

Avian malaria parasites (Haemosporida: Plasmodiidae) in mosquitoes (Diptera: Culicidae) of the Curonian Spit (South-East coast of the Baltic Sea)

Elena Platonova¹, Alexander Davydov¹, Maria Erokhina^{1,2}, and Andrey Mukhin¹

¹ Biological Station Rybachy, Zoological Institute, Russian Academy of Sciences, ul. Pobedy, 32, Rybachy, Kaliningrad Region, 238535, Russian Federation

² Department of Vertebrate Zoology, Faculty of Biology, Lomonosov Moscow State University, ul. Kolmogorova, 1, Moscow, 119234, Russian Federation

Address correspondence and requests for materials to Elena Platonova, plat.992@gmail.com

Abstract

In recent years, numerous studies have shown an increasing prevalence of avian haemosporidian parasites in Europe. However, little is known about the vectors of these parasites, particularly specific dipterian species transmitting malaria parasites to birds. This study aims to identify vectors of avian malaria parasites on the South-East Baltic coast. Mosquito females were collected from 2020 to 2021 on the Curonian Spit of the Baltic Sea using traps with birds as bait. All insects were identified to the species level through morphological features and the PCR method. Subsequently, they were dissected to extract salivary glands for studying the presence of avian malaria infecting stages (the sporozoites). The remaining mosquito parts were used later for molecular analysis to detect haemosporidian parasite DNA. A total of 596 mosquitoes belonging to 8 species were collected. The analysis revealed that *Culex pipiens* is a competent vector of avian *Plasmodium relictum* (genetic lineages pGRW11 and pSGS1) and a potential vector for *Plasmodium vaughani* (genetic lineage pSYAT05) on the Curonian Spit of the Baltic Sea.

Keywords: Avian malaria parasites, *Plasmodium*, mosquitoes, *Culex pipiens*, transmission, vectors.

Introduction

Avian malaria parasites of genus *Plasmodium* (Haemosporida, Plasmodiidae) belong to a very diverse group of parasitic protists that infect the internal organs and blood cells of birds and transmitted by blood-sucking female mosquitoes (Diptera, Culicidae) (Atkinson, 2008). Over 55 species of this genus have been documented up to date, with more likely to be discovered in the future (Valkiūnas and Iezhova, 2018; Fecchio, Chagas, Bell, and Kirchgatter, 2020).

Due to the capacity of *Plasmodium* to induce severe malaria in birds, these parasites have been extensively studied in relation to their interactions with vertebrate hosts, encompassing the development of infection, specificity, and virulence (Žiegytė and Valkiūnas, 2014). However, information on potential and competent vectors remains insufficient, primarily due to challenges in conducting vector studies in the wild. The developmental stages of avian malaria parasites in mosquitoes lack species-specific morphological traits, leading to the majority of vector competence surveys being conducted in laboratory conditions through experimental infection of various mosquito species with different *Plasmodium* spp. (Valkiūnas, 2005). While these experiments have confirmed some species, such as those from genera *Aedes*, *Coquillettidia*, *Culiseta*, *Culex*, *Mansonia*, and others, as vectors of bird malaria, they may not accurately reflect the actual transmis-

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Authors' information: Elena Platonova, PhD, Researcher, orcid.org/0000-0002-9425-8998; Alexander Davydov, Researcher Intern, orcid.org/0000-0001-8577-503X; Maria Erokhina, Research Assistant, orcid.org/0000-0002-9905-8338; Andrey Mukhin, PhD, Senior Researcher, orcid.org/0000-0003-1238-4163

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sion dynamics in the wild. Some of these potential vectors may not feed on birds in their natural habitat and, therefore, cannot be considered competent transmitters of avian malaria parasites.

The application of molecular methods, particularly PCR, has opened new avenues for the identification of avian *Plasmodium* in wild mosquito populations. Routine screening of mosquitoes sampled by light traps has revealed dipteran species that could be exposed to these parasites (Ejiri et al., 2009; Ventim et al., 2012; Zitra et al., 2015). However, PCR alone can only detect the DNA of the infection agent and does not confirm if the parasite completes its sporogony in an insect, developing infective stages for a vertebrate host — namely, sporozoites. Relying solely on PCR analysis may lead to errors, as a species may be erroneously recognized as a competent vector when the parasite's DNA is found in the mosquito's intestines due to feeding on an infected vertebrate host. Recent studies combining PCR for parasite identification and sporozoite detection in dipteran insects have described competent vectors for different haemosporidians, such as *Haemoproteus* (Bernotienė, Žiegytė, Vaitkutė, and Valkiūnas, 2019; Žiegytė et al., 2021; Žiegytė, Bernotienė, and Palinauskas, 2022) and *Leucocytozoon* (Njabo et al., 2009; Žiegytė and Bernotienė, 2022; Odagava et al., 2022). However, available data only describes a few vectors for small numbers of *Plasmodium* parasites.

The Curonian Spit, an almost 100-kilometer-long peninsula, features a varied mosaic landscape, including sand dunes, dry pine and wet mixed forests, and reeds (Valkiūnas, 2005). Every spring and autumn, thousands of birds utilize the spit as a stopover during their annual seasonal migration along the White Sea-Baltic flyway (Sokolov, Markovets, and Shapoval, 2017). Numerous avian species also breed in different habitats of this peninsula. Long-term studies conducted on the birds at the Biological Station “Rybachy” of the Zoological Institute of the Russian Academy of Sciences have revealed a high prevalence and diversity of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* in both local and migrating birds (Valkiūnas, 2005; Križanauskienė et al., 2006). Despite the substantial circulation of haemosporidian parasites, precise information regarding which species act as transmitters on the spit is lacking. The first field study conducted by Žiegytė et al. (2021) helped clarify potential vectors of *Haemoproteus* in the Paridae birds. Nevertheless, only indirect data, such as the analysis of infected birds, indicates which species of avian malaria parasites are transmitted in local passerines. Bernotienė (2012) identified a high diversity and abundance of mosquitoes on the Curonian Spit, with some of these species potentially capable of transmitting avian malaria.

The aim of the present study is to identify ornithophilic species of Culicidae that may be involved in the

transmission of avian malaria parasites on the Curonian Spit. Using bird-baited traps to attract mosquitoes, we collected specimens and analyzed them for the presence of DNA and sporozoites of avian malaria parasites. The results of this study contribute valuable information regarding the transmission of avian *Plasmodium* in the South East Baltic region.

Materials and methods

Study site

Ornithophilic mosquitoes were collected during the summer months (June — August) in 2020 and 2021 on the Curonian Spit of the Baltic Sea, Russia (55°16'28" N, 20°58'15" E). The Curonian Spit is a 98 km long peninsula that separates the Curonian Lagoon from the main body of the Baltic Sea and encompasses diverse natural habitats, including sandy dunes covered with willow bushes, mixed and coniferous forests, and patchy reed beds.

Various avian species nest on the Curonian Spit, and millions of European migrating birds use the peninsula as a stopover site for resting and feeding on their way to wintering and breeding grounds. The presence of a freshwater lagoon and a few small and medium-sized lakes and ponds near Rybachy village provides an excellent opportunity for the breeding of many mosquito species (Bernotienė, 2012). All these factors make the Curonian Spit a promising location for studying the interaction between blood parasites, vectors, and their vertebrate hosts

Collection of ornithophilic mosquitoes

To catch ornithophilic mosquitoes, we designed and set up a bird-baited trap. Briefly, the trap consisted of three different-sized cages, each placed inside the others. The smallest metallic one (37.5 × 33 × 25 cm) housed an adult Eurasian siskin (*Spinus spinus*). The second one was made of polyester with a mesh size of 1 mm draped over a wooden box-shaped frame (60 × 49 × 40 cm). Each side of this cage had several funnel-type plastic entrances for mosquitoes with an outer diameter of 10 cm and an inner one of 2 cm. Attracted by the siskin, mosquitoes entered the second cage and accumulated there. Additionally, to protect the bird from any threats, a big metal cage (76 × 66 × 53 cm) was built over the others. The mesh size of the metal net did not prevent mosquitoes from flying through the bars.

One trap was hung up on a tree branch about 2 meters above the ground in the wet mixed forest with an alder tree prevailing near Chaika Lake (55°14' N, 20°82' E). The second trap was established on a wooden platform 4 meters above the ground in reed and willow

shrub in the vicinity of the Rybachy bird trapping site (55°15' N, 20°86' E).

We checked the siskin used as a bait for the presence of blood parasites using PCR and examination of blood smears before and after the study. During the examination, the bird turned out to be uninfected.

Collecting and mosquito proceeding

The traps were checked daily to collect mosquitoes and feed the bird. Insects were extracted from the second cage of the setups using an electric aspirator (BugBaster, Sonic Technology Products, USA) and transferred to the laboratory. There, each female mosquito was identified to the species level based on its morphological features (Becker et al., 2010). Subsequently, all individuals were dissected in 1–2 hours after sampling to prepare their salivary glands following Valkiūnas (2005). Mosquitoes were euthanized with ethanol vapor, placed on a glass slide in a drop of normal saline, and gently decapitated. Afterward, their salivary glands were removed from the thorax and isolated in a tiny drop of saline. Using two sterile dissecting needles, the glands were carefully grinded and smeared.

The remnants of the mosquitoes were stored individually in 96% ethanol for further molecular analysis (as described below). The smears (each slide with individually prepared mosquito salivary glands) were air-dried, fixed in absolute methanol, and then stained with a 4% Giemsa solution for one hour. The stained preparations were screened at 1000× magnification under a light microscope (Olympus CX23, Japan) for the presence of sporozoites.

Molecular analysis

The molecular analysis of samples was conducted to detect: 1) DNA of avian malaria parasites in collected mosquitoes; 2) the accuracy of mosquito species identification. Total DNA was extracted from the remaining parts of dissected mosquitoes using the ammonium-acetate extraction protocol (Žiegytė et al., 2021).

A standard nested PCR protocol was employed to amplify a fragment of the parasite's mitochondrial cytochrome *b* gene with outer primers HaemNFI and HaemNR3, and inner primers HaemF and HaemR2 (Hellgren, Waldenström, and Bensch, 2004). To control for false positive results in the sample analysis, a negative control (dd H₂O) was included in each PCR run.

To validate the identification of mosquito species, mitochondrial DNA cytochrome *c* oxidase subunit 1 was amplified using primers LCO1490 and HCO2198 (Folmer et al., 1994).

The amplified DNA fragments were visualized on a 2% agarose gel. Positive samples were sequenced and

analyzed using the BioEdit program (Hall, 1999). To identify the genetic lineages of *Plasmodium* and mosquito species, sequences were compared against the genetic databases MalAvi and NCBI (<http://mbio-serv2.mbioekol.lu.se/Malavi> and <https://blast.ncbi.nlm.nih.gov/Blast.cgi>), respectively.

Results and discussion

In total, 596 mosquitoes were collected from bird-baited traps on the Curonian Spit: 200 insects in 2020 and 396 in 2021. All individuals did not contain any blood in their midguts thus they were unfed. We identified 8 mosquito species from 5 genera of Culicidae: *Culex pipiens*, *Coquillettidia richiardii*, *Culiseta annulata*, *Cs. morsitans*, *Cs. alaskaensis*, *Aedes geniculatus*, *Aedes cinereus*, *A. vexans*. The most abundant species at both sampling sites was *Cx. pipiens* (Supplementary material, Table 1), constituting about 95.2/96.9% in reeds/forest in 2020 and 81.5/87.4% in reeds/forest in 2021. In 2021, the second most abundant mosquito collected in the reeds was *Cs. spp.* with 18.5% of mosquitoes, in the forest — *Cq. richiardii*, comprising 11.4% of identified insects. The abundance of the remaining species was very low, representing less than 5%.

Overall, the abundance of mosquitoes in the forest was similar with sampled in the reeds in 2020 and higher than in the reeds in 2021 (Fig. 1A, B), with 96 out of 200 insects collected in 2020 and 342 out of 396 in 2021. The highest number of mosquitoes sampled from the bird-baited traps was at the end of June to the beginning of July in 2020 (121 of 200 mosquitoes from June 18 to July 4) and at the end of June to the middle of July in 2021 (232 of 396 insects from June 21 to July 10).

DNA of avian *Plasmodium* was detected in 9 individuals of *Cx. pipiens*, with 4 infected mosquitoes found in 2020 and 5 — in 2021. The detected genetic lineages (Table 1) belonged to two species of avian malaria parasites — *Plasmodium (Haemamoeba) relictum* (3 individuals with the genetic lineages pSGS1 and 4 individuals with pGRW11) and *Plasmodium (Novyella) vauhani* (2 mosquitoes with the genetic lineage pSYAT05). All infected mosquitoes were sampled in the forest site. In 4 preparations of mosquito salivary glands, sporozoites of *P. relictum* (2 of pSGS1 and 2 of pGRW11), both lineages, were found at the end of June and the beginning of August (Supplementary material, Table 1). Additionally, in two preparations, *Trypanosoma* sp. (Kinetoplastida, Trypanosomatidae) were detected, with one in coinfection with *P. relictum* (pSGS1).

According to Bernotienė (2012), at least 16 species of Culicidae inhabit the Curonian Spit. In our study, we found only eight species, with a predominance of *Cx. pipiens* in our samplings and rare recordings of those species that had shown high abundance in Bernotienė's

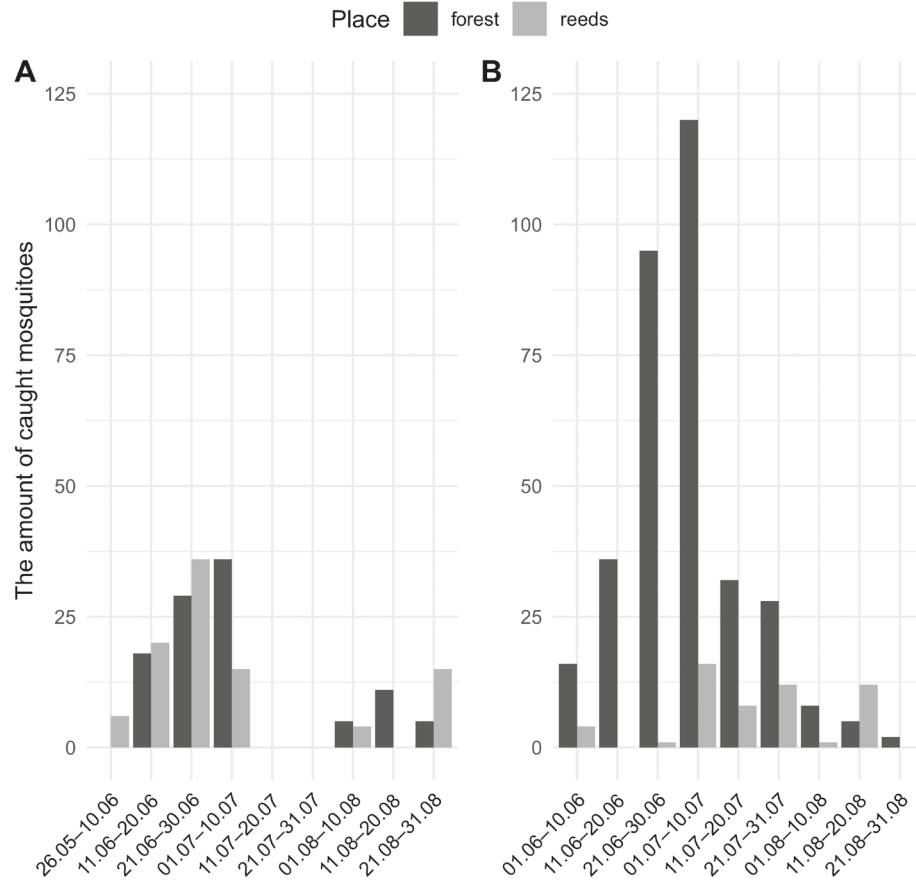


Fig. 1. The abundance of mosquitoes collected from bird-baited traps on the Curonian Spit in 2020 (A) and 2021 (B).

Table 1. Blood parasites found in mosquitoes *Culex pipiens* of the Curonian spit in 2020–2021

	2020		2021	
	Forest	Reeds	Forest	Reeds
June	<i>P. relictum</i> (pSGS1) 1; (pGRW11) 1	—	<i>P. relictum</i> (pSGS1) 1; (pGRW11)/ <i>Trypanosoma</i> sp. 1; <i>P. vaughani</i> (pSYAT05) 1	—
July	<i>P. vaughani</i> (pSYAT05) 1	—	—	—
August	<i>P. relictum</i> (pGRW11) 1; <i>Trypanosoma</i> sp. 1	—	<i>P. relictum</i> (pSGS1) 1; (pGRW11) 1	—

study. This difference can be explained by the methods used for mosquito acquisition. In the work conducted by Bernotienė, light-traps and active catching were used, while we employed a bird-baited trap method, which should attract only mosquitoes with a prevailing preference to feed on avian hosts. *Culex pipiens* is known to be primarily ornithophilic (Vinogradova, 2000), and, generally, it is a dominant species in many samplings using bird-baited traps (Černý, Votýpka, and Svobodová, 2011; L'Ambert, Ferré, Schaffner, and Fontenille, 2012; Ventim et al., 2012; Brugman et al., 2018). The rest of the recorded species have an opportunistic preference and feed on birds only occasionally (Becker et al., 2010); therefore, their proportion in our collections is minor. Also, we did not detect *Aedes annulipes*, *A. cantans*, *A. cataphylla*, *A. excrucians*, and *A. intrudens* in our study, which were

previously recorded to feed on the birds of the Curonian Spit (Bernotienė, 2012).

The number of collected *Cx. pipiens* changed for three months (Fig. 1A, B). The lowest number of attracted insects we observed from the middle of July until the end of August in both years; obviously, this situation is associated with the cycles of mosquito generations (Becker et al., 2010). Our observations of dipterans on the Curonian Spit from previous years show that the number of mosquitoes usually decreases from the end of June until the end of July-beginning of August (unpublished data).

Our results show that *Cx. pipiens* is a main vector of avian malaria on the Curonian Spit, consistent with data obtained in other field surveys suggesting that, in Europe, this mosquito species is involved in the trans-

mission of *Plasmodium* genetic lineages such as *P. relictum* of pSGS1, pGRW11 (Glaizot et al., 2012; Lalubin, Delédevant, Glaizot, and Christe, 2013; Martínez-de la Puente et al., 2015), *Plasmodium elongatum* of pGRW6 (Zele et al., 2014; Schoener et al., 2017), *Plasmodium matutinum* of pLINN1 (Ferraguti et al., 2013; Martínez-de la Puente et al., 2015), *Plasmodium cathemerum* of pPADOM02 (Glaizot et al., 2012; Lalubin, Delédevant, Glaizot, and Christe, 2013), *P. vaughani* of pSYAT05 (Synek, Munclinger, Albrecht, and Votýpka, 2013; Schoener et al., 2017), and others (Glaizot et al., 2012; Köchling, Schaub, Werner, and Kampen, 2023). However, all these results were obtained only by PCR analysis without confirmation of the presence of sporozoites in the salivary glands of infected insects, which cannot exclude the possibility of abortive development of infection in a mosquito (Žiegytė and Valkiūnas, 2014).

The sporozoites of *P. relictum* pGRW11 and pSGS1 were detected in mosquitoes *Cx. pipiens* in Japan (Kim and Tsuda, 2015). However, in Europe, there were only few findings of the parasite's DNA, and experimental confirmation that the infection completes its development in this mosquito species (Kazlauskienė et al., 2013). Our results confirm that *Cx. pipiens* is a competent vector for these two lineages of *P. relictum* in Europe. This avian malaria parasite is one of the most widely distributed in the world, infecting numerous bird species of different families and orders (Valkiūnas et al., 2018) and is able to cause severe disease in birds (Palinauskas, Žiegytė, Šengaut, and Bernotienė, 2018).

Plasmodium vaughani of pSYAT05 was detected twice in our samplings, but we did not find sporozoites of this parasite in the salivary glands of infected mosquitoes. This parasite is also widely distributed in Europe and is often found in birds and *Cx. pipiens* females (Mal-Avi database; Bensch, Hellgren, and Pérez-Tris, 2009). There is inconsistent experimental data on whether this parasite is able to complete its sporogony in *Cx. pipiens* mosquitoes or not. *Culiseta morsitans* showed its susceptibility to *P. vaughani* with the development of sporozoites in experimentally infected mosquitoes (Valkiūnas, 2005), but the competitiveness of these species to transmit the pSYAT05 genetic lineage should yet be confirmed.

Interestingly, we found the presence of *Trypanosoma* sp. together with sporozoites of avian malaria parasites in two smears of the salivary glands. Parasites of this genus are worldwide distributed and usually have a high prevalence in populations of wild birds. *Trypanosoma* has low specificity to the vectors and can use various blood-sucking species of Diptera (biting midges, black flies, louse flies, mosquitoes) and arachnids (mites) (Bernotienė et al., 2020). Co-infection of different blood parasites is often detected in wild birds, and the parasites can show antagonistic relationships (Aželytė et al.,

2022). However, apparently, both avian malaria and *Trypanosoma* parasites could complete their development in the salivary glands of *Cx. pipiens* mosquitoes.

According to the obtained results, the sporozoites of *P. relictum* on the Curonian Spit were recorded in June–August in 2020–2021 for both genetic lineages pGW11 and pSGS1. However, the circulation of this parasite could also occur in spring and autumn when the air temperature is relatively suitable for infection development, and the host, vector, and parasite are present. Recent studies showed that DNA of avian *Plasmodium* could be detected in temperate zones of Europe from April (Lalubin, Delédevant, Glaizot, and Christe, 2013) until November (Köchling, Schaub, Werner, and Kampen, 2023). The maximum density of birds on the Curonian Spit is observed in April–May and September–November during the seasonal migrations when thousands of passerines belonging to many species from different regions of Europe pass over the Spit (Sokolov, Markovets, and Shapoval, 2017). At this time, the possibility of avian malaria transmission in cold months cannot be excluded, especially since some haemosporidian parasites can still complete their sporogony in low temperatures (Valkiūnas et al., 2015; Platonova and Palinauskas, 2021).

Additionally, the second most abundant mosquito species in 2021 on the Curonian Spit in our study was *Cq. richiardii*. We did not find avian malaria infection in female individuals of this species. However, during recent years, more data have been accumulated to support the view that mosquitoes of this genus can play a significant role in the transmission of *Plasmodium* in birds (Njabo et al., 2009). The competence of *Cq. richiardii* as a vector of avian malaria has not been proven yet. Blood-sucking mosquitoes can acquire DNA of different haemosporidian parasites while feeding on birds occasionally. However, the development of the parasite can be aborted in inappropriate mosquito species, while traces of DNA still could be registered by PCR. Further experimental and field studies are needed to confirm the ability of *Cq. richiardii* to transmit avian malaria.

Conclusion

Our study revealed that mosquitoes of *Culex pipiens*, apparently, were the main vectors of avian malaria parasites on the Curonian Spit, transmitting at least one of the most widely distributed parasites in the world, *P. relictum*, of two genetic lineages (pSGS1 and pGRW11), whose sporozoites were found in the salivary glands of local mosquitoes, and probably *P. vaughani*, whose DNA was also found in several unfed mosquito females. *Coquillettidia richiardii* could be taken into account as a potential vector of avian *Plasmodium*, considering new records of its ornithophilic preferences.

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