Experimental study of newly described avian malaria parasite Plasmodium (Novyella) collidatum n. sp., genetic lineage pFANTAIL01 obtained from South Asian migrant bird

Elena Platonova (plat.992@gmail.com)  
Gamtos tyrimu centro Geologijos ir geografijos institutas https://orcid.org/0000-0002-9425-8998

Justė Aželytė  
Nature Research Centre

Tatjana Iezhova  
Nature REsearch Centre

Mikas Ilgūnas  
Nature Research Centre

Andrey Mukhin  
Highlands Biological Station of RAS

Vaidas Palinauskas  
Nature Research Centre

Research

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Abstract

Background

Avian malaria parasites are common and worldwide distributed microorganisms parasitizing erythrocytes and various tissues of the birds. These parasites are known to infect birds of different taxa and be the cause of the bird’s deaths in wildlife and captivity. A particular interest possesses the species of parasites with the ability to colonize new territories and infect local non-migratory birds. This scenario is likely in temperate zones of Europe because of the present climate change and its contribution to the spreading of southern origin vectors which can be involved in the transmission of malaria parasites. In the present study, a tropical Plasmodium parasite from a naturally infected long-distance migrant bird was isolated and tested for its ability to develop in common species of mosquitoes and European short-distance migrant bird.

Methods

Plasmodium sp. (pFANTAIL01) was isolated from the naturally infected Common rosench, Carpodacus erythrinus. The parasite was described based on the morphological features of its blood stages, the partial mitochondrial cytochrome b gene and development after experimental infection of birds and mosquitoes. The parasite was inoculated into Eurasian siskins, Carduelis spinus. Parasitemia, hematocrit value and weight of birds were monitored. At the end of the survey, internal organs were collected to study exoerythrocytic stages of this parasite. Experimental infection of mosquitoes Culex pipiens pipiens form molestus and C. quinquefasciatus was applied to study sporogonic development of the parasite.

Results

Based on morphological features, the new parasite was described as Plasmodium collidatum n. sp. and attributed to subgenus Novyella. It was revealed that the obtained pFANTAIL01 lineage is a generalist parasite infecting a wide range of avian hosts and most likely is transmitted in South and Southeast (SE) Asia and Oceania. In Europe this strain was recorded only in adult migratory birds wintering in South Asia. This parasite developed high parasitemia in experimentally infected siskins and caused 25% mortality. Exoerythrocytic stages of pFANTAIL01 were found in the lungs, liver, spleen and kidney of the deceased birds. Sporogonic development did not occur in C. p. p. f. molestus and C. quinquefasciatus mosquitoes.

Conclusion

Plasmodium collidatum is a highly virulent for Eurasian siskin and completes its development in these birds, which can be considered as a potential vertebrate host if the transmission of the infection starts occurring in Europe and temperate zones.

Background
Every year billions of European breeding birds migrate to their wintering ground [1].

The distances the individuals cover along their routes can reach thousands of kilometers and inevitably these movements involve birds as reservoirs in transporting and potentially spreading other organisms to the new territories [2, 3]. One group of parasites whose spreading can be enforced by migrating birds causes avian malaria. These parasites belong to Plasmodiidae (order: Haemosporida), are distributed worldwide and are diverse [4]. More than 100 years of studies on Plasmodium infecting birds show that some species of these parasites are virulent to their vertebrate hosts and cause severe disease [4–10]. Extensive molecular screening of juvenile and adult birds as well as migrant and non-migrating species of birds in Europe reveals which avian malarial parasites are transmitted within Europe and which are exotic species persisted only in birds after their return from wintering quarters [11]. Among the latter are Plasmodium delichoni (genetic lineage pCOLL6), P. homonucleophilum (pSW2), P. homocircumflexum (pCOLL4), P. ashfordi (pGRW2) and some others [12–15]. This is only a small fraction of all recorded Plasmodium genetic lineages, which are linked to morphologically described species and contains information about their development and virulence for a vertebrate host. In most of the cases, natural vectors are still unknown.

Transmission of the largest number of potentially invasive avian Plasmodium lineages found in Europe, occurs in Africa as this is the main wintering ground for the European long-distance migrant birds [4]. Instead of Africa, several bird species migrate to South and SE Asia, these are: Common rosenfinch (Carpodacus erythrinus), Rosy starling (Pastor roseus), Little bunting (Emberiza pussila), Read-breasted flycatcher (Ficedula parva), Blyths reed warbler (Acrocephalus dumetorum) and a few others [16].

According to the MalAvi database [17], there are more than 20 genetic lineages of the avian malarial parasites found in breeding European birds which migrate to South and SE Asia, but only few morphologically described lineages of Plasmodium were identified e.g. P. circumflexum, pTURDUS1 [18, 19] and P. relictum, pSGS1 [20, 21] and pGRW4 [22]. At present, there are no described malarial parasite lineages linked to Plasmodium species, which are transmitted only in South or SE Asia and annually brought to Europe with migrating birds. However, these parasites should be of the prime interest as they might be the main threat to local bird populations in the near future. According to some calculations, the prevalence of avian malaria will increase by two-threefold due to the global warming [23]. Ecological changes and invasive mosquito species may play the main role in the appearance of new interactions between invasive mosquito species and exotic Plasmodium parasites causing the transmission of these parasites on local, non-migrating or within Europe migrating birds. For instance, according to Medlock et al. and Martínez-de la Puente et al. [24, 25], invasive mosquito species Asian tiger mosquito (Aedes albopictus), originated from SE Asia is spreading in some parts of Europe already. The presence of Plasmodium vaughani (genetic lineage pSYAT05) DNA was recorded in these mosquitoes collected in Italy [25] and according to Martínez-de la Puente et al. [26], this mosquito could also be a potential vector for other avian malaria parasites, especially those originated from SE Asia.

The development and virulence in a vertebrate host and insect vector vary between different Plasmodium species and therefore identification of the parasite species and knowledge about their biology is crucial.
for better understanding the epizootiology and potential spread of avian malaria. In recent years, the
description of newly found avian malaria parasites includes both morphological and phylogenetic
information obtained from the molecular examination [13, 15, 27, 28]. Some studies go further and
provide information about the development patterns in a vertebrate host, erythrocytic and exoerythrocytic
stages, the virulence and information about potential vectors [9, 10, 12].

In the present study, a new species of malaria parasite obtained from a naturally infected long-distance
migrant Common rosenfinch (wintering in South Asia) in Northern Europe was described. Using
morphological and molecular methods the detailed description of blood stages and the phylogenetic
relationships of this lineage with other previously described avian malaria parasites is provided. The
development in the red blood cells (RBCs) and various tissues together with the caused virulence to the
vertebrate hosts was studied in experimentally infected juvenile siskins *Carduelis spinus*. Sporogonic
development of the newly described species was studied in blood sucking mosquitoes *Culex pipiens f.
molestus* and *C. quinquefasciatus*.

The obtained data can help understanding new parasite-host associations and impact on the host health
in regard to parasite spread, brought on by global climate change and newly formed ecological
conditions.

**Materials And Methods**

**Study site and design of the experiment**

In June of 2019, one adult male Common rosenfinch was caught in mist-nets at the Biological station
“Rybachy” of the Zoological Institute of the Russian Academy of Science. The blood was collected in
heparinized microcapillary by puncturing a brachial vein. Two blood smears were prepared and stained
as described by Valkiūnas et al. [4]; about 25-30 μL of blood was stored in SET-buffer (0.05 M Tris, 0.15 M
NaCl, 0.5 M EDTA, pH 8.0) for a later molecular analysis. Microscopic examination showed a
*Plasmodium (Novyella)* sp. infection. The strain was multiplied in one Eurasian siskin by subinoculation
of infected blood as described below. For studying experimental infection in the vertebrate host. Juvenile
Eurasian siskins, a common and widespread in Europe passerine bird, were used. In all, 18 juvenile
siskins were captured and randomly allocated to experimental (8 birds) and control (10 birds) groups
after microscopically proving the absence of *Plasmodium* parasites in their blood samples. Birds were
checked by using microscopic and later molecular examinations as described in below chapters.
Experimental birds were housed in individual cages (60×40×40 cm, Joko GmbH, Germany) and kept in a
vector-free aviary under controlled laboratory conditions (room temperature 22 ±1° C, photoperiod 17:7 of
light:dark). Food and water were provided *ad libitum* during the entire period of the experiment.

To infect experimental birds, standard protocol in accordance with Palinauskas et al. [29] was used. Each
experimental bird was subinoculated with a mixture (0.10 mL) of infected blood, 3.7% sodium citrate and
0.9% saline in proportion 4:1:5 into the pectoral muscles. Intensity of meronts in donor birds was 0.03%
and 0.05%. Two birds were inoculated with approximately $5 \times 10^4$ number of mature meronts, and six birds received about $8.3 \times 10^4$ meronts. Birds from the negative control group were inoculated using the same procedure and blood mixture as the experimental group, but with blood obtained from an uninfected siskin. The duration of the experiment was 36 days. To estimate the development of parasites in the blood the exposed birds were examined every 4 days by taking blood from the brachial vein as was described above. Small drop of blood was used to make smears for microscopy, a fraction of blood (20-30 μL) was placed in SET-buffer for the molecular analysis and the rest (about 30 μL) was used to measure hematocrit level. To measure the hematocrit level, blood collected in capillary was centrifuged using ELMI CM-70 (ELMI Ltd., Latvia) centrifuge for 5 minutes at 7000 r. p. m. Also, the body mass was measured in both experimental and control siskins.

At the end of the experiment all experimentally infected birds were euthanized. Their internal organs (the brain, heart, lungs, spleen, liver, kidneys, pectoral muscle) were extracted and placed to a 10% neutral buffered formalin solution for fixation. Fixed and parafilm-embedded tissues were cut in 4 μm, stained with hematoxylin-eosin (H&E) and examined microscopically under 1000x magnification [4,10] for parasite's exoerythrocytic stages. Also smears of the bone marrow from bird's femurs were prepared. Air-dried films were fixed in absolute methanol for 3 min and stained using Romanowski-Giemsa protocol to check the presence of phanerozoites [4].

**Experimental infection of mosquitoes**

To study the development of the new malarial parasite in an invertebrate host, two species of potential vectors, *Culex pipiens pipiens* f. *molestus* and *C. quinquefasciatus* were used.

Experimental colonies of mosquitoes were established at the Biological Station Rybachy in April 2019. The eggs of mosquitoes were obtained from P. B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania. Insects were kept in isolated laboratory under controlled conditions (room temperature 23 ± 1° C; humidity 75-80%; photoperiod 17:7 light:dark). Mosquitoes were kept in a nylon netted cage (45×45×45 cm, BugDorm, UK). Food for mosquitoes was provided in the form of cotton wools saturated with 5% saccharose solution [30].

For experimental infection an infected donor bird with approximate 1% parasitemia (gametocytemia around 0.3%) was used. A bird was carefully immobilized and fixed in a paper tube, leaving only its legs exposed for the mosquitoes [30]. This tube was placed into a separate mosquito cage with about 100 uninfected female mosquitoes taken from the main colony. After one hour engorged mosquitoes were separated into small cages (17.4 × 17.5 × 17.5 cm) and kept there up to 22 days post exposure (dpe). The same procedure was applied for control group mosquitoes, where a non-infected siskin was used to feed the females obtained from the main colony. Experimental mosquitoes were dissected gradually for preparations of different sporogonic stages. For ookinete preparations mosquitoes were dissected 1-3 dpe, for oocysts 8-22 dpe and for sporozoite preparations 12-22 dpe. Before dissection, all insects were
euthanized in an entomological aspirator with cottonwool moistened with 96% ethanol. The preparations of all sporogony stages were made according Žiegytė et al. [31].

In total, 26 *C. p.p. f. molestus* and 26 *C. quinquefasciatus* mosquitoes were infected. In control group, 25 *C. p.p. f. molestus* mosquitoes were engorged with uninfected blood.

**Microscopic examination of blood smears and species identification**

For blood smears screening, examination of sporogonic stages and parasitemia calculation in experimental individuals an Olympus CH2O light microscope with x40 and x100 magnifications was used. Pictures for measurements of the parasite at its different blood stages were prepared by using the Olympus BX61 light microscope equipped with digital camera DP70. Visualization of pictures was performed using the software AnalySIS FIVE (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Blood smears obtained from wild birds were examined about 15-20 min with x100 magnification. To evaluate the intensity of parasitemia, numbers of infected erythrocytes per 10 000 red blood cells were counted [29, 32].

**Molecular examination and phylogenetic analysis**

Total DNA was extracted from whole blood stored in SET-buffer, using the ammonium-acetate protocol [33]. The standard nested PCR protocol was used to amplify 478 bp fragment of the mitochondrial cytochrome b gene (cyt b) of *P. collidatum* [34, 35]. To control for a false amplification one positive control (DNA of *Plasmodium relictum* pSGS1) and one negative control (nuclease-free water) were used every10 samples. Final PCR-products were checked for the success of amplification by running them on 2% agarose gel. Obtained fragments were sequenced from both 5 and 3 ends using an ABI PRISM TM 3100 capillary sequencing robot (Applied Biosystems, USA). Obtained sequences were aligned in BioEdit software [36] and identified using BLAST-program of GenBank [37] and MalAvi database [17].

Phylogenetic analysis of the pFANTAIL01 with 28 additional sequences of haemosporidian parasites was conducted using the Bayesian method and performed in MrBayes v.3.1 [38]. The General Time Reversible Model with a proportion of invariable sites and variation among sites (GTR+I+G) was selected by the mrModeltest 3.7 program [39] as the best fitting model. In total, 3 million generations were run with a sample frequency of every 100th generation. Twenty five percent of obtained trees representing the burn-in phase were discarded. Remaining trees were used for the determination of the consensus tree. The final phylogenetic tree was visualized using FigTree software 1.4.4. [40].

The sequence divergence between different lineages was calculated by applying the Jukes–Cantor model of substitution implemented in the program MEGA 6.0 [41].

**Statistical analysis**
The statistical analysis was performed by using the RStudio interface based on R software [42]. The normality of distribution in the experimental dataset was evaluated by employing the Shapiro-Wilk test. Wilcoxon rank-sum test was used for the analysis of differences in hematocrit and body mass values between experimental and control groups of siskins. P-value above or equal to 0.05 was considered as significant.

**Results**

**Description of parasite**

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

**DNA-sequence:** Mitochondrial cyt b lineage pFANTAIL01 (478 bp, GenBank accession no. MW175901)

**Type host:** The Common rosench *Carpodacus erythrinus* (Passeriformes, Fringillidae).

**Additional hosts:** According to literature data the lineage pFANTAIL01 (synonym codes AP63, GenBank accession no. AY714196; C028, DQ212193 and ASI-2012, JX418225) have been recorded in 18 species of 15 families in 7 orders of naturally infected birds (see Table 1). Eurasian siskin was susceptible to experimental infection and could be a competent host.

**Type locality:** The Curonian Spit of the Baltic Sea (55°05'N, 20°44'E).

**Prevalence:** 71 Common rosenches were collected and examined for haemosporidian infection on Curonian spit in 2010-2019 years. About 7% of all rosenches were infected with different species of blood parasites. *P. collidatum* was reported in one bird individual in 2019. According to MalAvi database, this lineage is rare in Europe.

**Site of infection:** Erythrocytic meronts and gametocytes developed in mature red blood cells (Fig. 1); no other data.

**Vectors:** Natural vectors are unknown. Mosquitoes of *C. pipiens pipiens* form molestus and *C. quinquefasciatus* were not susceptible to *P. collidatum*.

**Distribution:** According to the molecular data, *Plasmodium collidatum* pFANTAIL01 has been reported in South, SE Asia, Australia, and neighboring islands as well as Europe (Table 1). In South, SE and Australia, *P. collidatum* has been found in numerous species of resident and migratory birds. Among infected European birds in the temperate zone, this lineage was confirmed only in adult birds of those species which are wintering in South Asia (*Carpodacus erythrinus, Pastor roseus*).

**Type specimens:** Hapantotype (accession nos. 49242-49244 NS, intensity of parasitemia is approximately 0.3%, *Carpodacus erythrinus*, the Curonian spit, Kaliningrad district, Russia, 36° 44' N, 119° 29' W, collected 6 June 2019 by E. Platonova) is deposited in the Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (accession nos. G466222, G466223 [1149b/19C, 1220b/19C]), the intensity
of parasitemia is approximately 10% and 3.3% respectively, of experimentally infected *C. spinus*, collected 26-30 July 2019 by E. Platonova) are deposited in the Queensland Museum, Queensland, Australia.

**Additional material:** Blood films from experimentally infected *C. spinus* (accession nos. 1149/19C, 1219/19C, 1220/19C, 1367/19C) and blood samples fixed in SET-buffer (accession nos. 1149/19C, 1219/19C, 1220/19C, 1367/19C) are deposited in the Nature Research Centre, Vilnius, Lithuania.

**Etymology:** The specific name reflects the morphological feature – markedly indented (lobular-like) appearance of the pellicle, which is typical for advanced gametocytes.

**Exoerythrocytic meronts:** Primary exoerythrocytic merogony (cryptozoites and metacryptozoites) was not investigated and remain unknown. Numerous secondary exoerythrocytic meronts (phanerozoites) were found in the lungs (Fig. 2a-c) of the infected individuals and appeared elongated (Fig. 2a), oval (Fig. 2b) or irregularly shaped (Fig. 2c). Phanerozoites in the lungs contained over 30 roundish merozoites. In smaller numbers, phanerozoites were also seen in the liver (Fig. 2d-f), spleen (Fig. 2g-i) and kidneys (Fig. 2j-l) of the infected individuals. In these organs phanerozoites appeared roundish or slightly oval and contained less than 30 merozoites.

**Trophozoites** (Fig. 1a, b) are most often seen in mature erythrocytes, however, rarely can be observed in polychromatophilic red blood cells. Round or oval trophozoites are located at the poles of infected erythrocytes. As trophozoites mature, they attach to the nucleus of the infected erythrocyte and this contact is maintained.

**Erythrocytic meronts** (Fig. 1c-j; Table 2) found only in mature erythrocytes. Growing meronts contain little, but readily visible, blue in color cytoplasm and small pigment granules (Fig. 1c); in mature meronts cytoplasm is rarely seen. Both, in growing and mature meronts a small bluish roundish and non-refractive globule is often seen (Fig. 1f, h). Meront with developing merozoites touches the erythrocyte nucleus, located laterally or close to the pole of the infected cell and this contact is maintained during the whole time of maturation (Fig. 1c-i). Grown meronts are oval or irregularly shaped and contain 4-8 merozoites (Fig. 1e-i). Pigment granules in mature meronts are small and clumped, however, scattered granules can also be seen in some cells (Fig. 1j). Effect of the gametocyte on the infected erythrocyte is not expressed. Mature merozoites contain prominent irregular-shaped nuclei, and each contains a small portion of readily visible cytoplasm (Fig. 1g, j).

**Macrogametocytes** (Fig. 1k-y; Table 2) found only in mature erythrocytes. The cytoplasm is markedly heterogeneous and is unevenly stained: more dense stained portions of the cytoplasm are intermediated with pale stained portions, which look like large vacuole-like pale-stained spaces (Fig. 1q-s, w, y), a characteristic feature of this species. Young forms usually found in the poles of infected erythrocytes. In the earliest growing gametocytes, long outgrowths often appear (Fig. 1l). Gametocytes vary in outline from amoeboid in growing to wavy in mature parasites. Gametocytes are located laterally to the nucleus of the infected erythrocyte; maturing and mature gametocytes are strictly nucleophilic, however, growing forms not touching the nucleus can also be seen occasionally (Fig. 1o). A central part of the pellicle of
some growing gametocytes does not extend to erythrocyte envelope causing a ‘dip’, which gives dumbbell-shaped form (Fig. 1q, r). Typically, the growing gametocytes are asymmetric in appearance, with one end being broader than the other one (Fig. 1p, s, t, x). Fully-grown gametocytes often do not adhere to the envelope of the erythrocyte and do not fill the poles of erythrocytes (Fig. 1x, y). Markedly wavy lobular-like appearance of the pellicle (on the opposite side to erythrocytes nucleus) of mature gametocytes is an important distinctive feature of this species (Fig. 1u, w). The cytoplasm stains more densely in lobules than in indented areas. The nucleus of the gametocyte is diffused and of unclear outline; it consists of several chromatin clumps, which visually look to be non-connected with each other in Giemsa-stained preparation and can be seen closer to center or anywhere in the gametocyte, a characteristic feature of this species (Fig. 1s-y). Due to the pale cytoplasm staining and diffuse nucleus, macrogametocytes are difficult to distinguish from microgametocytes. Pigment granules are roundish, of small (<0.5µm) size, most often grouped in one relatively large distinct spot, but occasionally also were seen scattered in the cytoplasm (Fig. 1s-x). The area of the pigment granule groups is relatively large, and this feature attracts attention during microscopic examination (Fig. 1y). Individual pigment granules do not change size and shape during the development of gametocytes, a rare feature in avian malaria parasites. Effect of the gametocyte on the infected erythrocyte is not expressed.

**Microgametocyte** (Fig. 1z-dd; Table 2) are difficult to distinguish from the macrogametocytes based on the intensity of the cytoplasm staining and appearance of parasite nuclei. The cytoplasm is relatively paler in the microgametocytes, all other characters are as in macrogametocytes. In the blood of type host, microgametocytes were seen extremely rare, and this prevented the complete picture of the morphometry of parasite.

**Taxonomic summary**

Morphology of *P. collidatum* – small meronts, a small amount of cytoplasm in meronts and elongated shape of grown gametocytes allows linking this species with the subgenus *Novyella*. Nucleophilic stages developing in the blood of the infected host allows to discriminate this species from other non-nucleophilic species of *Novyella*. This parasite can be readily distinguished from all nucleophilic *Novyella* species due to the following morphological characteristic of its mature gametocytes: i) the cytoplasm consists of intermediated readily visible large pale-stained and dense-stained areas, providing markedly heterogeneous appearance, ii) the majority of advanced gametocytes possesses the markedly indented (lobular-like) appearance of pellicle and iii) macro- and microgametocytes are difficult to distinguish based on their size and morphology of their nuclei. Additionally, the presence of a relatively large loose group on small pigment granules is also helpful during this parasite identification (see our description and [12, 13, 55, 56, 57]). The described parasite morphologically is the most similar to *P. delichoni* however, some features, like small roundish granules clearly distinguish this parasite from *P. delichoni*. Other mentioned nucleophilic species also have additional features, which are not found in the described parasite. Growing meronts of *P. nucleophilum* often displace the nuclei of the infected erythrocytes and its macrogametocytes have a compact nucleus; *P. paranucleophilum* possesses gametocytes, which push the nucleus in infected erythrocyte laterally; gametocytes of *P. homonucleophilum* are not strictly
nucleophilic. None of the above-mentioned features is characteristics of the newly described species. The main morphological features of described parasite blood stages, which were observed in type host (C. erythrinus) also maintained in Eurasian siskins after our experimental infection. Very occasionally meronts with 9 and 10 merozoites appeared in experimentally infected siskins, but not in type host.

**Phylogenetic analysis**

According to the phylogenetic analysis, the lineage pFANTAIL01 clusters together with Plasmodium nucleophilum lineage pDENPET03 (Fig. 3). The genetic distance between these two lineages is 4.31%. Both lineages cluster within a bigger clade with other two Novyella parasites e.g. P. ashfordi (pGRW2) and P. delichoni (pCOLL6). However, the genetic difference between pFANTAIL01 and the latter two cyt b lineages is 8.43% and 10.45%, respectively. P. collidatum (pFANTAIL01) morphologically is the most similar to pCOLL6, but distribution and host range of these lineages differ.

**Development and caused virulence in experimentally infected birds**

All experimentally exposed siskins were susceptible to P. collidatum (pFANTAIL01) and showed the complete development of all blood stages of the parasite.

Prepatent period of infection, when the first infected erythrocytes were detected in the blood smears varied between 12-20 days post infection (dpi), (Fig. 4). Dynamics of parasitemia were highly variable between individuals (Fig. 4); in some reaching up to 70-80%, while in others only < 1%. In half of infected birds after sharp increase of parasitemia, there was typical bell-shaped form of primary parasitemia with clearly decreasing slope after the sharp initial increase. High parasitemia maintained in most of the experimental birds up to 36 dpi, reaching up to 11-75% (Fig. 4-5). Two birds died with parasitemias of 15% and 18% on 32 dpi. All control birds survived until the end of the experiment.

The average body mass of the infected birds did not differ significantly from the control group throughout the experiment (W = 4415, p-value = 0.6502, Fig. 6a). Hematocrit value was decreasing gradually during the experiment in all infected birds. However, significant differences between infected and control siskins were not detected until 28 dpi (W = 4427, p-value = 0.4631; Fig. 6b). On day 32 the difference between two groups of birds was the biggest (W = 53, p-value = 0.08689)

**Development in experimentally infected mosquitoes**

After screening C. p. p. f. molestus and C. quinquefasciatus midguts and salivary glands preparations it was determined that ookinetes, oocysts and sporozoites in all exposed to P. collidatum (pFANTAIL01) mosquitoes within 22 days after blood feeding were absent. Authors also did not detect any zygotes within 48 h in preparations of mosquito midgut contents.

**Discussion**
pFANTAIL01 lineage from the Common rosefinch was identified as *Plasmodium (Novyella) collidatum* n. sp. The morphological analysis of the erythrocytic stages of the parasite showed that *P. collidatum* has typical features associated with *Novyella* subgenera: elongated gametocytes, small size meronts and development of the parasite only in mature red blood cells [4]. Based on several unique morphological features of blood stages of pFANTAIL01 lineage it is possible to distinguishable it from other *Novyella* parasites and to define this parasite as a separate species (see Taxonomic Summary). The phylogenetic relationships based on the mitochondrial cytochrome b gene revealed that the closest parasite lineage to pFANTAIL01 is *P. (Novyella) nucleophilum* pDENPET03 with the genetic difference of 4.25%.

Analysis of accumulated molecular data shows that *P. collidatum* has been recorded predominantly in Oceania and SE Asia and in migrant birds wintering in South Asia (Table 1). It was recorded not only in migrant, but also in resident bird species. The transmission, apparently, takes place in these regions. Several records of pFANTAIL01 were reported in breeding bird species in Europe, Common rosefinch [18] and Rosy starling [51] and in one of *Milvus* sp. [46]. All above mentioned passerines were adults after their spring backward migration returning from South Asia wintering areas. It is highly probable that transmission of this parasite does not occur in Europe as this parasite has never been recorded in juvenile birds before their first autumn migration or in any non-migrant bird species.

Interestingly, there is one case of *P. collidatum* in Spain, from a bird of prey belonging to the *Milvus* genus [46], the species is not defined. There might be two species of *Milvus* in Europe, one is *M. milvus*, mostly within Europe migrant and the second one, *M. migrans*, which could migrate to tropical Africa [46]. However, as this is a single record in raptor bird and the bird species was not clearly defined, it is difficult to make a definite conclusion where and how the bird was infected [58].

According to the MalAvi and GenBank databases, pFANTAIL01 lineage has been reported in wide range of birds of 17 species, 14 families and 7 orders. The most species infected with pFANTAIL01 belong to the order Passeriformes, but the DNA of this parasite was recorded in Anseriformes, Accipitriformes, Bucerotiformes, Charadriiformes, Strigiformes and Psittaciformes (Table 1). Apparently, *P. collidatum* is of low specificity for the vertebrate host and, therefore, can be considered as a generalist species. Although, it is unclear if *P. collidatum* develops gametocytes in all of the mentioned birds as the presence of infection in blood was confirmed only by molecular methods and the possibility of abortive parasite development could not be excluded [9, 59].

The damage caused by malaria parasites can be by the tissue stages of exoerythrocytic merogony or by the pathogenic effect due to severe anemia caused by erythrocytic stages of the parasite [10, 60].

The pathogenic effect of the exoerythrocytic meronts was described in bird species of different families and orders [4, 6, 60]. In most of cases the harm is caused by phanerozoites and partially by metacryptozoites. These large meronts often block capillaries of different organs causing a severe oedema, hemorrhage and necrosis of the surrounding tissue [4, 6]. Exoerythrocytic development and pathogenicity of tissue stages in genera *Novyella* is poorly studied [12, 61]. In general, it is believed that the development of exoerythrocytic stages occurs in accordance with the general scheme of the life cycle.
of avian malaria parasites [4]. The exoerythrocytic stages were described in *Plasmodium nucleophilum toucani* with huge infestation of internal organs with phanerozoites and caused mortality in experimentally infected canaries [62]. Phanerozoites were also seen in different internal organs of birds infected by *P. vaughani* [4], *P. paranucleophilum* [57] and *P. bertii* [4].

The pathogenic effect of the exoerythrocytic stages of *P. collidatum* was reported only in two species of cockatoo, a Yellow-tailed black cockatoo *Calyptorhynchus funereus* and Glossy black cockatoo *C. lathami* [53]. Both birds were infected in captivity and died despite of the medical treatment. The death of these birds was preceded by symptoms such as depletion, apathy and diarrhea. Histological preparations of tissues from different organs showed a large infestation of numerous schizonts in their liver, spleens, lungs and intestines together with hemorrhage and necrosis of other tissues [53]. According to the authors, these Australian species live in habitats where the presence of potential vectors is restricted. Both housing cockatoo birds were kept not in their species-specific conditions thus they most likely were exposed to parasite vectors. Birds died, apparently, due to the extensive damage of their internal organs by the tissue stages of *P. collidatum*, authors mention the change of biochemical parameters of the host blood due to liver damage [53]. In present experiment, numerous phanerozoites were observed in liver, lungs, spleen and kidney tissue of experimentally infected siskins. Two of eight birds died at the end of experiment when parasitemia started to decrease. Two mutually non-exclusive factors, the depleted immune system and pathologies caused by phanerozoites could trigger the death of the host. Similar cases have been reported by Ilgūnas et al. [63] in experimentally infected crossbill (*Loxia curvirostra*), siskin and starling (*Sturnus vulgaris*) which were inoculated with a highly virulent avian malaria parasite *Plasmodium* (*Giovannolaia*) *homocircumflexum* (lineage pCOLL4) and mortalities of birds were observed when parasitemia was decreasing.

The negative impact of erythrocytic stages of *Plasmodium* is most noticeable when the parasite damages a big number of blood cells. Several experimental studies showed this effect during a primary infection stage [29, 64, 65, 66]. The only limited information about the development of *Plasmodium* parasites from *Novyella* subgenus is obtained up to now comparing to some species belonging to *Haemamoeba* or *Giovannolaia*. It was considered that *Novyella* parasites are mainly of low virulence to birds [67]. However, the experimental studies with infection of tropical origin, *Plasmodium ashfordi* (pGRW2) and *P. delichoni* (pCOLL6) showed that these *Novyella* parasites develop high intensities of parasitemia in experimental birds [12, 15, 68, 69]. According to the present study, all infected siskins were susceptible *P. collidatum*. The prepatent period varied between individuals but was relatively long in all experimental birds (Fig. 4). This data is in consistent manner with the information about the development of other species of *Novyella* e.g. *Plasmodium vaughani* where the prepatent period lasts from one to six weeks [4, 70], in *P. ashfordi* − 2−4 weeks [15], in *P. delichoni* − 2−3 weeks [12]. The dynamic of parasitemia varied among individuals reaching peak values up to 0.42-80% (Fig. 4) but was rather extended in time comparing to other species from the most studied parasites from subgenus *Haemamoeba* which characterized by rapid increase of parasitemia and rapid decrease to chronic values within 36 dpi [29, 71].
During the study, the impact of *P. collidatum* on body mass and hematocrit level of infected birds was measured (Fig. 6a-b). The negative effect on body mass of the infected siskins was not detected comparing to control birds (Fig. 6a). This data agrees with former experimental studies where even severe malaria infection did not affect the body mass of infected individuals, probably, because birds kept in laboratory conditions were receiving food *ad libitum* and were able to compensate the energy loss [10, 29, 66, 72]. Hematocrit level slightly decreased in infected birds, but the differences between infected and control birds were insignificant (Fig. 6b). It is worth mentioning that although the parasitemia was high, in average >15% for a long period starting from 20 dpi, the hematocrit values only slightly decreased much later when the average parasitemia was reaching approximately 25%. This is not a typical case for parasites belonging to other subgenera, because the increase in intensity of parasitemia usually causes the decrease in the number of RBCs [6; 29, 73]. During the infection with *Haemamoeba* parasite *P. relictum* (pSGS1), the quick raise of parasitemia is immediately followed by a sharp drop of hematocrit value [29]. On the other hand, Palinauskas et al. [9] showed that during single infection with low parasitemia (less than 1%) by *Huffia* subgenus parasite *Plasmodium elongatum* (pERIRUB01), hematocrit value in experimentally infected siskins decrease dramatically. The similar situation was observed in canaries experimentally infected with *Novyella* species *P. paranucleophilum*, the bone marrow of infected birds had heavy invasion at low parasitemia but anemia was clearly manifested by the decrease in hematocrit values [57]. Apparently, a huge infestation of bone marrow by phanerozoites reduces erythropoiesis and cause the decrease in the number of RBCs and therefore decrease hematocrit values. At the present study, the exoerythrocytic stages in the bone marrow of the infected siskins were not detected. Probably, the erythropoietic system was compensating the loss of erythrocytes until its depletion on 32 dpi when slight decrease of hematocrit values in infected birds. It is worth noting that species of *Novyella* subgenus are the most deficiently studied avian malaria parasites [61] and further experimental studies are needed to clarify this point.

The annual migration of birds is an important factor for a possible invasion of new haemosporidian species [4, 74]. However, to complete the life cycle on new territories parasites need a competent vector (Culicidae mosquitoes) and suitable environmental conditions. At the present study, *C. p. p. f. molestus* and *C. quinquefasciatus* of the *Culex pipiens* complex mosquitoes were used for the experimental investigations. *Culex p. p. f. molestus* is the common mosquito species distributed around the world and was confirmed as a natural and potential vector for a number of *Plasmodium* species [9, 25, 31, 75, 76]. *Culex quinquefasciatus* is more distributed in subtropical and tropical regions with warmer and moister climates and is known to transmit avian malaria parasites [77, 78] as well. However, mosquitoes of both species experimentally exposed to infection of *P. collidatum* were not susceptible to this parasite. Neither ookinetes, oocysts or sporozoites were detected in any of the exposed insects. Also, zygotes were not seen in blood smears from engorged mosquitoes. Apparently, sporogonic development was aborted on the stage of forming gametes. Further studies are needed to identify a competent vector species for this parasite. Knowledge about natural vectors of pathogens causing lethal diseases is a cornerstone for the basic understanding of epizootiology of any disease and possible threats in the future. The introduction of competent vector species could lead to the establishment of the tropical *P. collidatum* (pFANTAIL01) in
Europe and that could further lead to an outbreak of new malarial infection in non-migrant or short-distance migrant birds which did not co-evolve with the introduced parasite. In the present study it was experimentally demonstrated that susceptible avian species which could enhance the transmission of tropical pathogen exist in Northern Palearctic.

Despite the fact, that \( P.\ collidatum \) did not develop in \( C.\ p.\ p.\ f.\ molestus \), there are other mosquitoes, especially invasive species, which potentially, could serve as a vector of this parasite. The anthropogenic activity and the global warming are the main factors contributing to the increased numbers of invasive species of mosquitoes and other vectors coming from southern regions [23, 24, 79, 80]. Since recent decades, there are 6 species of mosquitoes and 1 species of biting midges of tropical origin colonizing different parts of Europe. Most of these species are involved in the transmission of various human and animal diseases and could be responsible for the introduction of some of these infections in Europe. For example, the tropical biting midges \( Culicoides imicula \) introduced the bluetongue virus of ruminants widely throughout Europe [81]. Introduced new mosquito species could serve as competent vectors both for locally already transmitting \( Plasmodium \) spp. and for exotic blood parasite species carried by long-distance avian migrants. One of such examples could be an Asian tiger mosquito, a species which is spreading throughout Europe and which was listed in 100 of the world’s worst invasive species [82]. Several molecular studies indicated that the tiger mosquito could be a potential vector for some avian malaria parasites [25, 83]. However, precise experimental and field studies are needed to determine the possibility of such assumptions.

**Conclusion**

In conclusion, a new avian malaria parasite \( P.\ collidatum \) n. sp. (pFANTAIL01) was described, which is an incongruous species for European local birds and it’s transmission takes place in South, SE Asia and Oceania regions. \( P.\ collidatum \) completes the development in the Eurasian siskin, which is a short-distance migrant within Europe. The parasite develops high intensities of parasitemia and exoerythrocytic stages in the lungs, liver, spleen and kidneys of the vertebrate host. The negative effect on host health is not expressed in changed body mass and hematocrit values of experimentally infected birds, but this \( Plasmodium \) species is highly virulent causing the death of infected birds. Although common vector species of avian malaria, \( C.\ p.\ p.\ f.\ molestus \) and \( C.\ quinquefasciatus \) were not susceptible to \( P.\ collidatum \), this parasite should be considered as potential threat to siskins and likely to other non-migrating European birds if suitable vectors and ecological conditions appear.

**Abbreviations**

1. SE – Southeast Asia.
2. RBC – red blood cell.
3. Dpi – days post inoculation.
4. Dpe – days post exposure.
Declarations

Ethics approval

All parts of this study were carried out in strict accordance with the current laws of Lithuania and Russia. The catching of wild birds, collection of samples and experiment procedure 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). The permission for performing the experiment with birds in Russia was obtained from the ethical committee of Zoological Institute of Russian Academy of Science (permission number 2019-02-05-2/1). All effort was put in when handling birds and insects were made to reduce their suffering.

Availability of data and materials

All obtained data are available after email inquiry.

Consent for publication

Not applicable.

Competing interests

The Authors declare no competent interests.

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Authors’ contributions

Design of the experiment: EP, VP, JA, AM; Fieldwork: VP, EP, JA, AM; Birds caring and feeding: JA; Experiment performing: EP, VP, JA, AM; Morphological description of the parasite: TI; Mosquitoes infection and sporogony preparations analysis: EP; Histology preparations and description: MI; Molecular work and phylogenetic analysis: EP, JA; Statistical analysis: EP, AM; Paper writing: EP, VP, MI, AM. Paper reading and approval: All authors.

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Tables

Table 1 Host range and distribution of *P. collidatum* n. sp. (lineage pFANTAIL01) based on molecular examination (places in Results/Description of parasite chapter)
| Order and family of the avian host | Species of the avian host       | Locality            | Reference |
|-----------------------------------|---------------------------------|---------------------|-----------|
| **Anseriformes**                  |                                 |                     |           |
| Anatidae                          | *Dendrocygna javanica*          | Unknown             | [43]      |
| **Bucerotiformes**                |                                 |                     |           |
| Bucerotidae                       | *Penelopides panini*            | Philippines         | [44]      |
| **Charadriiformes**               |                                 |                     |           |
| Scolopacidae                      | *Calidris tenuirostris*         | Australia           | [45]      |
| **Falconiformes**                 |                                 |                     |           |
| Accipitridae                      | *Milvus* sp.                    | Spain               | [46]      |
| **Passeriformes**                 |                                 |                     |           |
| Acanthizidae                      | *Sericornis magnirostris*       | Australia           | [47]      |
| Fringillidae                      | *Carpodacus erythrinus*         | Czech Republic      | [18]      |
| Maluridae                         | *Malurus coronatus*             | Australia           | [48]      |
|                                   | *Malurus melanocephalus*        | Australia           | [48]      |
| Pachycephalidae                   | *Pachycephala simplex*          | Australia           | [47]      |
| Petroicidae                       | *Poecilodryas albispecularis*   | Australia           | [48]      |
| Rhipiduridae                      | *Rhipidura rufifrons*           | Australia           | [43]      |
| Sturnidae                         | *Acridotheres tristis*          | Singapore, Australia| [49], [50]|
|                                   | *Pastor roseus*                 | Bulgaria            | [51]      |
| Turdidae                          | *Turdus merula*                 | India               | [52]      |
| Zosteropidae                      | *Zosterops lateralis*           | Australia           | [50]      |
| **Psittaciformes**                |                                 |                     |           |
| Cacatuidae                        | *Calyptorhynchus funereus*      | Australia           | [53]      |
|                                   | *Calyptorhynchus lathami*       | Australia           | [53]      |
| **Strigiformes**                  |                                 |                     |           |
| Strigidae                         | *Glaucidium cuculoides*         | Thailand            | [54]      |
Table 2 Morphometry of host cells, mature gametocytes and erythrocytic meronts of *Plasmodium collidatum* n. sp. (lineage pFANTAIL01) (n=21) (places in Results/Description of parasite chapter)
| Feature                      | Measurements (μm) |
|------------------------------|-------------------|
| **Uninfected erythrocyte**   |                   |
| Length                       | 10.4-12.4 (11.5±0.5) |
| Width                        | 6.1-7.2 (6.5±0.3) |
| Area                         | 52.8-67.8 (60.5±4.3) |
| **Uninfected erythrocyte nucleus** |       |
| Length                       | 5.1-6.2 (5.5±0.3) |
| Width                        | 2.1-2.8 (2.3±0.2) |
| Area                         | 9.5-14.8 (10.8±1.1) |
| **Macrogametocyte**          |                   |
| **Infected erythrocyte**     |                   |
| Length                       | 11.2-13.6 (12.4±0.6) |
| Width                        | 5.3-7.38 (6.1±0.5) |
| Area                         | 52.4-71.5 (60.4±4.7) |
| **Infected erythrocyte nucleus** |            |
| Length                       | 4.6-5.8 (5.2±0.3) |
| Width                        | 1.6-2.5 (2.1±0.2) |
| Area                         | 7.0-10.8 (9.0±1.1) |
| **Gametocyte**               |                   |
| Length                       | 9.6-13.2 (11.5±1.1) |
| Width                        | 0.9-2.5 (1.5±0.4) |
| Area                         | 18.2-28.9 (21.8±2.6) |
| **Gametocyte nucleus**       |                   |
| Length                       | -                 |
| Width                        | -                 |
| Area                         | -                 |
| Pigment granules             | 7.0-14.0 (8.2±1.7) |
|**Microgametocyte** (n=6) |
|--------------------------|
|**Infected erythrocyte**  |
| Length                   | 10.7-14.1 (12.3±1.3) |
| Width                    | 6.0-6.6 (6.2±0.2)    |
| Area                     | 53.0-70.5 (61.6±7.3) |
|**Infected erythrocyte nucleus** |
| Length                   | 4.9-5.8 (5.3±0.4)    |
| Width                    | 2.0-2.6 (2.2±0.2)    |
| Area                     | 9.1-10.6 (9.9±0.6)   |
|**Gametocyte**            |
| Length                   | 10.5-13.0 (12.2±1.0) |
| Width                    | 1.8-2.3 (2.0±0.2)    |
| Area                     | 17.5-25.5 (21.6±3.0) |
|**Gametocyte nucleus**    |
| Length                   | 1.3-3.8 (2.8±1.0)    |
| Width                    | 0.5-0.7 (0.6±0.1)    |
| Area                     | 0.9-1.2 (1.0±0.2)    |
|**Pigment granules**      | . b                  |
|**Meront**                |
| Length                   | 2.8-5.6 (4.4±0.7)    |
| Width                    | 1.0-2.2 (1.7±0.3)    |
| Area                     | 3.7-8.0 (5.5±1.3)    |
| No. of pigment granules  | . b                  |
| No. of merozoites        | 4.0-8.0 (5.8±1.2)    |

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

b Pigment granules are clamped and are difficult to calculate.