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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 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).

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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 Plasmod*ium* 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  $(20 \pm 1 \,^{\circ}C; 50-60\%$  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  $6 \times 10^5$ ) 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  $\mu$ l 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  $\mu$ m 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 ( $65 \times 65 \times 65$  cm) under standard laboratory conditions ( $23 \pm 1$  °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  $(12 \times 12 \times 12 \text{ cm})$ , 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 oocvsts 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 ( $\times$ 100,  $\times$ 200 and  $\times$ 600) and then at high magnification ( $\times$ 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.  $P \leq 0.05$  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<sub>2</sub>, 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 ervthrocytic 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  $\mu$ m), 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  $\mu$ m. 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 =  $10 \mu m$ .



**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) Giemsastained preparations; (C, D) midgut preparation stained with Erlich's hematoxylin. Scale bar = 10  $\mu$ m.

#### 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 <sup>a</sup> (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	$1.2-1.7(1.4\pm0.2)$	$1.1-1.5(1.3\pm0.1)$
Area	6.2-11.5 (8.8 ± 1.8)	$6.1-9.8$ ( $8.4 \pm 1.4$ )
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	$0.7-1.2~(0.9\pm0.2)$	$0.8 - 1.2 (0.9 \pm 0.1)$
Area	7.5-11.9 (9.2 ± 1.1)	6.8-13.9 (9.7 ± 1.8)

<sup>a</sup> 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  $\mu$ m. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and S.D.

blood. Morphological features of gametocytes and meronts of pERI-RUB01 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  $\ge 0.7$  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. elonga-tum*-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 (Bernotiene 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. guinguefasciatus mosquitoes was documented (Raffaele, 1934; Micks, 1949). However, Micks (1949) obtained less than three oocvsts per midgut and did not observe any sporozoites in *C. auinauefasciatus*. 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 oocvsts 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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