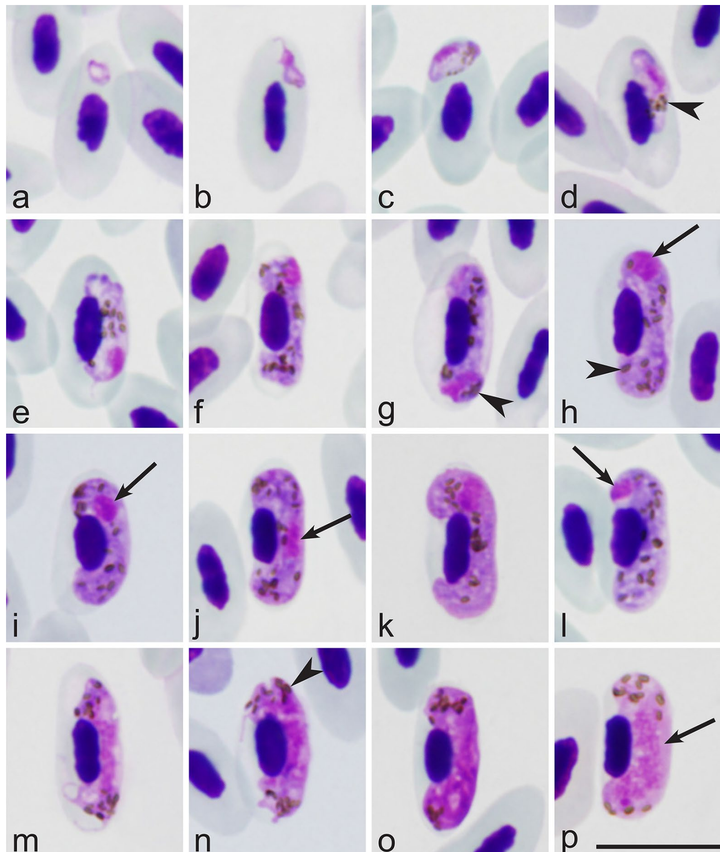


Fig. 3 Gametocytes of *Haemoproteus majoris* (lineage hPHYBOR04) from the blood of the fieldfare *Turdus pilaris*. **a**–**d** Young gametocytes. **e**–**l** Macrogametocytes. **m**–**p** Microgametocytes. Arrows point to parasite nuclei; arrowheads point to pigment granules. Giemsa-stained thin blood films. *Scale-bar*: 10 μm

Microgametocytes

General configuration as for macrogametocytes with usual haemosporidian sexually dimorphic characters: pale stained cytoplasm and large, markedly diffuse centrally located nuclei. Ameboid growing microgametocytes (Fig. 3m) common, but fully-grown microgametocytes usually with even outline (Fig. 3o, p). Pigment granules aggregated in nucleus-free tips of gametocytes (Fig. 3p).

Remarks

The main diagnostic characters of the reported gametocytes of lineage hPHYBOR04 in the fieldfare are indistinguishable from those of the lineage hPARUS1 belonging *H. majoris* in its type vertebrate host, the great tit. Parasites of both these lineages certainly belong to the same morphospecies.

(See figure on next page.)
Fig. 4 Megalomeronts of *Haemoproteus majoris* (lineage hPHYBOR04) from the kidneys of the fieldfare *Turdus pilaris* at four different magnifications. **a**, **b** 100×. **c**, **d** 200×. **e**, **f** 400×. **g**, **h** 1000×. Note that each megalomeronts is surrounded by a thick capsule-like wall and contains numerous cytomeres of irregular-shape, in which merozoites develop. Long simple arrows: megalomeronts; short simple arrows: capsule-like wall of megalomeront; simple arrowhead: cytomeres; triangular arrowheads: merozoites. Haematoxylin-eosin stained histological sections. *Scale-bar*: 20 µm

Exoerythrocytic development of *Haemoproteus majoris* (lineages hPHYBOR04 and hPARUS1)

Exoerythrocytic meronts were seen only in the histological preparations of kidneys in fieldfare infected with *H. majoris* (lineage hPHYBOR04, 0.2% parasitaemia intensity) (Fig. 4). These were big (up to 360 µm in diameter) roundish bodies (Fig. 4a, b), each covered with a prominent capsule-like wall (Fig. 4c–f, h), which was up to 6 µm in width. Developing megalomeronts contained numerous irregularly shaped cytomeres (Fig. 4c, d), in which merozoites (Fig. 4f, h) develop. The smallest cytomeres were roundish, with nuclear material aggregated on their periphery (Fig. 4e, g). Mature megalomeronts contained numerous uninuclear merozoites (Fig. 4f, h). Host cell nucleus was not visible. Inflammatory reaction was not seen around the megalomeronts.

Numerous morphologically similar megalomeronts were also observed in the internal organs of the great tit infected with *H. majoris* (lineage hPARUS1, intensity of parasitaemia of 4%) (Fig. 5). In this bird, single megalomeronts were seen in the liver, lungs, spleen; megalomeronts were especially numerous in the kidneys where up to 18 megalomeronts were observed in a single histological section (histological preparations of liver, lungs, spleen and kidneys of the *P. major* are deposited at Nature Research Centre, Vilnius, Lithuania (accession number 49155–49158 NS, respectively)).

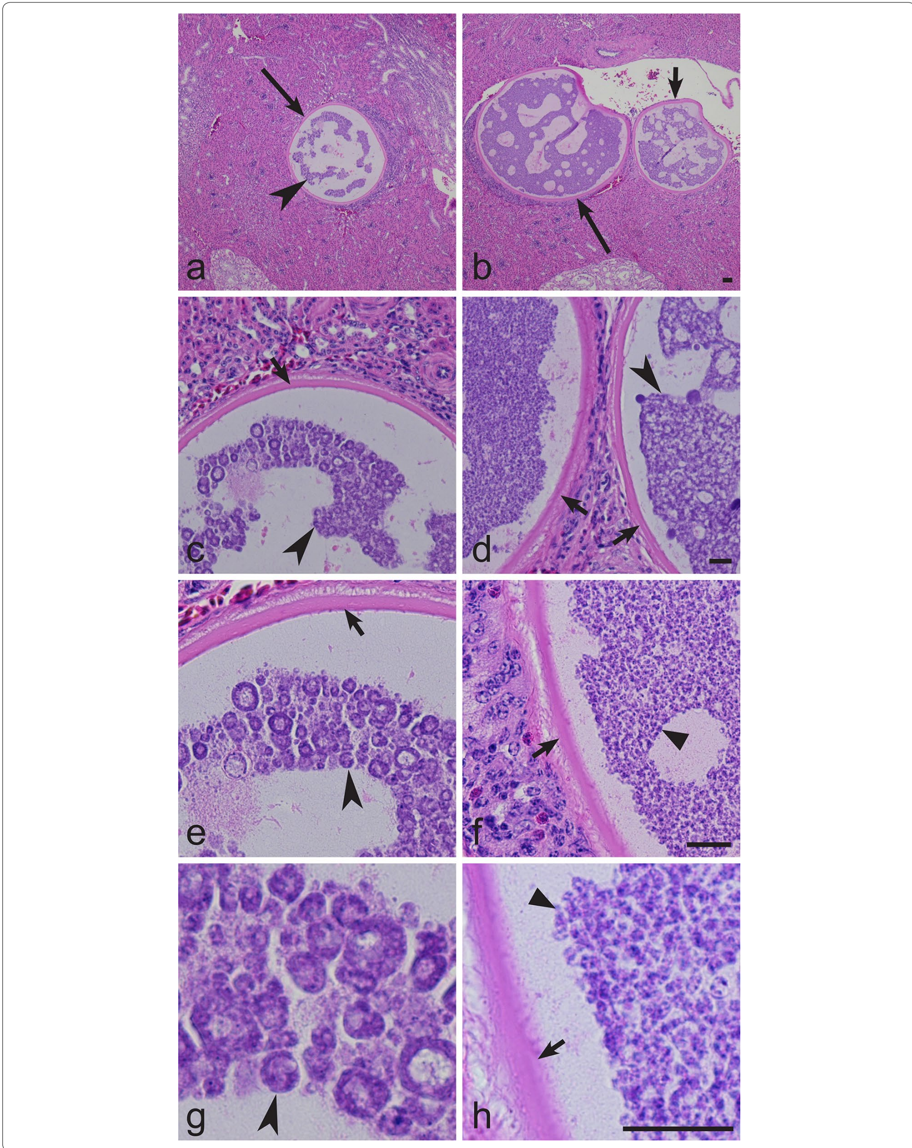
Laser microdissection of single megalomeronts from the great tit (Fig. 1) followed by DNA extraction, PCR and sequencing, confirmed that the observed megalomeronts belong to *H. majoris*. Three individual megalomeront preparations were analysed using laser microdissection and the reported sequence (194 bp) coincided with the hPARUS1 lineage of genus *Haemoproteus* in all three cases.

Sporogonic development of *Haemoproteus majoris* (lineage hPHYBOR04)

All pools of wild-caught *C. impunctatus* females (controls) tested by PCR were negative for haemosporidian infections, indicating that the insects sampled in the wild population did not carry natural infections and thus could be used for experimental exposure and experimental sporogony research of *H. majoris*. *Haemoproteus majoris* (lineage hPHYBOR04) completed sporogony in the experimentally exposed biting midge *C. impunctatus*. Ookinetes, oocysts and sporozoites were observed (Fig. 6a–c). Ookinetes were detected 12 hpe (Fig. 6a). Mature ookinetes were elongated bodies with clearly visible prominent nuclei and vacuoles. The ookinetes ($n=6$) measured 11.1–15.4 (mean 13.6 ± 1.3) µm in length and 1.9–2.8 (mean 2.3 ± 0.3) µm in width and 16.6–33.8 (mean 24.7 ± 5.7) µm^2 in area. Developing oocysts were observed 4 dpe (Fig. 6b). Sporogonic development completed successfully and sporozoites were observed 6–9 dpe in the salivary gland preparations (Fig. 6c), indicating that these biting midges are likely the natural vector of this parasite. Sporozoites are fusiform bodies with slightly off-centre located nuclei. Measurements of the sporozoites are given in Table 1.

Discussion

The key result of this study is the discovery of the exoerythrocytic stages in two lineages (hPHYBOR04 and hPARUS1) of *H. majoris*, a widespread blood parasite of passeriform birds. We have shown that these stages are megalomeronts (Figs. 4, 5). These data completed the information on the exoerythrocytic part of the life-cycle of this pathogen. To date, megalomeronts have been found in eight species of avian haemoproteids, but they were reported sporadically usually as case reports in non-passeriform birds [8]. Previous studies have detected megalomeronts in parrots [20, 24, 54], turkeys [55, 56], house sparrows [57–59], sacred kingfishers [60], bobwhite quails [17] and pigeons [61, 62] but parasite lineage identity has not been determined. As a result, the origin of the *Haemoproteus* species, which develop megalomeronts in avian hosts, remained unclear as well, except for *H. minutus* (lineages hTUPHI01 and hTURDUS2), which have been reported to cause lethal disease in parrots [24]. Particularly, it remained unclear if megalomeronts develop only when sporozoites are injected in non-adapted ("wrong") avian hosts, as was the case in parrots, or do these stages develop normally in the life-cycles of *Haemoproteus* species. The present study shows that megalomeronts are a normal part of exoerythrocytic development in common avian haemoproteids because the lineages hPHYBOR04 and hPARUS1 of *H. majoris* are widespread and their gametocytes have been often reported during natural infections (Fig. 3) [5], indicating a complete life-cycle in avian hosts. Due to the huge size of the megalomeronts and high intensity in some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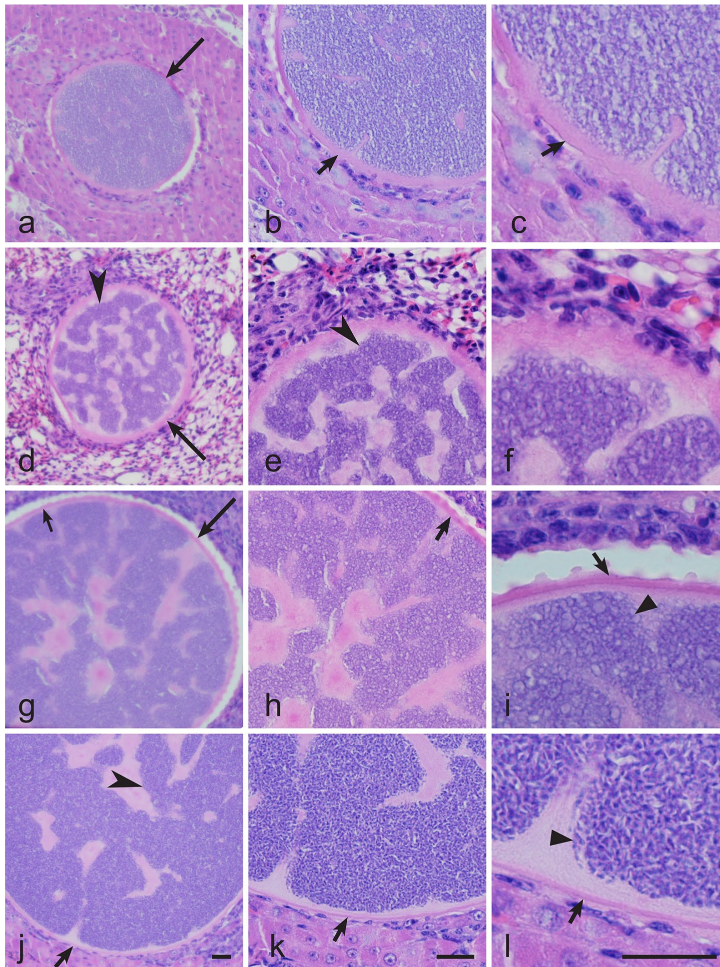

Fig. 5 Megalomeronts of *Haemoproteus majoris* (lineage hPARUS1) from the internal organs of the great tit *Parus major*. **a**–**c** Liver. **d**–**f** Lungs. **g**–**i** Spleen. **j**–**l** Kidneys. Same megalomeronts are shown at three different magnifications: **a**, **d**, **g**, **j**, 200×; **b**, **e**, **h**, **k** 400×; **c**, **f**, **i**, **l** 1000×. Note that the structure of the megalomeronts was similar in different organs, i.e. the parasites were covered with prominent capsule-like walls and contained numerous irregularly-shaped cytomeres, in which merozoites develop. Long simple arrows: megalomeronts; short simple arrows: capsule-like wall; simple arrowhead: cytomeres; triangular arrowhead: merozoites. Haematoxylin-eosin stained histological sections. *Scale-bar*: 20 µm

organs (kidneys), megalomeronts of *Haemoproteus* parasites are worthy of more attention for better understanding of the pathologies caused during haemoproteosis. Unfortunately, the information on history of the infections reported here (freshly acquired or relapsed infection) in examined birds is unknown because the birds

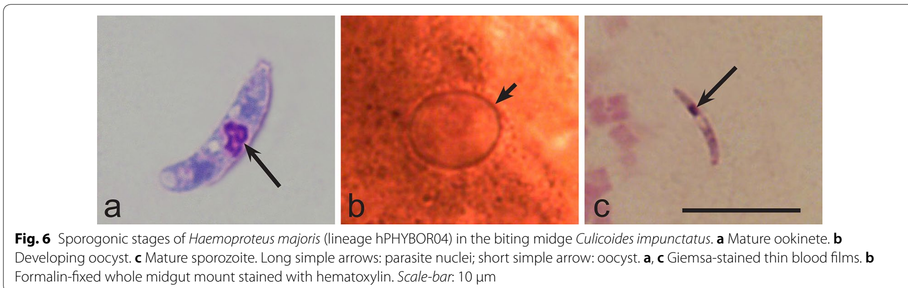

Fig. 6 Sporogonic stages of *Haemoproteus majoris* (lineage hPHYBOR04) in the biting midge *Culicoides impunctatus*. **a** Mature ookinete. **b** Developing oocyst. **c** Mature sporozoite. Long simple arrows: parasite nuclei; short simple arrow: oocyst. **a**, **c** Giemsa-stained thin blood films. **b** Formalin-fixed whole midgut mount stained with hematoxylin. *Scale-bar*: 10 μm

Table 1 Morphometry of sporozoites of *Haemoproteus majoris* (lineage hPHYBOR04) in biting midges *Culicoides impunctatus*

Feature	Measurements[a]
Length	6.7–9.2 (8.0 ± 0.6)
Width	0.9–1.6 (1.1 ± 0.2)
Area	4.4–8.5 (6.5 ± 1.0)

[a] Measurements ($n = 21$) are given in micrometres. Minimum and maximum values are provided, followed in parenthesis by the arithmetic mean and standard deviation

were naturally infected and wild-caught, so that the timing of infection cannot be specified. Infected individuals were active and looked non-exhausted in captivity.

Exoerythrocytic stages of avian haemoproteids were reviewed by Valkiunas & Iezhova [8]. Morphological features of *H. majoris* megalomeronts are unique among the avian haemoproteids described to date, particularly due to the presence of a prominent thick capsule-like wall covering the parasites and the markedly developed irregularly shaped cytomeres (Figs. 4, 5). Due to these features, the parasites reported here were similar to megalomeronts of *Leucocytozoon* spp. [2] or *Besnoitia* spp. [16]. To prove that the parasite stages detected in our study truly belong to haemoproteids, laser microdissection of single megalomeronts was carried out and DNA was isolated, amplified and sequenced. Because the detected *cytb* partial sequence coincided with the corresponding segment of the lineage hPARUS1, it was clear that the megalomeronts are stages of this parasite. This method was useful in species identification of oocysts in malaria parasites [63] and can be recommended in megalomeront studies. Additionally, *in situ* hybridization using specific probes can be used for distinguishing *Haemoproteus* infections from other parasites inhabiting tissues of birds [15].

The origin of the host cells of megalomeronts remains unclear. Host cell nucleus was not visible inside or close to megalomeronts, as is the case in megalomeronts of other avian haemoproteids but is characteristic to *Leucocytozoon* species [8]. Because megalomeronts of the lineage hPHYBOR04 were detected in numerous different internal organs, it seems probable that they might develop in non-specialised cells such as reticuloendothelial cells; further studies are needed to answer this question.

Nine closely related *Haemoproteus* lineages with genetic divergence of 0.2–1.3%, were grouped in a well-supported clade (Fig. 2, Clade A); of these, four have previously been characterised by morphology of their gametocytes as belonging to *H. majoris* [64]. This study showed that the lineage hPHYBOR04 also belongs to this species group. It is important to note that two recent studies [65, 66] have attributed the hCWT4 lineage to *H. majoris*. This is in agreement with our phylogenetic analysis; however, morphological characterization of the hCWT4 lineage gametocytes is still needed for final proof of this conclusion because some morphologically distinct *Haemoproteus* species differ just in few nucleotides in their partial *cytb* gene lineages [4, 29, 67].

Two lineages of *H. majoris* (hPHYBOR04 and hPARUS1) completed sporogonic development in *C. impunctatus* ([14]; this study). Previous studies have shown that *C. impunctatus* supports the complete sporogonic development of *H. balmorali* (lineage hSFC9), *H. belopolskyi* (hHIICT1), *H. minutus* (hTURDUS2), *H. motacillae* (hYWT1), *H. noctuae* (hCIRCUM01), *H. pallidus* (hPFC1) and unknown lineages of *H. dolniki*, *H. fringillae*, *H. lanii*, *H. parabelopolskyi* and *H. tartakovskyi* [2, 14, 30, 68, 69]. *Culicoides impunctatus* has been formerly considered to be mainly mammalophilic [70]; however, recent experimental observations and molecular testing indicate that this species also willingly feeds with bird blood and thus is worthy of attention in haemosporidiosis epidemiology research.

It is important to note that the lineages hPHYBOR04 and hPARUS1 of *H. majoris* are characterised by two

similar features, i.e. these parasites complete sporogonic development in *C. impunctatus* and develop similar megalomeronts (Figs. 4, 5). This finding provides an opportunity to speculate that phylogenies based on the partial *cytb* gene can be used for prediction of life-cycle patterns in avian haemoproteids. In other words, it is probable that other lineages of Clade A (Fig. 2) would also complete sporogonic development in the biting midge *C. impunctatus* and develop megalomeronts in the vertebrate hosts. Testing of this hypothesis is of theoretical interest for better understanding of possible application of molecular phylogenies in studies of the biology of haemosporidian parasites.

Conclusions

The complete life-cycle of *H. majoris* was uncovered, including the exoerythrocytic development, growth of gametocytes and sporogony from ookinetes to sporozoite stage. This study extended the knowledge about the genetic diversity of *H. majoris* by identification of one new lineage of this widespread blood parasite. We have shown that the lineage hPHYBOR04 of *H. majoris* completes sporogony in the biting midge *C. impunctatus*, as is the case with the lineage hPARUS1 of *H. majoris*. Importantly, these two lineages of *H. majoris* were found not only appearing in the same clade in the phylogeny, but also producing morphologically similar megalomeronts in different naturally infected avian hosts. In other words, the well-supported clades in phylogenies based on partial *cytb* gene are worthy of attention because they might indicate similar exoerythrocytic development in closely related parasites. This study shows that the megalomeronts of *Haemoproteus* parasites appear not only during abortive infection in non-adapted ("wrong") avian hosts, but also develop during natural infections in the competent avian hosts. It is possible that megalomeronts often develop in various avian *Haemoproteus* infections thus, are worthy of more attention due to their large size, damage of internal organs and possible negative impact on the host health.

Abbreviations

cytb: mitochondrial cytochrome *b*; dpe: days post-exposure; H&E: haematoxylin-eosin; hpe: hours post-exposure; PCR: polymerase chain reaction.

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Authors' contributions

Conceptualization: GV and MI. Funding acquisition: RB and CRFC. Morphological investigation of blood stages: GV and TI. Histological investigation: MI. Molecular investigation: MI, RB. Phylogenetic analysis: MI. Laser microdissection: MI. Vector research: DB and CRFC. Statistical analysis: DB. Supervision: GV. Figure preparation: MI and TI. Writing (original draft preparation): MI and GV; Writing (review and editing): CRFC, DB, RB and TI. All authors read and approved the final manuscript.

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Availability of data and materials

Representative preparations of blood, exoerythrocytic and vector stages were deposited in the Nature Research Centre, Vilnius, Lithuania (accessions 49045-49057 NS and 49152-49158 NS). Additionally, preparations of blood and exoerythrocytic stages were deposited in the Queensland Museum, Queensland, Australia (accessions G466217 and G466218, respectively). A representative sequence was submitted to the GenBank database under the accession number MN219405. The datasets used and/or analysed during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study complies with the current laws of Lithuania, was performed by licenced researchers and was approved by the Lithuania and Environmental Protection Agency, Vilnius (2018-04-13, no. 24). None of the experimental birds suffered apparent injury during sampling.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Clark NJ, Clegg SM, Lima MR. A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): new insights from molecular data. Int J Parasitol. 2014;44:329–38.
2. Valkiūnas G. Avian malaria parasites and other haemosporidia. Boca Raton: CRC Press; 2005.
3. Dimitrov D, Iezhova TA, Zehtindjiev P, Bobeva A, Ilieva M, Kirilova M, et al. Molecular characterisation of three avian haemoproteids (Haemosporida, Haemoproteidae), with the description of *Haemoproteus* (*Parahaemoproteus*) *palloris* n. sp. Syst Parasitol. 2016;93:431–49.
4. Levin I, Valkiūnas G, Iezhova TA, O'Brien SL, Parker PG. Novel *Haemoproteus* species (Haemosporida: Haemoproteidae) from the swallow-tailed gull (Laridae), with remarks on the host range of hippoboscid-transmitted avian hemoproteids. J Parasitol. 2012;98:847–54.
5. Bensch S, Hellgren O, Pérez-Tris J. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome *b* lineages. Mol Ecol Resourc. 2009;19:1353–8.
6. Bensch S, Canbäck B, DeBarry JD, Johansson T, Hellgren O, Kissinger JC, et al. The genome of *Haemoproteus tartakovskyi* and its relationship to human malaria parasites. Genome Biol Evol. 2016;8:1361–73.
7. Ricklefs RE, Medeiros M, Ellis VA, Svensson-Coelho M, Blake JG, Loiselle BA, et al. Avian migration and the distribution of malaria parasites in New World passerine birds. J Biogeogr. 2017;44:1113–23.
8. Valkiūnas G, Iezhova TA. Exo-erythrocytic development of avian malaria and related haemosporidian parasites. Malar J. 2017;16:101.
9. Bukauskaitė D, Iezhova TA, Ilgūnas M, Valkiūnas G. High susceptibility of the laboratory-reared biting midges *Culicoides nubeculosus* to *Haemoproteus* infections, with review on *Culicoides* species that transmit avian haemoproteids. Parasitology. 2019;146:333–41.

10. Perkins SL. Malaria's many mates: past, present and future of the systematics of the order Haemosporida. J Parasitol. 2014;100:11–25.
11. Sehgal RN. Manifold habitat effects on the prevalence and diversity of avian blood parasites. Int J Parasitol Parasites Wildl. 2015;4:421–30.
12. Videvall E, Cornwallis CK, Ahrén D, Palinauskas V, Valkiūnas G, Hellgren O. The transcriptome of the avian malaria parasite *Plasmodium ashfordi* displays host-specific gene expression. Mol Ecol. 2017;26:2939–58.
13. Ellis VA, Sari EHR, Rubenstein DR, Dickerson RC, Bensch S, Ricklefs RE. The global biogeography of avian haemosporidian parasites is characterized by local diversification and intercontinental dispersal. Parasitology. 2019;146:213–9.
14. Žiegytė R, Markovets MY, Bernotienė R, Mukhin A, Iezhova TA, Valkiūnas G, et al. The widespread biting midge *Culicoides impunctatus* (Ceratopogonidae) is susceptible to infection with numerous *Haemoproteus* (Haemoproteidae) species. Parasit Vectors. 2017;10:397.
15. Himmel T, Harl J, Kübber-Heiss A, Konicek C, Fernández N, Juan-Sallés C, et al. Molecular probes for the identification of avian *Haemoproteus* and *Leucocytozoon* parasites in tissue sections by chromogenic *in situ* hybridization. Parasit Vectors. 2019;12:282.
16. Bennett GF, Peirce MA, Ashford RW. Avian haematozoa: mortality and pathogenicity. J Nat Hist. 1993;27:993–1001.
17. Cardona CJ, Ihejirika A, McClellan L. *Haemoproteus lophortyx* infection in bobwhite quail. Avian Dis. 2002;46:249–55.
18. Ferrell ST, Snowden K, Marlar AB, Garner M, Lung NP. Fatal hemoprotozoal infections in multiple avian species in a zoological park. J Zoo Wildl Med. 2007;38:309–16.
19. Donovan TA, Schrenzel M, Tucker TA, Pessier AP, Stalis IH. Hepatic hemorrhage, hemocoelom, and sudden death due to *Haemoproteus* infection in passerine birds: eleven cases. J Vet Diagn Invest. 2008;20:304–13.
20. Olias P, Wegelin M, Zenker W, Freter S, Gruber AD, Klopfleisch R. Avian malaria deaths in parrots. Europe. Emerg Infect Dis. 2011;17:950–2.
21. Cannell BL, Krasnec KV, Campbell K, Jones HI, Miller RD, Stephens N. The pathology and pathogenicity of a novel *Haemoproteus* spp. infection in wild little penguins (*Eudyptula minor*). Vet Parasitol. 2013;197:74–84.
22. Dunn JC, Goodman SJ, Benton TG, Hamer KC. Avian blood parasite infection during the non-breeding season: an overlooked issue in declining populations? BMC Ecol. 2013;13:30.
23. Groff TC, Lorenz TJ, Crespo R, Iezhova T, Valkiūnas G, Sehgal RNM. Haemoproteosis lethality in a woodpecker, with molecular and morphological characterization of *Haemoproteus velans* (Haemosporida, Haemoproteidae). Int J Parasitol Parasites Wildl. 2019;10:93–100.
24. Ortiz-Catedral L, Brunton D, Stidworthy MF, Elsheikha HM, Pennycott T, Schulze C, et al. *Haemoproteus minutus* is highly virulent for Australasian and South American parrots. Parasit Vectors. 2019;12:40.
25. Bukauskaitė D, Bernotienė R, Iezhova TA, Valkiūnas G. Mechanisms of mortality in *Culicoides* biting midges due to *Haemoproteus* infection. Parasitology. 2016;143:1748–54.
26. Martínez-de la Puente J, Martínez J, Rivero-de-Aguilar J, Herrero J, Merino S. On the specificity of avian blood parasites: revealing specific and generalist relationships between haemosporidians and biting midges. Mol Ecol. 2011;20:3275–87.
27. Santiago-Alarcon D, Palinauskas V, Schaefer HM. Diptera vectors of avian haemosporidian parasites: untangling parasite life cycles and their taxonomy. Biol Rev Camb Philos Soc. 2012;87:928–64.
28. Bobeva A, Zehtindjiev P, Bensch S, Radrova J. A survey of biting midges of the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) in NE Bulgaria, with respect to transmission of avian haemosporidians. Acta Parasitol. 2013;58:585–91.
29. Chagas CRF, Bukauskaitė D, Ilgūnas M, Iezhova T, Valkiūnas G. A new blood parasite of leaf warblers: molecular characterization, phylogenetic relationships, description and identification of vectors. Parasit Vectors. 2018;11:538.
30. Žiegytė R, Palinauskas V, Bernotienė R, Iezhova TA, Valkiūnas G. *Haemoproteus minutus* and *Haemoproteus belopolskyi* (Haemoproteidae): complete sporogony in the biting midge *Culicoides impunctatus* (Ceratopogonidae), with implications on epidemiology of haemoproteosis. Exp Parasitol. 2014;145:74–9.
31. Atkinson CT, Thomas NJ, Hunter DB. Parasitic diseases of wild birds. Oxford: Wiley-Blackwell; 2008.
32. Boorman J. The maintenance of laboratory colonies of *Culicoides variipenniss* (Coq.), *C. nubeculosus* (Mg.) and *C. riethi* Kieff (Diptera, Ceratopogonidae). Bull Entomol Res. 1974;64:371–7.
33. Pages N, Bréard E, Urien C, Talavera S, Viarouge C, Lorca-Oro C, et al. *Culicoides* midge bites modulate the host response and impact on bluetongue virus infection in sheep. PLoS ONE. 2014;9:e83683.
34. Ishtiaq F, Guillaumot L, Clegg SM, Phillimore AB, Black RA, Owens IP, et al. Avian haematozoan parasites and their associations with mosquitoes across southwest Pacific islands. Mol Ecol. 2008;17:4545–55.
35. Kim KS, Tsuda Y, Sasaki T, Kobayashi M, Hirota Y. Mosquito blood-meal analysis for avian malaria study in wild bird communities: laboratory verification and application to *Culex sasai* (Diptera: Culicidae) collected in Tokyo, Japan. Parasitol Res. 2009;105:1351–7.
36. Njabo KY, Cornel AJ, Bonneaud C, Toffelmier E, Sehgal RN, Valkiūnas G, et al. Nonspecific patterns of vector, host and avian malaria parasite associations in a central African rainforest. Mol Ecol. 2011;20:1049–61.
37. Ferraguti M, Martínez-delaPuente J, Ruiz S, Soriguer R, Figuerola J. On the study of the transmission network of blood parasites from SW Spain: diversity of avian haemosporidians in the biting midge *Culicoides circumscriptus* and wild birds. Parasit Vectors. 2013;6:208.
38. Bernotienė R, Žiegytė R, Vaitkutė G, Valkiūnas G. Identification of a new vector species of avian haemoproteids, with a description of methodology for the determination of natural vectors of haemosporidian parasites. Parasit Vectors. 2019;12:307.
39. Valkiūnas G, Ilgūnas M, Bukauskaitė D, Žiegytė R, Bernotienė R, Jusys V, et al. *Plasmodium delichoni* n. sp.: description, molecular characterisation and remarks on the exoerythrocytic merogony, persistence, vectors and transmission. Parasitol Res. 2016;115:2625–36.
40. Hellgren O, Waldenström J, Bensch S. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol. 2004;90:797–802.
41. Godfrey RD, Fedynich AM, Pence DB. Quantification of hematozoa in blood smears. J Wildl Dis. 1987;23:558–65.
42. Sambrook J, Fritsch EF, Mamiatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
43. Bensch S, Stjernman M, Hasselquist D, Ostman O, Hansson B, Westerdahl H, et al. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc Biol Sci. 2000;267:1583–9.
44. Palinauskas V, Dolnik O, Valkiunas G, Bensch S. Laser microdissection, microscopy, and single cell PCR of avian haemosporidians. J Parasitol. 2010;96:420–4.
45. Ciloglu A, Ellis VA, Bernotienė R, Valkiūnas G, Bensch S. A new one-step multiplex PCR assay for simultaneous detection and identification of avian haemosporidian parasites. Parasitol Res. 2019;118:191–201.
46. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.
47. Hall A. BioEdit: a user-friendly biological sequence alignment editor and analysis program of Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–8.
48. NCBI BLAST. Rockville Pike, Bethesda MD, USA. 2019. https://blast.ncbi.nlm.nih.gov/Blast.cgi.
49. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19:1572–4.
50. Nylander JAA. MrModeltest v2. Program distributed by the author. Uppsala: Evolutionary Biology Centre, Uppsala University; 2004.
51. Rambaut A. FigTree: tree figure drawing tool version 1.4.3. 2009. http://tree.bio.ed.ac.uk/software/figtree. Accessed 15 Nov 2017.
52. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870–4.
53. R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2013. http://www.R-project.org/.
54. Miltgen F, Landau I, Ratanaworabhan N, Yenbutra S. *Parahaemoproteus desseri* n. sp.; gamétogonie et schizogonie chez l'hôte naturel: *Psittacula roseata* de Thailande, et sporogonie expérimentale chez *Culicoides nubeculosus*. Ann Parasitol Hum Comp. 1981;56:123–30.
55. Atkinson CT, Greiner EC, Forrester DJ. Pre-erythrocytic development and associated host responses to *Haemoproteus meleagridis* (Haemosporina:

Haemoproteidae) in experimentally infected domestic turkeys. J Protozool. 1986;33:375–81.
56. Atkinson CT, Forrester DJ, Greiner EC. Pathogenicity of *Haemoproteus meleagridis* (Haemosporina: Haemoproteidae) in experimentally infected domestic turkeys. J Parasitol. 1988;74:228–39.
57. Wenyon CM. Protozoology: a manual for medical men, veterinarians and zoologists, etc. London: Bailliére Tindal & Cox; 1926.
58. Peirce MA. Haemoproteid parasites of *Passer* spp. Parasitology. 1976;73:407–15.
59. Burtikashvili LP. Blood parasites of wild birds in Georgia. (In Russian) Tbilisi: Metsniereba; 1978.
60. Peirce MA, Lederer R, Adlard RD, O'Donoghue PJ. Pathology associated with endogenous development of haematozoa in birds from southeast Queensland. Avian Pathol. 2004;33:445–50.
61. Farmer JN. Gizzard lesions associated with *Haemoproteus sacharovi* infections of pigeons. Proc Iowa Acad Sci. 1964;71:537–42.
62. Earlé RA, Bastianello SS, Bennett GF, Krecek RC. Histopathology and morphology of the tissue stages of *Haemoproteus columbae* causing mortality in Columbiformes. Avian Pathol. 1993;22:67–80.
63. Valkiūnas G, Žiegytė R, Palinauskas V, Bernotienė R, Bukauskaitė D, Ilgūnas M, et al. Complete sporogony of *Plasmodium relictum* (lineage pGRW4) in mosquitoes *Culex pipiens pipiens*, with implications on avian malaria epidemiology. Parasitol Res. 2015;114:3075–85.
64. Križanauskienė A, Hellgren O, Kosarev V, Sokolov L, Bensch S, Valkiūnas G. Variation in host specificity between species of avian hemosporidian parasites: evidence from parasite morphology and cytochrome *b* gene sequences. J Parasitol. 2006;92:1319–24.
65. Nilsson E, Taubert H, Hellgren O, Huang X, Palinauskas V, Markovets MY, et al. Multiple cryptic species of sympatric generalists within the avian blood parasite *Haemoproteus majoris*. J Evol Biol. 2016;29:1812–26.
66. Carbó-Ramírez P, Zuria I, Schaefer HM, Santiago-Alarcon D. Avian haemosporidians at three environmentally contrasting urban greensapaces. J Urban Ecol. 2017;3:1–11.
67. Hellgren O, Križanauskienė A, Valkiūnas G, Bensch S. Diversity and phylogeny of mitochondrial cytochrome *b* lineages from six morphospecies of avian *Haemoproteus* (Haemosporida: Haemoproteidae). J Parasitol. 2007;93:889–96.
68. Valkiūnas G, Križanauskienė A, Iezhova TA, Hellgren O, Bensch S. Molecular phylogenetic analysis of circumnuclear hemoproteids (Haemosporida: Haemoproteidae) of sylviid birds, with a description of *Haemoproteus parabelopolskyi* sp. nov. J Parasitol. 2007;93:680–7.
69. Bukauskaitė D, Žiegytė R, Palinauskas V, Iezhova TA, Dimitrov D, Ilgūnas M, et al. Biting midges (*Culicoides*, Diptera) transmit *Haemoproteus* parasites of owls: evidence from sporogony and molecular phylogeny. Parasit Vectors. 2015;8:303.
70. Blackwell AA, Mordue J, Mordue W. Identification of bloodmeals of the Scottish biting midge, *Culicoides impunctatus*, by indirect enzyme-linked immunosorbent assay (ELISA). Med Vet Entomol. 1994;8:20–4.

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PAPER VIII

The experimental study on susceptibility of common European songbirds to *Plasmodium elongatum* (lineage pGRW6), a widespread avian malaria parasite.

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RESEARCH

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The experimental study on susceptibility of common European songbirds to *Plasmodium elongatum* (lineage pGRW6), a widespread avian malaria parasite

Mikas Ilgūnas*, Vaidas Palinauskas, Elena Platonova, Tatjana Iezhova and Gediminas Valkiūnas

Abstract

Background: *Plasmodium elongatum* (cytochrome *b* lineage pGRW6) is a widespread avian malaria parasite, often causing severe disease in non-adapted hosts. This parasite lineage is of global distribution however, its virulence remains insufficiently understood, particularly in wild birds. Surprisingly, this infection has never been reported in Common starlings *Sturnus vulgaris* and Common crossbills *Loxia curvirostra*, common European songbirds which were extensively sampled across Europe. A hypothesis was proposed that these birds might be resistant to the pGRW6 infection. The aim of this study was to test this hypothesis.

Methods: Lineage pGRW6 was isolated from a naturally infected Eurasian reed warbler, multiplied in vivo and inoculated in Common starlings and Common crossbills. Experimental and control groups (8 birds in each) were maintained in controlled conditions and examined microscopically every 4 days. Haematocrit value and body mass were monitored in parallel. At the end of the experiment (44 days post exposure), samples of internal organs were collected and examined using histological methods for possible presence of phanerozoites.

Results: All control birds remained uninfected. Experimental starlings were resistant. All exposed crossbills were susceptible and survived until the end of this study. Prepatent period was 12–16 days post exposure. Light parasitaemia (< 0.7%) developed in all birds, and only few phanerozoites were seen in bone marrow cells of 5 of 8 experimentally infected crossbills. Significant changes were reported only in haematocrit value but not body mass in the exposed crossbills compared to controls.

Conclusion: *Plasmodium elongatum* (pGRW6) is of low virulence in Common crossbills and is unable to develop in Common starlings, indicating innate resistance of the later bird species. Low virulence in Common crossbills is likely due to the inability or low ability of this parasite lineage to develop phanerozoites resulting in light (if at all) damage of stem bone marrow cells. This study suggests that susceptibility of different bird species to the lineage pGRW6 is markedly variable. The global distribution of this parasite might be due to low virulence in wild adapted avian hosts, which survive this infection and serve as reservoirs host for non-adapted birds in whom this infection is often lethal.

Keywords: Avian malaria, *Plasmodium*, *Plasmodium elongatum*, Birds, Phanerozoites, Pathology

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Background

Malaria is burdening birds worldwide. In all, 55 morphologically readily distinct species of avian *Plasmodium* have been identified [1] and genetic data suggest that their number might be even greater [2]. However, virulence of the majority of *Plasmodium* infections remain insufficiently investigated, particularly in wildlife. Severe disease and mortality due to malaria have been often reported in zoos, aviaries and private collections worldwide. Non-adapted wild bird species also suffer dramatically [3–7]. Information about malaria influence on adapted wild birds is contradictory, with no certain pattern during development of the same *Plasmodium* species lineage in different species of avian hosts. For example, experimental data indicate that the cytochrome *b* lineage (*cytb*) pSGS1 of *Plasmodium relictum* might develop high parasitaemia resulting in severe anaemia in Common crossbills *Loxia curvirostra*, Eurasian siskins *Carduelis spinus* [8], but not in House sparrows *Passer domesticus* and Common chaffinches *Fringilla coelebs* [9]. The same is true for exo-erythrocytic development of the parasites. Mainly, *cytb* lineage pCOLL4 of *P. homocircumflexum* demonstrates markedly different ability to produce phanerozoites in different species of avian hosts resulting in different virulence and mortality rates [10].

Plasmodium elongatum (*cytb* lineage pGRW6) is one of the most widespread avian malaria agents, which has been reported in birds belonging to more than 15 avian families and 11 orders [2]. This parasite species was discovered by Clay G. Huff in 1930 in the USA, where it is widespread and prevalent. Since then, this infection has been reported in all continents, except Antarctica [11, 12]. However, intraspecies genetic variation of this pathogen was unclear. The lineage pGRW6 was originally determined by Beadell et al. [13], and it was assigned to *P. elongatum* approximately 10 years ago [14]. Several closely related lineages are available in GenBank and MalAvi database, and they probably belong to *P. elongatum*, but morphological evidence is lacking except for the lineage pERIRUB01. For unclear reasons, the latter parasite lineage is rare in wildlife [15].

Plasmodium elongatum was known to cause severe disease and even death in captive zoo birds around the world already during the "pre-molecular era" [16–21]. Parasitaemia is usually light (< 1%) both in naturally and experimentally infected birds [11, 16]. Thus, the primary reason of disease and death has been linked to distortion of stem bone marrow cells by exoerythrocytic meronts (phanerozoites) [11, 16]. Phanerozoites develop primarily in cells of the haemopoietic system, particularly in bone marrow, often resulting in the disruption of erythropoiesis and leading to anaemia even during light parasitaemia [12, 16, 22]. Recent molecular studies show that *P. elongatum* (pGRW6) is responsible for severe disease and even mortality in captive and wild non-adapted bird species worldwide [4–6].

Surprisingly, *P. elongatum* (pGRW6) infection has never been reported in Common starlings *Sturnus vulgaris* or Common crossbills *Loxia curvirostra*, common Holarctic songbirds which have been extensively sampled for haemosporidian parasites across Europe [2]. There are records of malaria parasites and other related haemosporidians in these two bird species (*Plasmodium ashfordi* lineage pGRW2), *Plasmodium relictum* (lineages pSGS1 and pGRW4), *Plasmodium homocircumflexum* (lineage pCOLL4), *Plasmodium unalis* (lineage pTUMIG03), *Haemoproteus tartakovskyi* (lineage hSISKIN1), *Haemoproteus pastoris* (lineage hLAMPUR01), but not of the lineage pGRW6 [2]. It is worth noting that Common startling is an invasive species spreading globally [23] in areas where *P. elongatum* has been often reported [2]. Common crossbills are also broadly distributed in the Holarctic zoogeographical region where transmission of *P. elongatum* also takes place. Both of these bird species likely have been exposed to this infection naturally and should be evolutionary adapted to this pathogen. A hypothesis was proposed that the lack of pGRW6 reports in crossbills and starlings might indicate their innate resistance to this infection. We aimed to test this hypothesis experimentally. *Plasmodium elongatum* (lineage pGRW6) was isolated from naturally infected reed warbler and experimentally passaged into juvenile starlings and crossbills, which were monitored in controlled laboratory conditions and examined using microscopic and histological methods.

Methods

Study site

Experimental work was carried out at the Biological Station of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea (55°05′ N, 20°44′ E) in July and August of 2016. Mist nets and Rybachy-type funnel traps were used to catch juvenile wild birds (< 7 months old). All birds were screened for haemosporidian infections using microscopic examination and only the non-infected individuals were selected. Negative result of prior haemosporidian infections of experimental birds was later confirmed using polymerase chain reaction (PCR)-based screening methods in the laboratory.

Experimental design

All birds were maintained under controlled conditions at a natural light–dark photoperiod. Control and

experimental groups of each bird species were maintained in separate cages (size of 90 × 50 × 90 cm) located close to each other. The Common crossbills were maintained indoors in a vector-free room. The Common starlings were kept in an outside aviary, in cages, covered with a fine-mesh bolting silk preventing penetration of blood-sucking insects.

A strain of *P. elongatum* (lineage pGRW6, GenBank accession no. DQ368381), isolated from a naturally infected Eurasian reed warbler *Acrocephalus scirpaceus* was multiplied in two uninfected Eurasian reed warblers and used to infect the recipient birds of each species. 50 µl of infected donor blood was mixed with 12.5 µl sodium citrate and 62.5 µl of 0.9% saline solution per recipient bird [24]. The prepared mixture was sub-inoculated into the pectoral muscle of the experimental birds of both species. Each bird was inoculated approximately 125 µl of the inoculum. In all, 16 Common starlings and 16 Common crossbills were used for this study: 8 birds of each species were inoculated with same isolate of *P. elongatum* while the remaining 8 birds of each species were maintained as controls to prove the absence of natural transmission of haemosporidians during this experiment.

All birds were maintained for 44 days post exposure (DPE). Birds of experimental and control groups were weighed and blood from brachial vein was collected for microscopic examination and haematocrit level measures every 4 days. Brachial vein was punctured with a sterile needle and approximately 50 µl of blood was collected in heparinized microcapillaries. A small drop of blood was used to make three blood films, which were air-dried, fixed by dipping in absolute methanol for 3 min., stained with Giemsa and examined microscopically [12]. Approximately 20 µl of the blood in the microcapillary was fixed in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for molecular analysis. These samples were maintained at −4 °C in the field and later at −20 °C in the laboratory. Remaining blood was used for centrifugation (10,000 rpm for 5 min.) and measuring of haematocrit value.

At the end of the experiment, all experimental birds were euthanized by decapitation, and the brain, heart, kidneys, liver, lungs, spleen, and a piece of the pectoral muscle were collected and fixed with 10% neutral formalin. Additionally, a smear of bone marrow was prepared; these smears were fixed with absolute methanol, stained with Giemsa keeping the same protocol as for blood films and examined using light microscope [12]. In the laboratory the collected tissues were embedded in paraffin blocks. Histological sections of 4 µm were prepared, stained with haematoxylin-eosin (H&E) and examined microscopically [12].

Morphological analysis

An Olympus BX51 light microscope equipped with the Olympus DP12 digital camera and imaging software Olympus DP-SOFT were used to examine preparations. Each blood film was examined for 15–20 min. at medium magnification (×400), and then at least 100 fields were studied at high magnification (×1000). Intensity of parasitaemia was calculated as a percentage by actual counting of the number of parasites per 1000 erythrocytes or per 10,000 erythrocytes if infections were light [25]. Histological preparations were examined at low magnification (×200) for 10–15 min., followed by examination at medium magnification (×400) for 10–15 min. and then at high magnification (×1000) for another 20–30 min.

Statistical analyses

Statistical analyses were carried out using the 'R' package [26]. Normality of data distribution was evaluated by applying the Shapiro–Wilk test. Differences between the means for data which were not distributed according to normal distribution were evaluated using the Wilcoxon test. Fisher's exact test was used to evaluate if there was a statistically significant difference between haematocrit levels and body mass between the control and experimental groups in each bird species.

Molecular analysis

Total deoxyribonucleic acid (DNA) was extracted from SET buffer fixed blood samples using an ammonium-acetate protocol [27]. Partial mitochondrial cytochrome *b* (*cytb*) sequences were amplified using a nested-PCR protocol [28, 29]. PCR mixes consisted of 12.5 µl of Dreamtaq Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 µl of nuclease-free water, 1 µl of each primer and 2 µl of template DNA. Primer pair HaemFNI/HaemNR3 was used for the first PCR according the protocol described by [29]. For the second PCR, the primer pair HAEMF/HAEMR2 was used according to the protocol by [28]. For the second PCR instead of genomic DNA, 2 µl of the first PCR product was used. PCR success was evaluated by performing electrophoresis on a 2% agarose gel. 2 µl of the second PCR product was used for this evaluation. Nuclease-free water (negative control) and a *Plasmodium* sample, which was positive in previous testing (positive control) were used to determine possible false amplifications. No case of false amplification was found. Positive PCR products were sequenced from the 5′ end with the HAEMF primer [28] using dye terminator cycle sequencing (Big Dye). Sequencing was carried out using an ABI PRISM TM 3100 capillary sequencing robot (Applied Biosystems, USA). Sequences of parasites were

edited and examined using the BioEdit program [30]. The 'Basic Local Alignment Search Tool' (megablast algorithm) was used to identify the amplified *cytb* sequences [31]. The 'Basic Local Alignment Search Tool' of the MalAvi database was used to double check the identified sequences [2].

Phylogenetic analysis

Phylogenetic tree was constructed using partial sequences (479 bp) of the mitochondrial *cytb* gene. In all, 37 sequences of *Plasmodium* and 9 sequences of *Haemoproteus* were used. One sequence of *Leucocytozoon* sp. (lineage lSISKIN1) was used as outgroup. The Bayesian phylogenetic tree was constructed using MrBayes version 3.1 software [32]. The best fitting model of evolution (GTR) was selected by software MrModeltest 3.7 [33]. Analysis was run for a total of 10 million generations with a sample frequency of every 100th generation. Before the construction of the consensus tree, 25% of the initial trees were discarded as the 'burn in' period. The tree was visualized using the software FigTree v1.4.3 [34].

Results

Both microscopic and molecular examinations showed that all birds used in this study were free of haemosporidian parasites prior to the experimental infections. All control birds remained uninfected during the entire study, indicating absence of transmission of haemosporidian parasites in captivity.

Microscopic examination showed that parasitaemia did not develop in any of the exposed Common starlings. That was confirmed by negative PCR tests in all experimental birds, indicating resistance of this avian host. All exposed Common crossbills were susceptible, with prepatent period ranging from 12 to 16 (on average 15) DPE (Fig. 1). Both microscopic (Fig. 2a–d) and PCR-based examinations showed presence of a single *P. elongatum* (lineage pGRW6) infection. Typical blood stages of *P. elongatum* were observed (Fig. 2a–d). Mainly, erythrocytic meronts occurred in immature erythrocytes; the parasites were small and contained readily visible pigment and elongate merozoites, which were arranged parallel to each other in a row (Fig. 2b). Gametocytes located in mature erythrocytes; the parasites were thin and elongate, with uneven margins and few small pigment granules present in the cytoplasm (Fig. 2c, d). Parasitaemia reached a small peak (0.3% average parasitaemia) on 20 DPE (Fig. 1). After this slight increase, the parasitaemia fluctuated in each individual bird, but did not reach the peak level again (Fig. 1). In spite of low parasitaemia, the decrease in average haematocrit value of the experimental group compared to the control group was significant ($p < 0.001$) (Fig. 1); that coincided with the small increase of parasitaemia (20 and 32 DPE) (Fig. 1). After the initial decrease (20 DPE), haematocrit value was restored and even surpassed the initial level, but after the next decrease (32 DPE) it remained low to the end of this study (Fig. 1).

No significant changes between the body mass of experimental and control groups of Common crossbills were observed during the experiment ($p = 0.184$) (Fig. 1). All exposed Common crossbills survived to the end of this study. 1, 2, 3, 5 and 55 phanerozoites were observed in the bone marrow only of 5 exposed bird individuals (Fig. 2 e–h). Phanerozoites were not seen in bone marrow preparations of 3 experimental birds, and they were not reported in other organs in all exposed birds.

Phylogenetic analysis showed that *P. elongatum* (pGRW6) groups with the lineage pERIRUB01 belonging to the same parasite species as well as 6 non-identified closely related lineages, 6 of which differ from both identified lineages of *P. elongatum* only by 1 bp (Fig. 3).

Discussion

This study supports the proposed hypothesis about innate resistance of Common starlings to the pGRW6 lineage of *P. elongatum*. This conclusion might explain why *P. elongatum* (pGRW6) has never been found in Common starlings worldwide [2]. This lineage of *P. elongatum* has been reported in birds belonging to 70 different species all over the world [2] thus, certainly it is a generalist malaria parasite. However, it is important to note that in the majority of these reports, *P. elongatum* (pGRW6) infection was detected using only molecular methods and it remains unclear if the parasite is able to complete its life cycle in all of the reported avian hosts to gametocyte stage, which is essential for natural transmission. This study indicates that some reports of *P. elongatum* (pGRW6) might be abortive infections, as is the case in the Common starlings, and PCR amplifications might be due to presence of circulating sporozoites, which are unable to initiate infections in resistant avian hosts [35]. Further studies are needed to specify this issue.

Interestingly, the Common starling is resistant or can tolerate several different malaria infections. For example, former experimental research with a cosmopolitan generalist parasite *P. relictum* (pSGS1) showed that this bird species was resistant as well [9]. *Plasmodium relictum* has been reported to infect a particularly big number of birds (> 300 species) [12, 36–38], however not a single report of the lineage pSGS1 or relative lineage pGRW11 have been from the Common starling so far. Additionally, a recent experimental study with *P. homocircumflexum* (lineage pCOLL4) showed that Common starlings were susceptible to this parasite and light parasitaemia

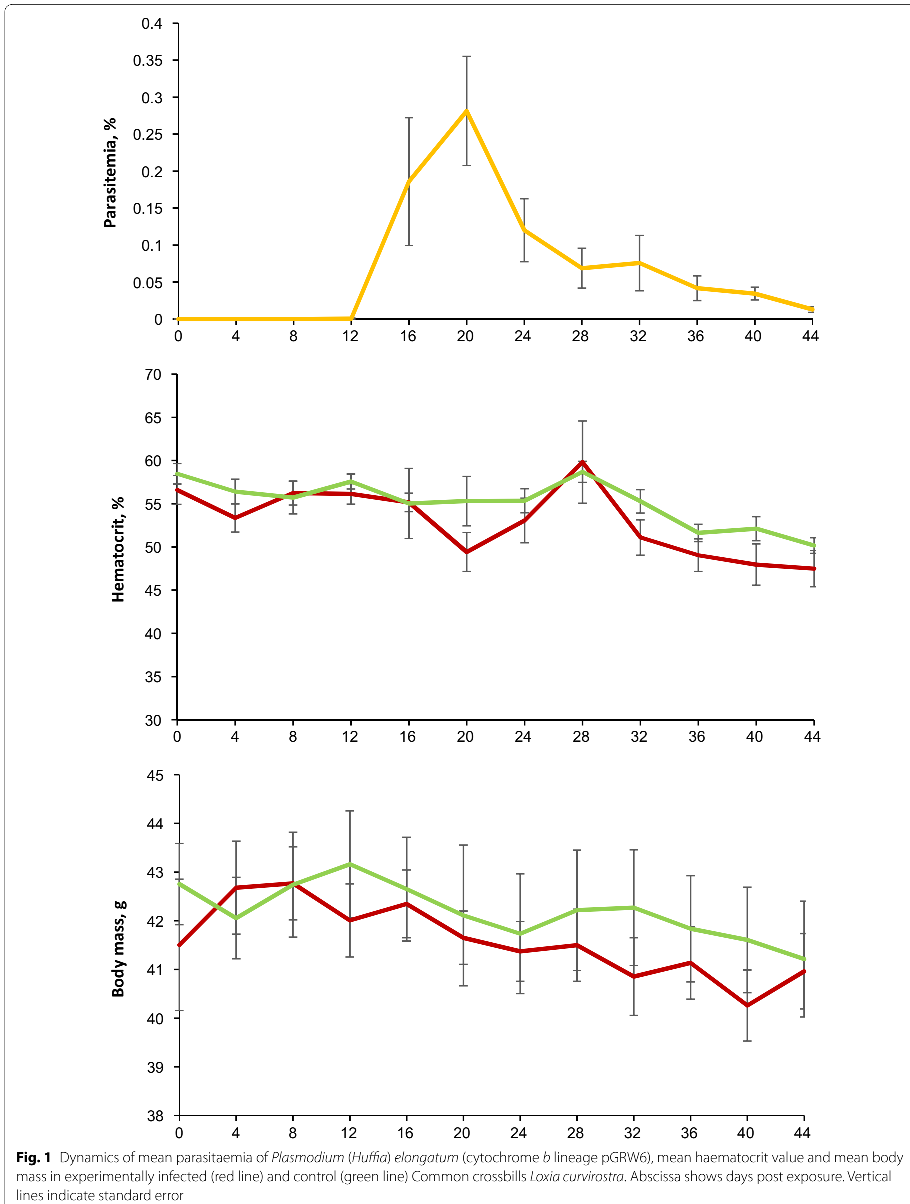

Fig. 1 Dynamics of mean parasitaemia of *Plasmodium* (*Huffia*) *elongatum* (cytochrome *b* lineage pGRW6), mean haematocrit value and mean body mass in experimentally infected (red line) and control (green line) Common crossbills *Loxia curvirostra*. Abscissa shows days post exposure. Vertical lines indicate standard error

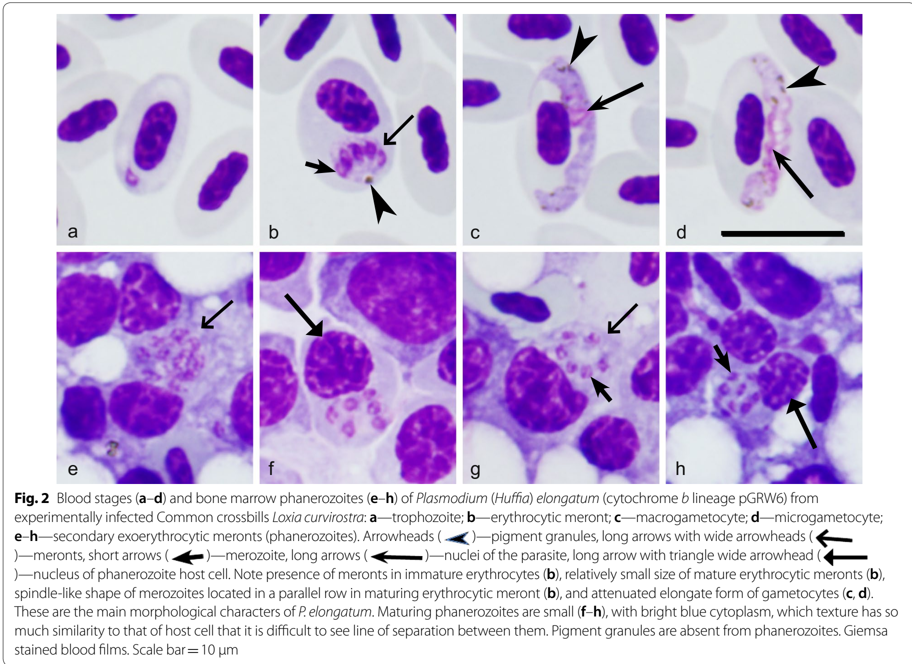

Fig. 2 Blood stages (**a**–**d**) and bone marrow phanerozoites (**e**–**h**) of *Plasmodium* (*Huffia*) *elongatum* (cytochrome *b* lineage pGRW6) from experimentally infected Common crossbills *Loxia curvirostra*: **a**—trophozoite; **b**—erythrocytic meront; **c**—macrogametocyte; **d**—microgametocyte; **e**–**h**—secondary exoerythrocytic meronts (phanerozoites). Arrowheads ( )—pigment granules, long arrows with wide arrowheads ( )—meronts, short arrows ( )—merozoite, long arrows ( )—nuclei of the parasite, long arrow with triangle wide arrowhead ( )—nucleus of phanerozoite host cell. Note presence of meronts in immature erythrocytes (**b**), relatively small size of mature erythrocytic meronts (**b**), spindle-like shape of merozoites located in a parallel row in maturing erythrocytic meront (**b**), and attenuated elongate form of gametocytes (**c**, **d**). These are the main morphological characters of *P. elongatum*. Maturing phanerozoites are small (**f**–**h**), with bright blue cytoplasm, which texture has so much similarity to that of host cell that it is difficult to see line of separation between them. Pigment granules are absent from phanerozoites. Giemsa stained blood films. Scale bar = 10 µm

developed, however, all individuals resisted the development of secondary exo-erythrocytic meronts (phanerozoites) resulting in low virulence and absence of any clinical signs of the infection [10].

Plasmodium homocircumflexum (lineage pCOLL4) is virulent and kills many bird species [10, 39, 40], but Common starlings readily tolerate this infection. Complete or partial resistance of Common starling to different species of avian malaria parasites may be one of the factors allowing this bird species to spread globally [41]. Mechanisms responsible for the Common starlings' resistance or/and tolerance to infections of different species of avian *Plasmodium* remain unclear, and the mentioned above host-parasite model organisms could be used for research aiming at better understanding of the molecular mechanisms of the innate resistance during avian malaria.

Experiments with Common crossbills showed different results. Mainly, all exposed birds were susceptible and developed light parasitaemia. However, phanerozoites were reported in bone marrow preparations of only 63% of the infected individuals. In the majority of the phanerozoite-positive birds, only between 1 and 5 parasites were seen in bone marrow, indicating low secondary exo-erythrocytic development. Because phanerozoites often cause severe pathology in birds [10, 11, 15, 40] it seems that the ability to resist the development of the phanerozoites may explain the low virulence of *P. elongatum* pGRW6 in this bird species, which is readily susceptible to *P. relictum* (pSGS1), *P. homocircumflexum* (pCOLL4) and some other avian malaria parasites [9, 10, 15, 40].

Prepatent period of *P. elongatum* infection both in sporozoite and blood-induced infections varied between 9 and 12 days in experimentally exposed Domestic canaries and ducklings [11, 12]. This study in accord with these data, however parasitaemia was reported 16 DPE in 7 of 8 Common crossbill individuals. This was probably because blood was tested not daily, but every 4 DPE.

Because parasitaemia was readily recognizable in exposed Common crossbills, it is unclear why the *P. elongatum* (pGRW6) infection has not been reported in wild populations of Common crossbills before [2]. It might be due to the fact that the majority of published studies of

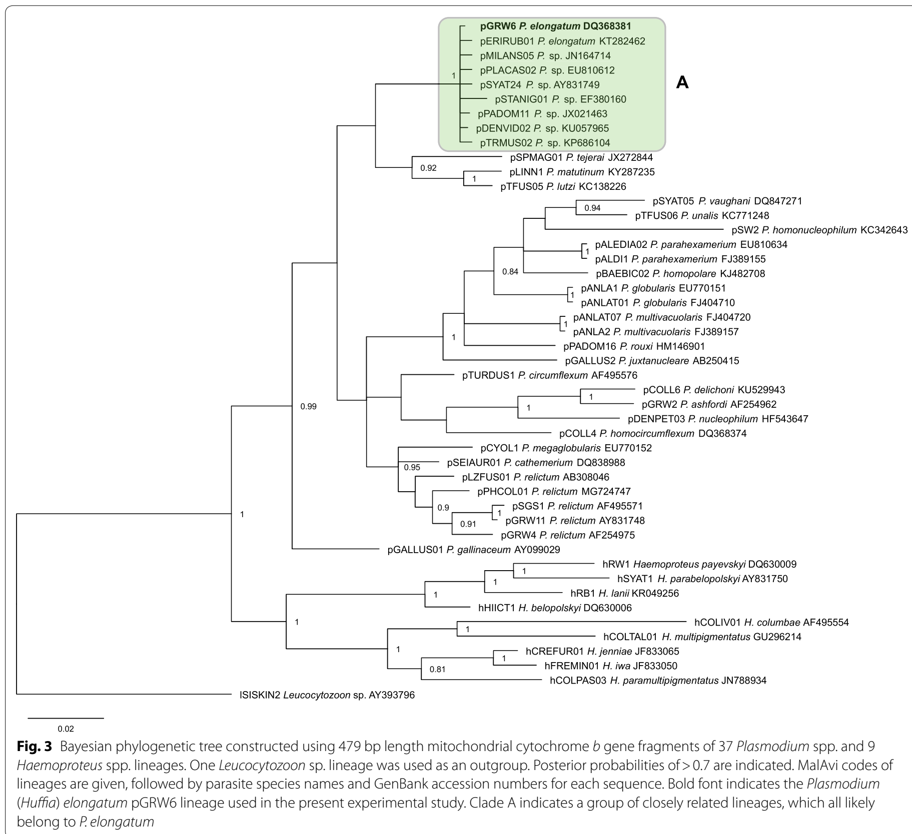

Fig. 3 Bayesian phylogenetic tree constructed using 479 bp length mitochondrial cytochrome *b* gene fragments of 37 *Plasmodium* spp. and 9 *Haemoproteus* spp. lineages. One *Leucocytozoon* sp. lineage was used as an outgroup. Posterior probabilities of > 0.7 are indicated. MalAvi codes of lineages are given, followed by parasite species names and GenBank accession numbers for each sequence. Bold font indicates the *Plasmodium* (*Huffia*) *elongatum* pGRW6 lineage used in the present experimental study. Clade A indicates a group of closely related lineages, which all likely belong to *P. elongatum*

haemosporidian parasites in crossbills were performed on the Curonian Spit during spring–summer migration or irruptions when mainly juvenile birds were sampled in May–June [8, 10, 40, 42]. This bird represents a unique ecological group of the Holarctic bird species that can breed in winter or in early spring [43] when mosquito vectors are inactive and transmission of malaria is absent, resulting in absence of malaria in Common crossbills during the sampling time [44]. This might explain the absence of *Plasmodium* and the paucity of other haemosporidian parasites in the 307 examined juvenile Common crossbills during the spring migration from the northern European breeding grounds to the southern latitudes on the Curonian Spit in June during a 3-year study [44]. Investigation of adult individuals in summertime is necessary to answer the question concerning the prevalence of *P. elongatum* in this bird species.

Former studies showed that *P. elongatum* parasitaemia is usually light [11, 12, 15, 45], and this is in accordance to this study. A small increase of parasitaemia was observed 20 DPE, however parasitaemia rapidly decreased and it was relatively stable during the remaining period of the observation, with gametocytes readily predominating in the circulation. Persistence by light parasitaemia might be an evolutionary adaptation, which should be beneficial for the parasite. Firstly, the impact of the parasites on the avian hosts is relatively low during light parasitaemia, and infected birds are more likely to survive [12, 46].

Experimental observations show that the exposed birds tend to be more active when they do not cope with symptoms of disease [47]. In other words, such avian hosts are less likely to be killed by predators, providing better opportunities for the parasite to be transmitted further. Secondly, high gametocytaemia might be virulent and have a negative effect on the vectors due to damage of their midgut by migrating ookinetes. However, data about the virulence of avian *Plasmodium* parasites on blood-sucking insects remain insufficient. Experimental observations showed that *Haemoproteus* species, a sister genus of haemosporidians, caused high mortality in mosquitoes and biting midges after infecting by blood meal with heavy gametocytaemia [48]. It is plausible that a similar effect might occur during the sporogonic development of *Plasmodium* parasites, therefore research should be conducted to test this hypothesis. Either way, low parasitaemia coupled with predominated gametocytaemia should contribute to parasite transmission from both, the hosts and the vectors, perspectives. That might explain why light parasitaemia of haemosporidian parasites predominate in the wild [47].

Haematocrit value dropped slightly during the small peak of parasitaemia, however it returned to normal levels several days after the peak when parasitaemia decreased (Fig. 1). The decrease in haematocrit level has been reported and is a common feature in other avian malaria infections during the parasitaemia peaks [9, 10, 49–51]. This is the first study which reports a decrease in haematocrit level during *P. elongatum* infection in relation to parasitaemia. It was believed that the decrease of haematocrit level during *P. elongatum* infections in domestic canaries and ducklings was mainly related to the damage caused by phanerozoites in the hematopoietic stem cells, which are responsible for erythropoiesis [2, 11, 15, 45]. It is important to note that, contrary to the infection of pERIRUB01 lineage of *P. elongatum* [15], few phanerozoites were observed in the bone marrow in this study. However, despite the low number or even absence of phanerozoites in the bone marrow of experimental birds, haematocrit value decreased slightly, but significantly compared to the control group (Fig. 1). These data indicate that the destruction of the infected erythrocytes also plays some role in the fluctuation of haematocrit value during *P. elongatum* infections, but it is not so obvious and is short-term in comparison to *P. relictum* [9, 52] or *P. homocircumflexum* [10, 40] infections. In other words, this study emphasizes that blood pathology also influences the haematocrit level fluctuations during *P. elongatum* infection.

One of the commonly accepted signs of degrading bird health is the decrease of the body mass during heavy haemosporidian infections, which might be accompanied with the decrease in host locomotion activity [12, 47]. However, recent studies show that there is no general pattern in regard of influence of different species of malaria parasites on the body mass of avian hosts: there are reports about not changed, decreased or even increased body mass in exposed birds compared to the control groups during different malarial infections [9, 10, 22]. Results of this study indicate that the changes in birds' body mass is not a direct indicator of bird health, at least in some haemosporidian parasite infections.

Plasmodium elongatum is known to be highly virulent in non-adapted birds. There is a number of reports of this infection causing lethal diseases in penguins in zoos and rehabilitation centres all over the world [4–6, 16–21]. It is interesting to note that virulence of this parasite can vary in different hosts remarkably—from complete resistance (starlings, present study), to complete susceptibility and low virulence (crossbills, present study) and complete susceptibility and high virulence (species of penguins and the Brown kiwi, [4–6]). This raises a question about mechanisms, which might be responsible for these differences in the virulence: are they related to the host and their ability to resist infections or the parasite and its ability to infect the host, or a combination of both? Available experimental data show that Common starlings are able to fully resist two (*P. relictum* and *P. elongatum*) and partially resist one species (*P. homocircumflexum*) of avian malaria parasite species [9, 10, present study]. It seems that this host species is able to cope with malaria infections. However, it remains unclear what mechanisms are responsible for this feature. Common crossbills, Eurasian siskins and Domestic canaries have been repeatedly shown to be good model organisms for avian malaria research [15, 53–55] as they are susceptible to many species of avian malaria and are seemingly genetically different from Common starlings in regard to the ability to resist avian malaria. Similar situation is in the case of the Magellanic penguin (*Spheniscus magellanicus*) and many other penguin species; based on the available data these birds can host different lineages of haemosporidian parasites [2], suggesting they might lack the resistance to these parasites. Based on the available molecular data, it is clear that *P. elongatum* is able to infect various avian hosts [2, 12]. The cosmopolitan *P. elongatum* and its various vertebrate hosts, in which the same lineage develops differently, is a good model system to access molecular mechanisms of resistance during avian malaria.

A group of lineages, which are closely related to *P. elongatum* (pGRW6) appeared in one well-supported clade in the phylogenetic tree (Fig. 3, clade A). Because genetic difference between these lineages is negligible, it is probable that all these parasites are intraspecies variants of *P. elongatum*. Morphological evidence is needed to prove

this hypothesis because some readily distinguishable haemosporidian species are very similar in *cytb* gene partial sequence and can differ just in few nucleotides [29, 56]. Only two lineages (pGRW6 and pERIRUB01) have been linked to this species so far. Because the lineages pERIRUB01 and pGRW6 are closely related and genetically similar in *cytb* but are different in regard of their virulence and ability to develop phanerozoites [15, this study], it is predictable that the lineages of the clade A also might be different in their biological features and the ability to cause disease in birds. Intraspecies variation in *cytb* gene is a common feature in avian haemosporidian parasites but remains insufficiently investigated in regard of the biology of parasites of these lineages. In other words, the biological meaning of negligible difference between closely related lineages of the same parasite morphospecies remain insufficiently understood. Due to the global distribution and the easy morphological species identification using blood stages, the parasites of different lineages of *P. elongatum* are convenient model organisms for research aiming at better understanding the virulence and other biological features of the parasites representing the closely related intraspecies variants of the same *Plasmodium* species. For example, the early studies of *P. elongatum* showed that the American and European isolates of same species are markedly different in the ability to cause severe disease in domestic canaries, but the genetic characterization was insufficiently developed at that time [11]. Recent molecular techniques provide opportunities for easier distinguishing and genetic characterization of different *P. elongatum* strains and opens opportunities for targeting experimental research on the biology of malaria parasites on their lineage levels.

Conclusion

The key result of this study is that the susceptibility of different bird species to the same *cytb* lineage of *P. elongatum* varies markedly. It might manifest itself from complete resistance to complete susceptibility with the ability to tolerate the infection as well as complete susceptibility with severe disease and high mortality. This should be taken into consideration in bird management and veterinary medicine. The global distribution of *P. elongatum* (pGRW6) might be due to the low virulence in some species of wild birds, which readily survive this infection, but serve as reservoirs hosts for non-adapted birds, in whom this parasite is often lethal. The complete resistance or ability to tolerate infections of common malaria agents in some avian hosts should be considered as the factors contributing to spread of invasive birds globally. This is likely the case with Common starlings and *P. elongatum* (pGRW6), and worth attention in biogeography and epidemiology, particularly of invasive species of hosts and parasites.

Abbreviations

cytb: mitochondrial cytochrome *b*; DNA: deoxynucleic acid; DPE: days post exposure; H&E: haematoxylin–eosin; PCR: polymerase chain reaction.

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Authors' contributions

Conceptualization: MI, VP, GV; Formal analysis: MI; Funding acquisition: GV, VP; Investigation: MI, VP, TI, EP; Resources: GV; Supervision: GV; Visualization: MI, TI; Writing—original draft preparation: MI; Writing—review and editing: VP, TJ, EP, GV. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical statement

The described experimental work of this study complies with the current laws of Lithuania and Russia. All experimental procedures were according to the All Union State standard (ГОСТ No P53434-2009 "Principles of good laboratory practice") of Russian Federation. All carried out experimental procedures were approved by the Biological Station Rybachy of the Zoological Institute, Russian Academy of sciences and are in accord with the International Research Co-operation Agreement between the Biological Station Rybachy and Nature Research Centre (Vilnius, Lithuania) (No 25-05-2010). Work with birds was also approved by the Forest and Nature Protection Agency of Kaliningrad Region, Russia (No 18, 5-05-2016). None of the experimental birds suffered apparent injury during experiments and all efforts were made to minimize handling time and potential suffering of birds.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Valkiūnas G, Iezhova T. Keys to avian malaria parasites. Malar J. 2018;17:212.
2. Bensch S, Hellgren O, Perez-Tris J. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome *b* lineages. Mol Ecol Resour. 2009;19:1353–8.
3. Murata K, Nii R, Sasaki E, Ishikawa S, Sato Y, Sawabe K, et al. *Plasmodium* (*Bennettinia*) *juxtanucleare* infection in a captive white eared-pheasant (*Crossoptilon crossoptilon*) at a Japanese zoo. J Vet Med Sci. 2008;70:203–5.
4. Howe L, Castro IC, Schoener ER, Hunter S, Barraclough RK, Alley MR. Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. Parasitol Res. 2012;110:913–23.
5. Vanstreels RET, Kolesnikovas CKM, Sandri S, Silveira P, Belo NO, Ferreira FC, et al. Outbreak of avian malaria associated to multiple species of Plasmodium in Magellanic penguins undergoing rehabilitation in southern Brazil. PLoS ONE. 2014;9:e94994.

6. Vanstreels RE, da Silva-Filho RP, Kolesnikovas CK, Bhering RC, Ruoppolo V, Epiphanio S, et al. Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. Vet Res. 2015;46:30.
7. Grilo ML, Vanstreels RET, Wallace R, Garcia-Parraga D, Braga EM, Chitty J, et al. Malaria in penguins—current perceptions. Avian Pathol. 2016;45:393–407.
8. Palinauskas V, Valkiūnas G, Bolshakov CV, Bensch S. *Plasmodium relictum* (lineage SGS1) and *Plasmodium ashfordi* (lineage GRW2): the effects of the co-infection on experimentally infected passerine birds. Exp Parasitol. 2011;127:527–33.
9. Palinauskas V, Valkiūnas G, Bolshakov VC, Bensch S. Plasmodium relictum (lineage P-SGS1): effects on experimentally infected passerine birds. Exp Parasitol. 2008;120:372–80.
10. Ilgūnas M, Bukauskaitė D, Palinauskas V, Iezhova T, Fragner K, Platonova E, et al. Patterns of *Plasmodium homocircumflexum* virulence in experimentally infected passerine birds. Malar J. 2019;18:174.
11. Garnham PCC. Malaria parasites and other haemosporidia. Hoboken: Blackwell Scientific Publications Press; 1966.
12. Valkiūnas G. Avian malaria parasites and other Haemosporidia. Boca Raton: CRC; 2005.
13. Beadell JS, Gering E, Austin J, Dumbacher JP, Peirce MA, Pratt TK, et al. Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. Mol Ecol. 2004;13:3829–44.
14. Valkiūnas G, Zehtindjiev P, Dimitrov D, Križanauskienė A, Iezhova TA, Bensch S. Polymerase chain reaction-based identification of *Plasmodium* (*Huffia*) *elongatum*, with remarks on species identity of haemosporidian lineages deposited in GenBank. Parasitol Res. 2008;102:1185.
15. Palinauskas V, Žiegytė R, Iezhova TA, Ilgūnas M, Bernotienė R, Valkiūnas G. Description, molecular characterisation, diagnostics and life cycle of *Plasmodium elongatum* (lineage pERIRUB01), the virulent avian malaria parasite. Int J Parasitol. 2016;46:697–707.
16. Fleischman RW, Squire RA, Sladen WJL, Melby EC. Malaria (*Plasmodium elongatum*) in captive African penguins (*Spheniscus demersus*). J Am Vet Med Assoc. 1968;153:928–35.
17. Herman CM, Kocan RM, Snyder EL, Knisley JO. *Plasmodium elongatum* from a penguin. Bull Wildl Dis Assoc. 1968;4:132.
18. Beier JC, Stoskopf MK. The epidemiology of avian malaria in black-footed penguins (*Spheniscus demersus*). J Zoo Animal Med. 1980;11:99–105.
19. Beier JC, Trpis M. Incrimination of natural culicine vectors which transmit *Plasmodium elongatum* to penguins at the Baltimore Zoo. Can J Zool. 1981;59:470–5.
20. Cranfield MR, Shaw M, Beall F, Skjoldager M, Ialeggio D. A review and update of avian malaria in the African penguin (*Spheniscus demersus*). Proc Am Ass Zoo Vet. 1990;243–8.
21. Graczyk TK, Cranfield MR, McCutchan TF, Bicknese EJ. Characteristics of naturally acquired avian malaria infections in naive juvenile African black-footed penguins (*Spheniscus demersus*). Parasitol Res. 1994;80:634–7.
22. Palinauskas V, Žiegytė R, Šengaut J, Bernotienė R. Different paths—the same virulence: experimental study on avian single and co-infections with *Plasmodium relictum* and *Plasmodium elongatum*. Int J Parasitol. 2018;48:1089–96.
23. Linz GM, Homan HJ, Gaukler SM, Penry LB, Bleier WJ. European starlings: a review of an invasive species with far-reaching impacts. Managing vertebrate invasive species. In: Proceedings of an International Symposium (eds G.W. Witmer, W.C. Pitt & K.A. Fagerstone). USDA APHIS / WS, National Wildlife Research Center, Fort Collins, CO. 2007;378–86.
24. Iezhova TA, Valkiūnas G, Bairlein F. Vertebrate host specificity of two avian malaria parasites of the subgenus *Novyella*: *Plasmodium nucleophilum* and *Plasmodium vaughani*. J Parasitol. 2005;91:472–4.
25. Godfrey RD, Fedynich AM, Pence DB. Quantification of hematozoa in blood smears. J Wildl Dis. 1987;23:558–65.
26. R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2013. http://www.R-project.org/. Accessed 18 May 2019.
27. Sambrook J, Fritsch EF, Mamiatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
28. Bensch S, Stjernman M, Hasselquist D, Ostman O, Hansson B, Westerdahl H, et al. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc Biol Sci. 2000;267:1583–9.
29. Hellgren O, Waldenström J, Bensch S. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol. 2004;90:797–802.
30. Hall A. BioEdit: a user-friendly biological sequence alignment editor and analysis program of Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–8.
31. NCBI BLAST. Rockville Pike, Bethesda MD, USA. 2019. https://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed 26 Jun 2019.
32. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19:1572–4.
33. Nylander JAA. MrModeltest v2. Program distributed by the author: Evolutionary Biology Centre, Uppsala University; 2004.
34. Rambaut A. FigTree: tree figure drawing tool version 1.4.3. 2009. http://tree.bio.ed.ac.uk/software/figtree. Accessed 15 Nov 2017.
35. Valkiūnas G, Iezhova TA, Loiseau C, Sehgal RN. Nested cytochrome b polymerase chain reaction diagnostics detect sporozoites of hemosporidian parasites in peripheral blood of naturally infected birds. J Parasitol. 2009;95:1512–5.
36. Bennett GF, Whiteway M, Woodworth-Lynas C. A host-parasite catalogue of the avian haematozoa. Mem Univ Nfld Occ Pap Biol. 1982;5:1–243.
37. Bishop MA, Bennett GF. Host-parasite catalogue of the avian haematozoa: supplement 1, and bibliography of the avian blood-inhabiting haematozoa. Mem Univ Nfld Occ Pap Biol. 1992;2:1–244.
38. Atkinson CT, Thomas NJ, Hunter DB. Parasitic diseases of wild birds. Oxford: Wiley-Blackwell; 2008.
39. Palinauskas V, Žiegytė R, Ilgūnas M, Iezhova TA, Bernotienė A, Bolshakov C, et al. Description of the first cryptic avian malaria parasite, *Plasmodium homocircumflexum* n. sp. with experimental data on its virulence and development in avian hosts and mosquitoes. Int J Parasitol. 2015;45:51–62.
40. Ilgūnas M, Bukauskaitė D, Palinauskas V, Iezhova TA, Dinhopl N, Nedorost N, et al. Mortality and pathology in birds due to *Plasmodium* (Giovannolaia) *homocircumflexum* infection, with emphasis on the exoerythrocytic development of avian malaria parasites. Malar J. 2016;15:256.
41. BirdLife International 2016. *Sturnus vulgaris*. The IUCN Red list of threatened species 2016:e.T22710886A87847984. Accessed 26 Jun 2019.
42. Hellgren O, Waldenström J, Pérez-Tris J, Szöllősi E, Hasselquist D, Križanauskiene A, et al. Detecting shifts of transmission areas in avian blood parasites—a phylogenetic approach. Mol Ecol. 2007;16:1281–90.
43. BirdLife International 2017. *Loxia curvirostra* (amended version of 2016 assessment). The IUCN Red List of Threatened Species 2017: e.T22720646A111131604. Accessed 26 Jun 2019.
44. Valkiūnas G, Iezhova T. A key for some puzzles in ecological parasitology. Ekologija. 2000;2:27–30.
45. Corradetti A, Neri I, Cavallini C. Non identiti di *Plasmodium paddae* e di *Plasmodium praecox*(*relictum*). Parassitologia. 1968;10:145–9.
46. Granthon C, Williams DA. Avian malaria, body condition, and blood parameters in four species of songbirds. Wilson J Ornithol. 2017;129:492–508.
47. Mukhin A, Palinauskas V, Platonova E, Kobylkov D, Vakoliuk I, Valkiūnas G. The strategy to survive primary malaria infection: an experimental study on behavioural changes in parasitized birds. PLoS ONE. 2016;11:e0159216.
48. Bukauskaitė D, Bernotienė R, Iezhova T, Valkiūnas G. Mechanisms of mortality in Culicoides biting midges due to *Haemoproteus* infection. Parasitol. 2016;143:1748–54.
49. Permin A, Juhl J. The development of *Plasmodium gallinaceum* infections in chickens following single infections with three different dose levels. Vet Parasitol. 2002;105:1–10.
50. Paulman A, McAllister MM. *Plasmodium gallinaceum*: clinical progression, recovery, and resistance to disease in chickens infected via mosquito bite. Am J Trop Med Hyg. 2005;73:1104–7.
51. Williams RB. Avian malaria: clinical and chemical pathology of *Plasmodium gallinaceum* in the domesticated fowl *Gallus gallus*. Avian Pathol. 2005;34:29–47.
52. Palinauskas V, Valkiūnas G, Križanauskienė A, Bensch S, Bolshakov CV. *Plasmodium relictum* (lineage P-SGS1): further observation of effects on experimentally infected passeriform birds, with remarks on treatment with Malarone™. Exp Parasitol. 2009;123:134–9.

53. Cellier-Holzem E, Esparza-Salas R, Garnier S, Sorci G. Effect of repeated exposure to *Plasmodium relictum* (lineage SGS1) on infection dynamics in domestic canaries. Int J Parasitol. 2010;40:1447–53.
54. Cornet S, Nicot A, Rivero A, Gandon S. Evolution of plastic transmission strategies in avian malaria. PLoS Pathog. 2014;10:e1004308.
55. Pigeault R, Vézilier J, Cornet S, Zélé F, Nicot A, Perret P, et al. Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of *Plasmodium*. Phil Trans R Soc B. 2015;370:20140300.
56. Valkiūnas G, Ilgūnas M, Bukauskaitė D, Chagas CRF, Bernotienė R, Himmel T, et al. Molecular characterization of six widespread avian haemoproteids, with description of three new *Haemoproteus* species. Acta Trop. 2019;197:105051.

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