

Article

Blood Parasites (Haemosporida, Trypanosomatida) in *Culex pipiens*: A Study and Review of Hibernating and Active Mosquitoes

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Abstract: *Culex pipiens* mosquitoes (Diptera: Culicidae) are widespread during warm periods and actively feed on blood while serving as vectors for various human and animal pathogens. *Culex* mosquitoes overwinter as adults in temperate zones, raising the question of whether hibernating *Cx. pipiens* can act as pathogen reservoirs. In this study, hibernating mosquitoes and mosquitoes collected during the warm season were tested for the presence of trypanosomatids and avian haemosporidian parasites using PCR. Midgut preparations were made from *Cx. pipiens* females in order to search for trypanosomatids morphologically. In total, 1037 *Cx. pipiens* mosquitoes (556 collected during the warm season and 481 overwintering mosquitoes) were investigated. The parasite prevalence differed for mosquitoes collected during the warm season and hibernating ones for both Haemosporida (2.9% in warm-season and no infections in overwintering mosquitoes) and Trypanosomatida (1.6% and 0.4%, respectively) parasites. A phylogenetic analysis confirmed that the trypanosomatids found in hibernating mosquitoes were monoxenous and were not parasites of vertebrates. The peak prevalence of Haemosporida parasites was detected in July (4.9%) and August (2.8%), and for Trypanosomatida, it was detected in May (3.5%). The results of the present study show that overwintering *Cx. pipiens* mosquitoes are questionable reservoirs for avian haemosporidian parasites, but some monoxenous trypanosomatids can be found in overwintering females.

Keywords: *Culex pipiens*; *Plasmodium*; *Trypanosoma*; hibernation; vector



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1. Introduction

Even though mosquitoes have been researched for over a century (ever since Sir Patrick Manson demonstrated in 1877 that they can transmit filarial worms [1] and Sir Ronald Ross discovered in 1897 that Culicinae mosquitoes are a part of the avian malaria life cycle [2]), there is still a significant gap in our knowledge regarding the vector competence of various mosquito species in Europe [3]. The mosquito species *Culex pipiens* is the most frequently encountered when searching for information on the transmission of various vertebrate pathogens [4–7]. West Nile (WNV), Ockelbo, Usutu, Sindbis and Equine encephalitis viruses are spread by *Culex* mosquitoes [6,8–11]. These mosquitoes are also known as vectors of several *Trypanosoma* species [4,5,12], avian haemosporidian parasites belonging to the genus *Plasmodium* [3,7], as well as pathogens causing lymphatic filariasis [13,14]. This is not a complete list of the various pathogens that are known to be spread by *Culex* mosquitoes [8–14]. Avian haemosporidian and *Trypanosoma* parasites are widespread and infect birds from a broad variety of avian families with diverse consequences, ranging from subclinical infections to severe and fatal disease [15]. The prevalence of *Plasmodium* and *Trypanosoma* parasites in passerine birds in Lithuania is about 5% and 26%

respectively [16], so these parasites can serve as model organisms to study the biology of vector-borne pathogens.

In a temperate climate, the females of *Culex*, *Anopheles* and *Culiseta* can hibernate as adults in various shelters (cellars, bunkers, abandoned buildings, tree holes) [11], and within *Culex* genera in our study area, *Cx. pipiens*, *Cx. torrentium* and *Cx. territans* hibernate as adult females. *Culex pipiens* f. *pipiens* females are anautogenous (they require a blood meal for their egg laying) and, depending on the climatic conditions, can develop several generations per year [11]. Males of this species die during winter, while females search for above-ground overwintering sites [17,18]. Hibernating mosquito females do not express host-seeking behavior and use the accumulated fat body as an energy source. Even though there are studies about *Cx. pipiens*'s inability to use blood as a source for lipid reserves for overwintering [19], other studies suggest that 40% of female *Cx. pipiens* who were subjected to warmer temperature periods took a full blood meal and did not undergo ovarian maturation, which gives them an equal chance of surviving [20]. There is evidence of blood-feeding in diapausing *Cx. pipiens* females in the wild [21]. Because of the fact that *Culex* females overwinter as adults, it raises the question if (1) overwintering mosquitoes can be winter reservoirs for some pathogens or if (2) mosquitoes are newly reinfected each spring by feeding on the blood of infected hosts. The answer to this question is especially important when studying the biology of pathogens that are transmitted by *Culex* mosquitoes and cannot be transmitted via vertical transmission.

There are studies molecularly confirming the presence of viruses in overwintering mosquitoes—WNV RNA was found in overwintering *Cx. tarsalis* [22] and *Cx. p. pipiens* females [23]. Unlike some arboviruses, Protozoa parasites transmitted by *Culex* mosquitoes cannot be transmitted via transovarial transmission. Many studies provide information on the prevalence of avian haemosporidian parasites in wild-caught mosquitoes collected during the warm season [3,7,24], but there is almost no knowledge of whether the parasites persist in hibernating mosquitoes. We tested hibernating *Cx. pipiens* females for the presence of avian *Trypanosoma* and haemosporidian parasite DNA and compared our results with results obtained from active *Cx. pipiens* mosquitoes caught during the warm period at similar localities. For this purpose, we employed microscopy and PCR-based methods. We aimed to understand if hibernating *Cx. pipiens* mosquitoes can be infected with avian *Trypanosoma* and haemosporidian parasites and in this way act as reservoirs for these parasites.

2. Materials and Methods

2.1. Collection of Mosquitoes

Hibernating mosquitoes were collected in December and January 2023–2024 using mouth aspirators in damp cellars in South Lithuania, Norkūnai village (54.500694, 23.940056), Panemuninkėliai village (54.431389, 24.068250), West Lithuania, Palanga (55.918278, 21.055639) and Vilnius (bunkers in Vilnius (54.700444, 25.332583 and 54.688764, 25.362232) and in the cellar of Verkiai water tower (54.749139, 25.291444)).

Culex pipiens mosquitoes were caught regularly during the warm period (when the average daily temperature was over 10 °C) from May to September using a sweeping net (2021) in Vilnius environs (Dvarčionys 54.736569, 25.384285, Belmontas 54.688764, 25.362232) and CDC trap-baited with CO₂ (2022–2023) in South Lithuania Puvočiai (54.112762, 24.302303) and in Vilnius environs (Kairėnai 54.733956, 25.403669, Verkiai 54.748405, 25.289150) and Brinkišės (54.798850, 25.059928). All investigated localities were mixed forests with small water bodies (ponds, puddles, marshes). Hibernating mosquitoes were collected at similar habitats with cellars as their hibernating places. The material was transported to the laboratory the same day after collection for further processing.

2.2. Mosquito Identification Dissection and Microscopic Examination of Samples

All collected mosquitoes were placed in a freezer for 10–15 min to make them inactive; after that, mosquito females were identified according to Becker et al. and Gunay et al.'s

identification keys [11,25] under a stereomicroscope, MOTIC SMZ-171 (Motic, Hong Kong, China). Only *Culex* mosquito females were further investigated.

Females were dissected immediately after identification for (a) salivary gland [24] and (b) midgut preparations [15,26]. Salivary glands were extracted from the anterior part of the thorax and were used to search for invasive stages of haemosporidian parasites (sporozoites) as described in [24]. Midgut preparations were used to search for *Trypanosoma* parasites [15,26]: the last two–three segments of a mosquito's abdomen were gently pulled together with the digestive tract. Half of the midgut was separated from the rest of the organs, pulled into pieces, spread in a thin layer on a microscopic slide, and set aside to dry at room temperature. For each dissection, a fire-sterilized pair of needles was used. Slides were then fixed with a drop of absolute methanol and stained for one hour with a 4% Giemsa solution [15]. The remaining parts of the mosquitoes were placed individually in an SET buffer (solution of Sodium, Ethylenediaminetetraacetic acid (EDTA) and Tris-HCl) for further DNA extraction (see Section 2.3). Data on the prevalence of haemosporidian parasites from the same mosquitoes collected during the warm period have been published as a part of another investigation [24].

The midgut preparations of mosquitoes that were determined to be PCR-positive for trypanosomatid DNA (see Section 2.3) were later examined microscopically. Microscopy was performed using an Olympus BX-43 microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP12 digital camera and image software, Olympus DP-SOFT v.3.2 (Olympus, Tokyo, Japan). Entire samples were screened at $\times 1000$ magnification. Positive slides (49805 NS–49807 NS) were deposited at the Nature Research Centre (Akademijos str. 2, Vilnius, Lithuania).

Culex pipiens and *Cx. torrentium* mosquito females can only be distinguished by molecular methods. To separate these two species, we used a method which was based on the use of restriction enzymes [27]. After DNA extraction (see Section 2.3) and PCR, the COI region of mitochondrial DNA is cleaved differently by two restriction enzymes, and the results are visualized on the agarose gel. The *SspI* enzyme cuts a COI fragment of *Cx. pipiens* into two (~620 pb and 210 bp), and the COI fragment of *Cx. torrentium* remains uncut. With the enzyme *FspBI*, this is the opposite of a previously described one [27]. This method enables the distinction of two sibling species, *Cx. pipiens* and *Cx. torrentium*, without sequencing.

2.3. DNA Extraction, PCR and Sequencing

We expected a lower prevalence of parasites in hibernating mosquitoes, so they were pooled (up to 10 individuals in one pool collected at the same locality on the same day) for molecular analysis, and active mosquitoes were investigated individually. The total DNA from pools of hibernating *Culex* females was extracted using an ammonium acetate protocol [28], and DNA from active mosquitoes was extracted using an ammonium acetate protocol or GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Vilnius, Lithuania) as described in [24]. Both DNA extraction methods have been used before and are equally effective.

For the detection of haemosporidian parasites, a nested PCR protocol with two pairs of primers (outer primers: HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3'); inner primers: HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3')) was used to amplify a fragment of the cytochrome *b* gene (*cytb*) [29,30]. For trypanosomatid detection, a nested PCR protocol with two pairs of primers (outer primers: Tryp763 (5'-180 CATATGCTTGTCAAGGAC-3') and Tryp1016 (5'-CCCCATAATCTCCAATGGAC-3'); inner primers: Tryp99 (5'-TCAATCAGACGTAATCTGCC-3') and Tryp957 (5'-182 CTGCTCCTTTGTATCCCAT-3')) was used to amplify a DNA fragment encoding SSU 18S rRNA [31,32]. For both types of PCR, a negative control (nuclease-free water) and a positive control (extracted DNA from bird blood, infected with *Plasmodium* (in the investigation of haemosporidian parasites) or *Trypanosoma* (in the search for tripanomatids) parasites

confirmed both by PCR and microscopy) were included in every 46 samples. The PCR products were visualized in a 2% agarose gel using MidoriGreen dye (NIPPON Genetics Europe, Düren, Germany). Samples with visible bands (~500 bp for haemosporidians and ~750 bp for trypanosomatids) were considered positive, and the PCR product was precipitated using an ammonium acetate protocol [28] and sent for sequencing. Purified PCR-positive samples were sequenced using an Applied Biosystems Genetic Analyzer 3500 in both strands with inner primers. The obtained sequences were deposited in GenBank (PP946099-PP946107; PP948731, PP948732).

2.4. Analysis of Sequences and Statistics

The obtained chromatograms were analyzed with Geneious Prime (2023.0.4), both strands were aligned, and contig sequences were formed. The analysis of the haemosporidian sequences was described in a previous article [24]. For the identification of trypanosomatid sequences, GenBank BLAST (National Centre of Biotechnology Information website: <https://www.ncbi.nlm.nih.gov/BLAST>, accessed on 4 June 2024) was used.

A phylogenetic tree was prepared using Geneious Prime (2023.0.4) software, while jModeltest-2.1.10 software suggested the best fit model (GTR+I+G) for further phylogenetic analysis. The phylogenetic tree was constructed using the MrBayes plugin v3.2.6. The analysis was run for 6 million generations (sampling frequency: every 100th generation), and 25% of the initial trees were discarded as a 'burn-in' period before the construction of the consensus tree.

Fisher's exact test was used to compare the prevalences of haemosporidian and *Trypanosoma* parasites between hibernating and active mosquitoes.

3. Results

Overall, 1037 *Cx. pipiens* females were processed: 556 individuals were collected during the warm season and 481 hibernating females from wintering places (Table S1). The peak month with the highest numbers of caught *Cx. pipiens* mosquitoes was July (39.5%), while in August and in June, this amount was 31.1% and 23.9%, respectively (Figure 1). May and September were the months with the least numbers of *Cx. pipiens* caught, with the percentages being 5.0% and 0.3%, respectively. Based on the results of the restriction enzyme tests [27], we did not find *Cx. torrentium*, and all investigated mosquitoes were *Cx. pipiens*. While collecting hibernating *Culex*, we also found hibernating *Culiseta* and *Anopheles* females, but they were not investigated, as the focus of the study was *Culex* mosquitoes.

3.1. Infections with Trypanosomatida Parasites

Overall, DNA of trypanosomatids was found in 11 (1.1%) *Cx. pipiens* females: 9 infected individuals were detected in the warm period (1.6%), and 2 infected individuals were collected from overwintering sites (0.4%). The difference between the prevalence of *Trypanosoma* parasites in hibernating and active mosquitoes was statistically significant ($p = 0.005$). Trypanosomatids were identified based on the similarity of obtained sequences. *Trypanosoma culicavium* (Figure 2A) (obtained sequence shows 99.14% similarity to a sequence deposited in GenBank—OM509727) DNA was found in five mosquitoes, *Trypanosoma theileri* was detected in three individuals, *Trypanosoma trinaperronei* (Figure 2B) (99.93% similarity to a sequence from GenBank—MN752212) was found in one mosquito, and two hibernating mosquito females were infected with unidentified monoxenous trypanosomatids (Figure 2C), as determined by the phylogenetic analysis and the similarity of obtained sequences (99.51% similarity to a sequence deposited in GenBank—OP748978; Table 1).

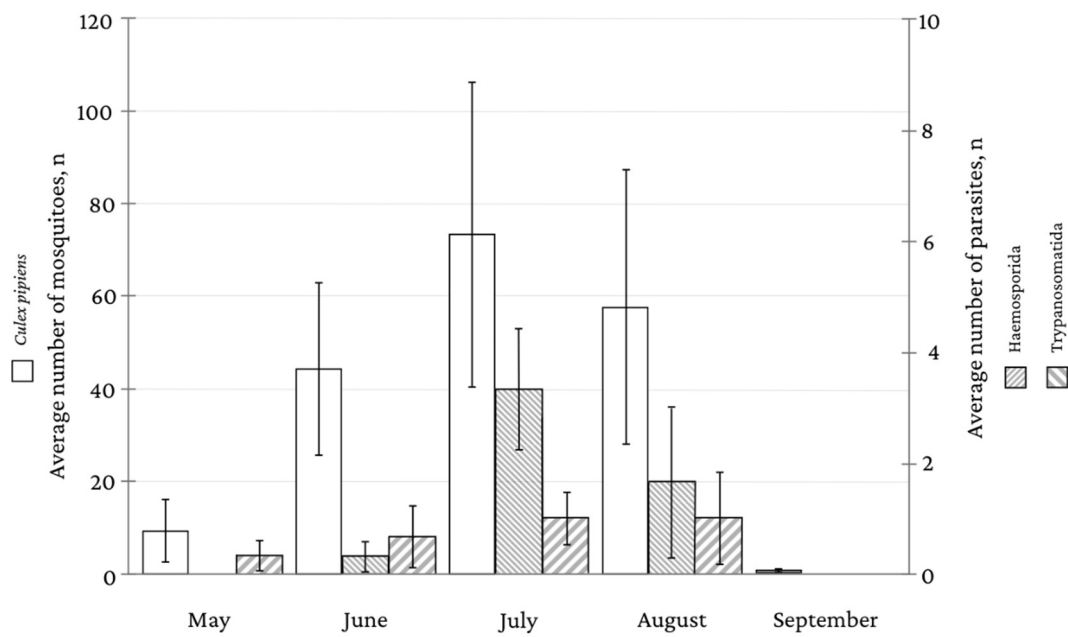


Figure 1. Average number of collected *Culex pipiens* females (left axis), with the average number of mosquitoes infected with Haemosporida and Trypanosomatida parasites (right axis) during the warm season in 2021–2023. Average $\pm$ standard error.

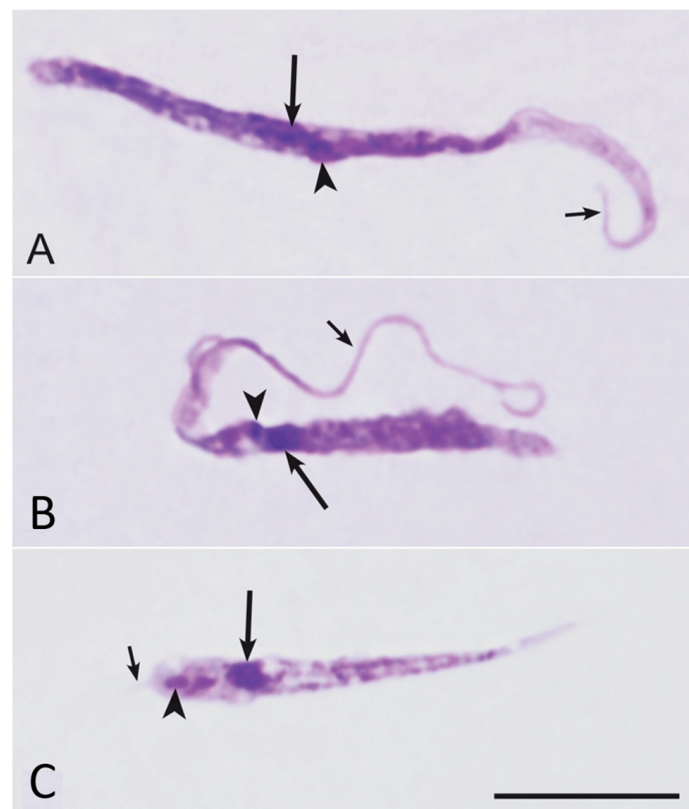


Figure 2. Trypanosomatids found in *Culex pipiens* midgut preparations: (A)—*Trypanosoma culicavium* (GenBank No. PP946100); (B)—*Trypanosoma trinaperronei* (PP946104); (C)—monoxenous trypanosomatid (PP948731). Long arrows—parasite nuclei; arrowheads—kinetoplast; short arrows—free flagellum. Scale bar = 10 μ m.

Table 1. Trypanosomatidae found in *Culex pipiens* mosquitoes, with collection date and site given.

Collection Date	Collection Site	Microscopy Positive	GenBank No	Trypanosomatid Species	Vertebrate Host
25 May 2022.	Verkiai	—	PP946099	<i>Trypanosoma culicavium</i>	Birds
29 June 2022.	Kairėnai	+	PP946101	<i>T. culicavium</i>	Birds
29 June 2022.	Kairėnai	—	PP946100	<i>T. culicavium</i>	Birds
14 July 2022.	Kairėnai	—	PP946102	<i>T. theileri</i>	Mammals
3 August 2022.	Kairėnai	+	PP946104	<i>T. trinaperronei</i>	Mammals
3 August 2022.	Kairėnai	—	PP946105	<i>T. theileri</i>	Mammals
30 August 2022.	Kairėnai	+	PP946107	<i>T. culicavium</i>	Birds
18 July 2023.	Kairėnai	+	PP946103	<i>T. culicavium</i>	Birds
18 July 2023.	Kairėnai	—	PP946106	<i>T. theileri</i>	Mammals
7 December 2023.	Verkiai ¹	+	PP948731	<i>Monoxenous trypanosomatid</i>	No vertebrate host
8 December 2023.	Vilnius	—	PP948732	<i>Monoxenous trypanosomatid</i>	No vertebrate host

¹ Cellar of water tower.

The phylogenetic analysis (Figure 3) was used to determine the phylogenetic position of unidentified trypanosomatids, and it showed that all detected trypanosomatids formed two clades: the *Trypanosoma* species clustered into clade A, and the monoxenous trypanosomatids (*Crithidia* and unidentified Trypanosomatidae) clustered into clade B. Within clade A, avian *Trypanosoma* (*T. culicavium*, *T. bennetti*, *T. everetti*, *T. thomasbancrofti*, *T. avium*) subclades stand apart from the *Trypanosoma* of mammals (*T. theileri*, *T. trinaperronei*).

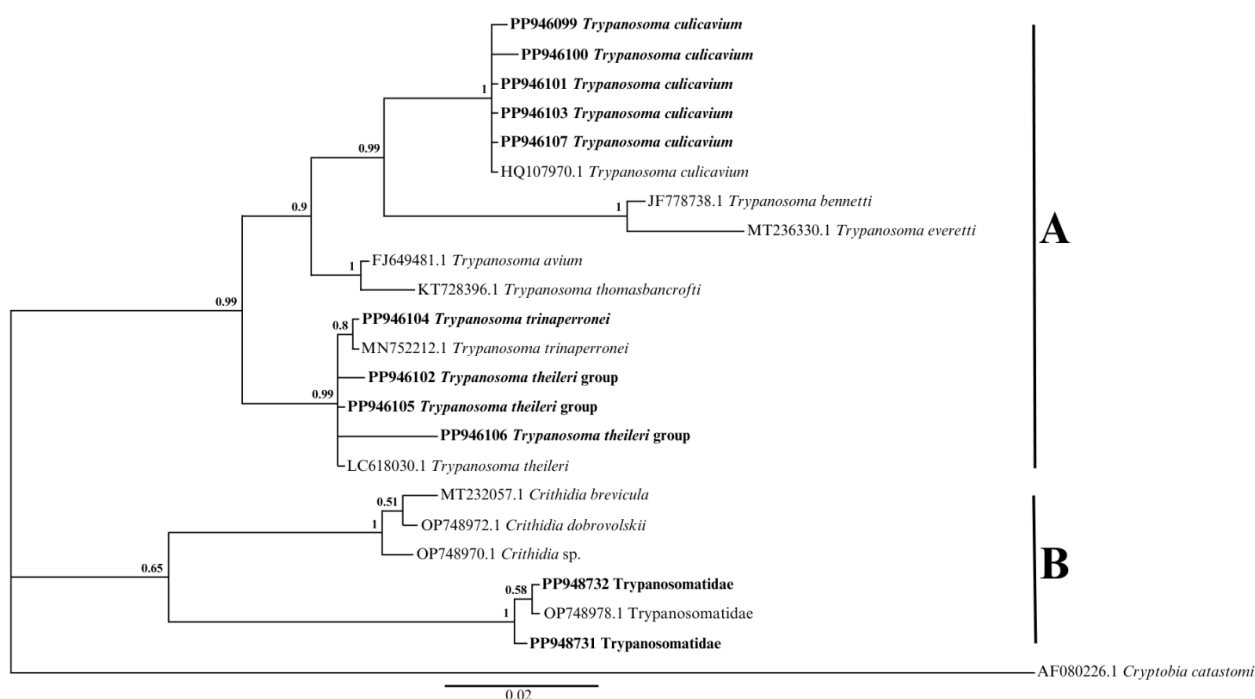


Figure 3. Bayesian phylogenetic tree of *Trypanosoma* (A) and other Trypanosomatidae (B) using fragments of 18S rRNA. The tree was rooted using *Cryptobia catostomi*. The Genbank accession numbers of samples obtained during this investigation are written in bold text.

All *Trypanosoma* species detected by PCR were obtained microscopically in the midgut preparations of investigated mosquitoes (Figure 2), except for *T. theileri*. The Trypanosomatida infection prevalence was the highest in May (3.5%), while during summer, it varied between 1.4% (July) and 1.7% (August).

3.2. Infections with Haemosporidian Parasites

No hibernating mosquitoes were detected to be infected with haemosporidian parasites. There were 16 infected individuals of *Cx. pipiens* (2.9%) collected during the warm period (Figure 1). The difference between the prevalence of haemosporidian parasites in hibernating and active mosquitoes was statistically significant ($p = 0.0002$). Three *Plasmodium* species (*Plasmodium homonucleophilum* (pSW2), *P. matutinum* (pLINN1), *P. vaughani* (pSYAT05)), two *Haemoproteus* species (*Haemoproteus brachiatus* (hLK03), *H. asymmetricus* (hTUPHI01)) and one mixed infection of *Leucocytozoon/Plasmodium* were identified in the collected mosquitoes.

The highest prevalence of haemosporidian parasites in *Cx. pipiens* mosquitoes was detected in July (4.9%) and August (2.8%).

4. Discussion

The main finding of this research was that when screening a similar number of *Cx. pipiens* mosquito females collected during the warm season and in winter for the presence of *Trypanosoma* and avian haemosporidian parasites, we found significantly different prevalences. Haemosporidian and *Trypanosoma* parasites were not detected in hibernating mosquitoes, while the prevalence of the same parasites in *Cx. pipiens* females collected during warm periods was 1.6% (nine positive mosquitoes) for *Trypanosoma* and 2.9% (16 positive mosquitoes) for haemosporidian parasites.

We detected three *Trypanosoma* species in the mosquitoes collected during the warm period (Table 1). Until now, quite a high diversity of trypanosomatids has been found in *Cx. pipiens* mosquitoes worldwide (Table 2). Two *Trypanosoma* species are known to be transmitted by these mosquitoes: *T. culicavium*, originally found and described from the gut of *Cx. pipiens* mosquitoes [5,33], and *T. thomashancofti* [4,12,33], for which *Cx. pipiens* was confirmed as a vector [4]. Other trypanosomatids have been periodically detected in individuals of this mosquito species, including *T. avium* [34–36] and *T. theileri* [37]. It was reported that among the avian trypanosomes transmitted by mosquitoes, the prevalence of *T. thomashancofti* in wild-caught mosquitoes, as well as in birds, was lower (0.13%) compared with the prevalence of *T. culicavium* (4.5%) [4]. The overall prevalence of *T. thomashancofti* in wild-caught mosquitoes in Europe is low [4,35,36], and it is not surprising that during our study, we did not detect this species in *Culex* mosquitoes.

Table 2. *Trypanosoma* spp. found in *Culex* mosquitoes in various studies. EI—experimental infections of mosquitoes; WM—wild-caught mosquitoes.

Trypanosomatid Species	Isolate	Origin of Isolate	EI	WM	Country, Source
<i>Trypanosoma thomashancofti</i>	CUL15, CUL98	<i>Culex pipiens</i>	+		Czech Republic [4,5]
	OF19	<i>Ornithomya fringilline</i> *	+		Czech Republic [4]
	PAS343	<i>Phylloscopus sibilatrix</i> *	+		Czech Republic [4]
<i>T. theileri</i>	—	<i>Cx. pipiens</i>		+	Czech Republic [4,37]
<i>T. culicavium</i>	CUL1, CUL6, CUL24, CUL28, CUL31	<i>Cx. pipiens</i>		+	Czech Republic [5]
	—	<i>Cx. pipiens</i> s.l./ <i>torrentium</i> , <i>Cx. modestus</i> , <i>Cx. spp</i>		+	Austria [35]
<i>Trypanosoma</i> sp.	CUL5, CUL2	<i>Cx. pipiens</i>		+	Czech Republic [33]
<i>T. avium</i>		<i>Cx. pipiens</i> s.l./ <i>torrentium</i>		+	Austria [35]
		<i>Cx. pipiens</i> , <i>Cx. tarsalis</i>		+	USA [38]

*—*Culex* mosquitoes were experimentally infected using these isolates; +—indication of research method (experimental infection (EI) or study of wild-caught mosquitoes (WM)); —the isolate was not determined or named.

Trypanosoma theileri was one of the first mammalian trypanosomatids described, and vectors of these parasites are known to be deer keds [39] and tabanids [40]. Information on the prevalence of this parasite in mosquitoes is scarce, with recent studies [4] indicating a prevalence of only 0.05% in *Culex*, while in *Aedes* species, it can reach nearly 22%. Our findings demonstrate a higher prevalence of *T. theileri* in *Culex* mosquitoes (0.72%), which was confirmed by PCR, although not microscopically (Table 1). Despite dissecting and preparing midgut preparations, it is possible that the preference of the *T. theileri* group trypanosomes to develop in the hindgut [37] may explain this discrepancy.

Currently, there are several species of *Megatrypanum* trypanosomes parasitizing deer: *Trypanosoma mazamarum* [41], *T. cervi* [42], *T. stefanskii* [43] and *T. trinaperronei* [39]. Together with *T. theileri*, *T. melophagium*, *T. cervi* and *T. trinaperronei* compose a *Trypanosoma theileri* complex [39,44,45]. Deer keds (*Lipoptena cervi* and *L. mazamae*) are known vectors of *T. trinaperronei* [39], and previously, this parasite has not been found in mosquitoes. We found one *Cx. pipiens* mosquito infected with *T. trinaperronei* (Table 1, Figure 2B), and the type of host of this trypanosome species is known to be *Odocoileus virginianus* (Ruminantia, Cervidae), white-tailed deer [39].

Various monoxenous trypanosomatids, such as several *Crithidia* species (*C. brevicula* [36], *C. dedva* [34], *C. fasciculata* [35] and *C. dobrovoliskii*), *Strigomonas* cf. *oncopelti*, *Paratrypanosoma* cf. *confusum* and an unknown lineage of Trypanosomatidae [22], have also been detected. Monoxenous trypanosomatids are generally considered non-pathogenic; however, there is some evidence suggesting negative effects on insect fitness, although this has only been investigated in a few insect species [46,47]. These insect parasites are not limited to blood-feeding habits and can infect hosts through numerous means, such as feeding on infected prey or ingesting contaminated substances like fresh feces (i.e., coprophagy) or contaminated substrates, such as sugar meals [46,47]. Therefore, their detection in overwintering mosquitoes does not indicate previous blood-feeding before the wintering period. However, reports on monoxenous parasites such as *Crithidia* spp. infecting humans and non-human mammalian hosts alone and in co-infections with *Leishmania* parasites are increasing in number and consistency each year [48]. Together with the reported cases of non-human trypanosome infections in humans [49], these findings underline the importance of investigating the transmission of trypanosomatids between hosts. Recently, Kostygov et al. [50] discovered an unknown lineage representing a new monoxenous trypanosomatid that has not previously been documented, detected in overwintering *Cx. torrentium* mosquitoes. Both *Cx. torrentium* and *Cx. pipiens* mosquitoes feed on birds and mammals, including humans, making them potential bridge vectors for the transmission of zoonotic pathogens from birds to humans [51]. During our study, two overwintering *Cx. pipiens* females were found to be infected with an unknown trypanosomatid showing a 98.16% and 99.51% similarity to a parasite found in *Cx. torrentium* mosquitoes [50].

There seem to be no data on the seasonality of avian trypanosomes, especially in vectors. In 1947, Vanderplank described the seasonal variation in the numbers of mammals with *Trypanosoma* infections in their blood: infections seem to be the highest during the raining periods, March–April and November–December [52]. Therefore, Pori et al. found no seasonal variations in the avian trypanosome infection prevalence from a total of 685 birds of 87 species [53].

There are at least 16 species and 50 genetic lineages of *Plasmodium* detected in *Cx. pipiens* mosquitoes, whereas sporozoites of only 10 *Plasmodium* species, as well as one genetic lineage not assigned to a species (pCXPIP09), have been found in the salivary glands of *Cx. pipiens* (Table 3). Sporozoites are the invasive stage of haemosporidian parasites, and the finding of sporozoites in the salivary glands of mosquitoes shows that the parasite has finished its development and can be transmitted to another host [54]. Solely PCR-based detection of parasite DNA shows that the mosquito had a blood meal on an infected host but does not show that the mosquito is a vector of a parasite, as parasite DNA can be detected from infected blood meal, indicating the abortive development of a parasite [54]. This is the case when *Haemoproteus* or *Leucocytozoon* parasites are detected in

Culex mosquitoes [24,54], as they were in this research. Even though mosquitoes are a dead end for parasites of other haemosporidian genera (*Haemoproteus* and *Leucocytozoon*) [15], the collection of such data provides new information on the mosquitoes' feeding preferences, as *Haemoproteus* parasites are specific to vertebrate hosts [15].

Table 3. Experimental infections of mosquitoes (EI) and molecular studies (PCR) of wild-caught mosquitoes (WM) showing what haemosporidian parasite DNA or parasites were detected in *Culex pipiens* mosquitoes. Information acquired from the MalAvi database and [3,55]. Cases with sporozoites detected in mosquito salivary glands are marked in bold and underlined.

<i>Plasmodium</i> Species	Lineages	EI	WM	PCR	Country/References
<i>P. cathemerium</i>	pPADOM02			+	Japan [56–63], Switzerland [64,65], USA [66]
	unknown lineage				USA [67,68]
<i>P. circumflexum</i>	pTURDUS1			+	Switzerland [64]
<i>P. durae</i>	unknown lineage				Africa [15]
<i>P. elongatum</i>	pGRW06			+	France [69] Austria [34,70]
	unknown lineage	+	+		USA [67], Italy [71,72]
<i>P. gallinaceum</i>	pGALLUS01			+	Japan [57,58,63]
<i>P. garnhami</i>	unknown lineage	+			Egypt [73]
<i>P. giovannolai</i>	unknown lineage	+			Italy [74,75]
<i>P. homonucleophilum</i>	pSW2			+	Lithuania [24], Switzerland [64]
<i>P. juxtannucleare</i>	pGALLUS02			+	Japan [63]
	unknown lineage	+			Japan [76]
<i>P. kemp</i>	unknown lineage	+			USA [77]
<i>P. lophurae</i>	unknown lineage				USA [78]
<i>P. matutinum</i>	pLINN1			+	France [69], USA [66], Italy [79], Spain [80], Austria [34]
			+	+	Lithuania [24]
	unknown lineage				USA [81–83]
<i>P. relictum</i>	pGRW04			+	Japan [62,63,84] , Madagascar [85]
	pGRW11			+	Italy [79], Japan [58,60,84], Switzerland [64,65], France [69]
		+			Lithuania[86]
	pSGS1			+	Romania [87], Japan [58,60–62,84], Austria [34,70], France [69], Portugal [88], Italy [79], Switzerland [64,65], Spain [80], Turkey [89]
		+			Lithuania [86]
<i>P. rouxi</i>	unknown lineage			+	Romania [87]
		+			Germany [90,91], Algeria [92], USA [93], Columbia [94,95]
<i>Plasmodium</i> sp.	unknown lineage		+		USA [81,83]
	pAFTRU5			+	Switzerland [65], Italy [79]
	pCOLL1			+	France [69], Switzerland [65], Spain [80]
	pCXPIP01, CXPIP02, pCXPIP03, pCXPIP04, pCXPIP05, pCXPIP06, pCXPIP07			+	USA [66]

Table 3. Cont.

<i>Plasmodium</i> Species	Lineages	EI	WM	PCR	Country/References
<i>Plasmodium</i> sp.	pCXPIP09			+	Japan [56–63,84]
	pCXPIP10, pCXPIP11, pCXPIP12, pCXPIP13, pCXPIP14			+	Japan [58,60,63]
	pCXPIP15			+	Japan [61]
	pCXPIP20, pCXPIP21, pCXPIP22, pCXPIP23			+	Turkey [89]
	pCXPIP24, pCXPIP25, pCXPIP26			+	France [69]
	pCXPIP30			+	Madagascar [85]
	pCXPIP31			+	Japan [96]
	pCXPIP32, pCXPIP33				Italy (Iurescia et al., unpubl)
	pCXQUI01			+	Japan [63]
	pDELURB4			+	Italy [79], France [69], Austria [34]
	pDELURB5, pDONANA03, pDONANA05			+	Austria [34], France [69]
	pPADOM01			+	France [69], Switzerland [65]
	pSPHUM05			+	Japan [59]
	pSPMAG10, pZOCAP03, pSYCON02			+	Japan [62]
<i>P. subpraecox</i>	unknown lineage	+			Italy [97]
<i>P. unalis</i>	pTUMIG03			+	USA [66]
<i>P. vaughani</i>	pSYAT05			+	France [69], USA [66], Lithuania [24], Italy [79], Japan [60], Switzerland [64,65], Turkey [89], Austria [34,70], Czech Republic [98]
	unknown lineage	+			Italy [99]

+—indication of research method (experimental infection (EI) or study of wild-caught mosquitoes (WM), data obtained using PCR methods (PCR)).

There are almost no data on the detection of haemosporidian parasites in hibernating mosquitoes. *Plasmodium matutum* (pLINN1) was found in two pools of mosquitoes collected in their hibernation sites in November using PCR [100]. November is just the beginning of the hibernation period for *Culex* mosquitoes in the temperate zone of the northern hemisphere, and mosquitoes harboring parasites would need to survive at least until April to be able to transmit the parasite to an avian host. We collected hibernating mosquitoes in December and January and collected them in similar parts (south and east) of the country as the active mosquitoes were collected in, except for one locality in western Lithuania. Avian haemosporidian parasites are investigated in the western part of the country, showing high prevalences in different bird species [15,26,46], so we expected to find at least some infected hibernating mosquitoes there. However, we did not find haemosporidian and *Trypanosoma* parasites in the hibernating mosquitoes. According

to different authors, the prevalence of haemosporidian parasites in active *Cx. pipiens* mosquitoes varies between 0.04% and 6.6% in different studies (0.04%—[88]; 0.52%—[56]; 0.61%—[98]; 3.08%—[60]; 6.6%—[64]), and a 2.9% prevalence was detected in *Cx. pipiens* active mosquitoes in Lithuania, which falls in the middle [24]. Although all sites of this investigation were similar habitats, the prevalence of haemosporidian parasites differed between study sites and was high in Verkiai (7%) during the warm period. Both active and hibernating mosquitoes were tested in this location, with no infected hibernating mosquitoes being found. The relationship between the study area and parasite prevalence in mosquitoes should be the focus of further research.

Culex pipiens mosquitoes are considered to be mainly ornithophilic [79,101]. The presence of avian haemosporidian DNA in the tested *Cx. pipiens* females show that these mosquitoes had a blood meal on birds, as this is the only way for mosquitoes to be infected with these parasites [54]. *Trypanosoma culicavium* is an avian trypanosomatid [5], further proving their ornithophilic tendencies. On the other hand, *T. theileri* [37] and *T. trinaperronei* [39] can only develop in mammals, indicating the mammalophilic or opportunistic behavior of *Cx. pipiens* mosquitoes. Such blood-sucking insects can serve as bridge vectors between different groups of animals, like *Cx. pipiens* being a bridge vector for WNV from birds to humans [102].

In summary, our investigation did not detect avian haemosporidian or avian *Trypanosoma* parasites in hibernating *Cx. pipiens* mosquitoes. In contrast, during the warm season, the prevalence of these parasites was 2.9% for haemosporidian parasites [20] and 1.6% for avian trypanosomatid parasites. Only monoxenous trypanosomatids were detected in overwintering *Cx. pipiens* mosquitoes. Studies conducted with a larger number of mosquitoes over several years may help to better assess the role of hibernating mosquitoes in avian blood parasite transmission. Given the potential for these parasites to survive in wintering mosquitoes, experimental studies are needed to determine whether the parasites fail to survive in mosquitoes or whether mosquitoes infected with parasites do not survive until spring.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microbiolres15040146/s1>, Table S1: Investigated *Culex pipiens* mosquitoes with collecting time and location.

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