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Trypanosomes transmitted by mosquitoes: Occurrence in hosts, transmission, and specificity

Trypanosomy přenášené komáry: výskyt v hostitelích, přenos a specifita

Ph.D. THESIS / DIZERTAČNÍ PRÁCE

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Abstrakt

Trypanosomy (*Trypanosoma*, Kinetoplastea) jsou dixenní krevní prvoci, kteří střídají v životním cyklu obratlovce a krev sající bezobratlé. Infekce obratlovců mohou probíhat bez zřejmých zdravotních komplikací, ale mohou způsobovat i závažná onemocnění ohrožující jak lidi, tak zvířata. Pozornost výzkumu se proto většinou obrací právě směrem k druhům trypanosom způsobujících lidská onemocnění jako Chagasova choroba a spavá nemoc, či nagana a surra u dobytka, a také k jejich přenašečům, kterými jsou mouchy tse tse a ploštice. Méně známý je fakt, že trypanosomy jsou přenášeny i komáry, konkrétně trypanosomy ptačí a s velkou pravděpodobností i savčí ze skupiny *T. theileri*. Nicméně role komárů v životním cyklu trypanosom je jen nedostatečně prozkoumána, a proto se tato disertace věnuje tomuto tématu.

V rámci experimentální práce jsme prokázali, že komáři rodu *Culex* jsou vnímavými hostiteli a pravděpodobnými přenašeči dvou druhů ptačích trypanosom: *T. thomasbancrofti* a *T. tertium* n. sp. Naopak pro *T. theileri* nejsou komáři rodu *Culex* vhodnými hostiteli, těmi se ukázali být komáři rodu *Aedes* a překvapivě i flebotomové rodu *Phlebotomus.* Všechny tři zkoumané trypanosomy se vyvíjely v zadní části střeva komárů a byly nalézány i v jejich prediuretické tekutině. Tato lokalizace trypanosom napovídá, že kromě přenosu pozřením infekčního vektora je možný i přenos skrze oční spojivku.

V terénních odchytech jsme se zaměřili na prevalenci těchto tří trypanosom mezi volně žijícími živočichy. Ptačí trypanosomy byly nalézány výhradně v ornitofilních komárech rodu *Culex,* ale s poměrně nízkou prevalencí, pohybující se jen okolo 0,1 %. Jejich prevalence je nízká i mezi hmyzožravými ptáky, kdy dosahovala u *T. thomasbancrofti* 3 % a u *T. tertium* 1,5 %. Naopak savčí *T. theileri* byla nalézána převážně u mamalofilních komárů rodu *Aedes* s prevalencí převyšující 20 % a v několika případech byla determinována i mezi komáry rodu *Culex.* Mimo komárů byla přítomnost savčích trypanosom potvrzena i u 44 % odchycených ovádů a poprvé demonstrována u muchniček. Fylogenetická studie potvrdila, že získané izoláty *T. theileri* spadají do dvou dříve popsaných skupin, a navíc jsme potvrdili i existenci třetí linie ve skupině *T. theileri*.

Abstract

Trypanosomes (*Trypanosoma*, Kinetoplastea) are dixenous blood protists that require not only a vertebrate host but also a blood-feeding invertebrate to complete their life cycle. Infection of vertebrates can be asymptomatic, but on the other hand can cause serious diseases affecting lives of humans and animals. Thus, researchers usually focus on *Trypanosoma* species causing Chagas disease and sleeping sickness in humans or nagana and surra in animals, and on their vectors: tsetse flies and kissing bugs. However, mosquitoes are able to transmit trypanosomes as well, specifically, avian trypanosomes and probably mammalian trypanosomes from the *T. theileri* group. Nevertheless, the role of mosquitoes in the life cycle of trypanosomes has substantial gaps, which are focused in this dissertation.

Within the experimental work, it has been demonstrated that mosquitoes of the genus *Culex* are susceptible hosts of two species of avian trypanosomes: *T. thomasbancrofti* and *T. tertium* n. sp. On the other hand, *Culex* mosquitoes were unsuitable hosts for *T. theileri*, while the genus *Aedes* and surprisingly even sand flies (*Phlebotomus perniciosus*) turned up to be competent vectors. All investigated trypanosomes were able to develop within the guts of mosquitoes and were also found in their prediuretic liquid. This localization of trypanosomes suggests that the transmission occurs by ingestion of infected vector and through the conjunctiva.

During field studies, we focused on determining the prevalence of these trypanosomes among free living animals. Avian trypanosomes were strictly found in ornithophilic mosquitoes of the genus *Culex*, but with a relatively low prevalence around 0.1%. Their prevalence was also lower among insectivorous birds, reaching 3% for *T. thomasbancrofti* and 1.5% for *T. tertium*. On the contrary, *T. theileri* trypanosomes were detected mainly in mammalophilic mosquitoes of the genus *Aedes* with a prevalence exceeding 20%, however in some cases was *T. theileri* detected even among *Culex* mosquitoes. Apart from mosquitoes, the presence of mammalian trypanosomes was confirmed in 44% of captured tabanid flies and demonstrated in black flies for the first time. Additionally, phylogenetic studies confirmed that obtained *T. theileri* isolates belong to two previously described lineages, and moreover, the existence of third lineage was confirmed.

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1 Introduction

Trypanosomes (Euglenozoa; Kinetoplastea; Trypanosomatida) (Adl et al., 2019) are blood parasites generally known for serious diseases they are causing in humans (Chagas disease, sleeping sickness) and animals (nagana, surra, dourine). Trypanosomes are dixenous parasites, requiring both vertebrate hosts and blood-sucking invertebrates to complete their life cycle. While vectors play a crucial role in sustaining trypanosomes in populations, research is focused primarily on vectors like tsetse flies or kissing bugs that transmit trypanosomes dangerous to humans and livestock. However, trypanosomes can be also transmitted by mosquitoes, which are connected with pathogens causing devastating diseases like malaria or dengue fewer.

Avian trypanosomes are common parasites of birds but, due to their low pathogenicity, usually neglected in studies. Although the life cycles of many avian trypanosome species are still unexplored, it is known that their vectors can be found among various species of blood-sucking insects. Mosquitoes were speculated as vectors of avian trypanosomes at the beginning of 20th century (Schaudinn, 1904); however, experimental confirmation was achieved more than 100 years later (Votýpka et al., 2012). Based on molecular data, it is evident that there are at least three species of avian trypanosomes transmitted by mosquitoes: *Trypanosoma culicavium*, *Trypanosoma thomasbancrofti* and *Trypanosoma* sp. lineage III (Zídková et al., 2012; Votýpka et al., 2012; Šlapeta et al., 2016).

Besides avian trypanosomes, there are indications that mosquitoes might play some role in the life cycle of mammalian trypanosomes, namely, the *Trypanosoma theileri* group. These usually non-pathogenic trypanosomes are transmitted between domestic cattle and wild ungulates by tabanid flies, and by keds between sheeps and goats (Hoare, 1972; Böse et al., 1987). Mosquitoes started to be considered as vectors only recently, following the discovery of high prevalence of *T. theileri* in naturally infected mosquitoes (Schoener et al., 2018), which was in congruence with our own field data obtained in parallel.

My study is thus focused on the neglected role of mosquitoes as vectors of avian as well as mammalian trypanosomes.

1.1 Avian trypanosomes

Avian trypanosomes are successful blood parasites occurring worldwide. These digenetic protists are well adapted to transmission in ecosystems across the world, from tropical continents to those with cold climates, everywhere where avian hosts and blood-sucking insects are present. Despite their wide distribution, avian trypanosomes are generally neglected due to their low pathogenicity. Only a few studies focussed on the negative health impacts of *Trypanosoma* infected birds (Baker, 1956; Molyneux, 1983; Cigler et al., 2023). Some effects of infection are indirect, like feather lightening in infected birds, having a potential negative impact on sexual selection (Scheuerlein and Rickles, 2004; Lumpkin et al., 2014).

Avian trypanosomes, their biodiversity and development in avian hosts are not completely understood. Following the infection of the bird, trypanosomes appear in peripheral blood, but their prepatent period significantly varies. They can be detected almost immediately (Baker, 1956; Svobodová and Rádrová, 2018), or after several weeks post-infection (Svobodová et al., 2017; Svobodová and Rádrová, 2018). Trypanosomes appear in the peripheral blood in the form of trypomastigotes, which are infective to vectors. Subsequently, trypanosomes retreat from peripheral blood to the bone marrow (Baker 1956; Kučera 1983). Infections are long-term and chronic, with occasional relapses into peripheral blood (Svobodová and Votýpka, 2004; Svobodová et al., 2017; Svobodová and Rádrová, 2018; Svobodová et al., 2023).

The first avian trypanosome was described over 130 years ago (Danilewsky, 1889) and named *Trypanosoma avium*. Since then, more than 100 species of avian trypanosomes have been described, primarily based on morphological differences or the occurrence in a new avian host. However, descriptions of trypanosomes based on presumed vertebrate-host specificity might not be valid, as trypanosome lineages are not strictly host-specific and can infect multiple avian orders (Sehgal et al., 2001; Votýpka and Svobodová, 2004; Zídková et al., 2012; Svobodová et al., 2023). Today, avian trypanosomes are divided into three groups comprising at least 16 lineages (Zídková et al., 2012; Santolíková et al., 2022). The group C is represented by *T. avium/T. thomasbancrofti* which includes isolates from passerines, raptors, black flies, mosquitoes, and louse flies. Group B includes two related species, *T. culicavium* and *T. corvi*, and includes isolates from passerines, mosquitoes, and louse flies. Group A, which includes *T. bennetti* and *T. everetti*, was known to include only isolates from

free-living birds while the vector remained unknown (Zídková et al., 2012). Recently, the role of biting midges in transmission of *T. bennetti/T. everetti* was confirmed, and naturally infected biting midges were found (Svobodová et al., 2017; Bernotienė et al., 2020). Lately, Kostygov et al. (2021) divided avian trypanosomes in to three subgenera defined by common morphological characteristics and 18S rRNA phylogenetic analyses: *Trypanomorpha* (*T. avium, T. gallinarum, T. thomasbancrofti*), *Avitrypanum* (*T. corvi, T. culicavium*), *Ornithotrypanum* (*T. anguiformis, T. bennetti, T. everetti*). In this thesis, we use the original groups of avian trypanosomes (Zídková et al., 2012) in the purpose of consistency, since it is used in the published articles.

The prevalence of trypanosomes in various avian orders ranges on a scale from 3 to 70% (Rintamaki, 1999; Sehgal et al., 2005; Oakgarove et al., 2014; Cooper et al., 2017; Pornpanom et al., 2019; Svobodová et al., 2023). These differences can be affected by factors as life history traits. Since one of the ways of avian trypanosomes transmission is through the ingestion of an infected vector, their prevalence is affected by the diet of birds (Votýpka et al., 2012). However, trypanosomes infect also raptors that do not feed on insects. In this case, the height of the nest comes into play, as vectors differ in height preferences (Černý et al., 2011). Black flies are dominant vectors reported from canopy, where many bird species build their nests and are exposed to vectors of *T. avium* s. s. which is transconjunctivally transmitted by black flies (Votýpka and Svobodová, 2004; Černý et al., 2011).

Moreover, published differences in prevalence can be caused by the use of different detection methods, as microscopic examination of blood has low sensitivity due to low parasitaemias. Therefore, molecular techniques or blood cultivation are generally considered more reliable (Kučera, 1982; Kirkpatrick and Sauthers, 1988; Sehgal et al., 2001; Valkiunas et al., 2011; Svobodová et al., 2023).

1.2 Vectors and transmission of avian trypanosomes

Trypanosomes are digenetic parasites, requiring avian hosts and blood-sucking arthropods to complete their life cycle. Known vectors of avian trypanosomes include black flies (Simuliidae, Bennett, 1961, Votýpka et al., 2002; Votýpka and Svobodová, 2004), hippoboscid flies (Hippoboscidae, Baker 1956; Votýpka et al., 2004; Santolíková et al., 2022), mosquitoes (Culicidae, Baker, 1976; Votýpka et al., 2012; Fialová et al., 2021), biting midges (Ceratopogonidae, Miltgen and Landau, 1982; Svobodová et al., 2017; Bernotienė et al., 2020), and sand flies (Psychodidae, Kato et al. 2011; Svobodová and Rádrová, 2018).

Trypanosomes are found in various parts within the gut of infected insects, which provides insights into their mode of transmission. Most commonly, avian trypanosomes colonize the hindgut and/or midgut, indicating that these species cannot be transmitted by inoculation during blood feeding. Among avian trypanosomes, *T. culicavium* is a species with a unique localization on the stomodeal valve, similar to the localization of leishmania in phlebotomine sandflies. It was assumed that *T. culicavium* shares a similar mode of transmission as *Leishmania*, involving destruction of the stomodeal valve and regurgitation of parasites during feeding (Volf et al., 2004). However, this type of transmission has not been confirmed (Votýpka et al., 2012). Thus, all avian trypanosomes with experimentally described life cycle are transmitted by ingestion of an infected vector (Bennett, 1961; Votýpka and Svobodová, 2004; Votýpka et al., 2012; Svobodová and Rádrová, 2018). Moreover, while taking a blood meal, the insect can excrete a prediuretic liquid containing metacyclic trypanosomes originating from the hindgut. These stages can infect the avian host through the conjunctiva since the area around eyes is frequently used for insect feeding (Votýpka and Svobodová, 2004; Fialová et al., 2021). Transconjunctival mode of transmission favours these trypanosome species since they do not rely solely on being eaten by a bird, but can infect birds that do not primarily feed on insects.

The specificity of avian trypanosomes to vectors varies. For example, *T. avium* was isolated from blackflies and their vectorial role was experimentally confirmed soon after (Votýpka et al., 2002; Votýpka and Svobodová, 2004). However, *T. avium* can also develop infection in biting midges (Svobodová et al., 2017; Bernotienė et al., 2020) and sandflies (Kato et al., 2011; Svobodová and Rádrová, 2018). On the other hand, *T. culicavium* is much more specific. This species was able to develop heavy infections only in *Culex* mosquitoes, but not in *Aedes* mosquitoes or *Culicoides* biting midges (Votýpka et al., 2012; Svobodová et al., 2017). *T. corvi,* a sister lineage to *T. culicavium* in group B, was found in hipposboscids only. The vectorial specificity of group A is also high because it fails to develop both in sandflies and in mosquitoes as well, and infects only biting midges (Svobodová et al., 2017; Svobodová and Rádrová, 2018).

Despite the undisputed role of vectors in the life cycles of trypanosomes, there is limited knowledge about the prevalence of trypanosomes among wild blood-sucking insects. Prevalence of avian trypanosomes ranging from 4% to 8% was reported in bloodsucking Diptera belonging to several families (Svobodová et al., 2015); prevalences do not exceed

11% (Kazak et al., 2023; Votýpka et al., 2012; Schoener et al., 2018). Additionally, parasites are frequently detected from pooled samples, so that some individuals can contain undigested bloodmeal. This can lead to potential misidentification of insect as vectors (Reeves et al., 2007) as insects can only be considered as vectors when parasites are still present in the gut after blood digestion and defecation.

1.3 Mosquitoes as vectors of avian trypanosomes

Mosquitoes (Diptera: Culicidae) are in focus as vectors of pathogens causing infections: viruses (dengue fever, yellow fever, West Nile virus), protists (malaria), and helminths (lymphatic filariasis). Mosquitoes are notoriously associated with parasites of the genus *Plasmodium.* Moreover, avian *P. relictum* played a pivotal role in the discovery made by Ronald Ross in 1897, confirming mosquitoes as vectors (Cox, 2010). However, the role of mosquitoes in relation to trypanosomes is still insufficiently explored. In the middle of 19th century, mosquitoes were identified as vectors of the anuran *T. rotatorium* (Mayer, 1843 in Desser et al., 1973). The role of mosquitoes as vectors of avian trypanosomes was experimentally confirmed much later (Votýpka et al., 2012). In addition to dixenous trypanosomes, mosquitoes also harbour monoxenous kinetoplastids (Wallace, 1943; Flegontov et al., 2013, Kostygov et al., 2021).

The first mention of the development of avian trypanosomes in mosquitoes is dated back to the early 20th century. Schaudinn (1904) described the division of trypanosomes in the midgut and hindgut of *Culex* mosquitoes. He also suggested transovarial transmission of trypanosomes after their finding in the ovaries. However, trypanosomes were probably mistaken with sperm, as these observations could not be confirmed (Bennett, 1970; Baker, 1976; Votýpka et al., 2012). The development of trypanosomes in *Aedes* mosquitoes was observed by Bennett (1961, 1970). He described massive multiplication of trypanosomes in the midgut when the peritrophic matrix was present. After the rupture of the matrix and blood digestion, trypanosomes were found only in the hindgut. Chatterjee (1977) described a similar development of infection in mosquitoes. Both authors concluded that avian trypanosomes are transmitted into the avian host by ingestion of the infected mosquito (Bennett, 1961; Chatterjee, 1977). Bennett also observed trypanosomes in the faeces of mosquitoes, suggesting transmission through damaged skin. He even tested the possible regurgitation of trypanosomes during blood feeding, but as their localization was in the hindgut, it is not surprising that regurgitation of trypanosomes did not occur (Bennett,

1961). Avian trypanosomes are only rarely described in the salivary glands of mosquitoes, and there is no evidence for successful transmission during blood feeding (David and Nair, 1955; Schoener et al., 2019). These historical discoveries support today's opinion that avian trypanosomes are naturally transmitted through the ingestion of an infected vector or via the conjunctiva.

Based on molecular data, it is currently assumed that there are at least three species of avian trypanosomes transmitted by mosquitoes: *T. culicavium*, *T. thomasbancrofti* and *Trypanosoma* sp. lineage III (Zídková et al., 2012; Votýpka et al., 2012; Šlapeta et al., 2016).

1.3.1 *Trypanosoma culicavium*

T. culicavium is closely related to *T. corvi* transmitted by hippoboscid flies, and both lineages belongs to group B in the phylogenetic tree of avian trypanosomes (Zídková et al., 2012). It is also the first mosquito species which life cycle has been experimentally proved. *T. culicavium* was able to develop heavy infections in *C. p. quinquefasciatus* mosquitoes with the prevalence reaching 85%. On the contrary, *Ae. aegypti* showed very low susceptibility to infection (Votýpka et al., 2012). The localisation of *T. culicavium* in the gut is on the stomodeal valve, but even so, there was no successful transmission into the bird host during the blood feeding of the infected mosquito. The lack of trypanosome regurgitation was confirmed by the absence of trypanosomes in the blood after membrane feeding and using forced feeding. Transmission did occur perorally after ingestion of the infected vector (Votýpka et al., 2012). Transmission through conjunctiva was not tested but given the localization in the anterior part of the gut, excretion of metacyclic stages into the prediuretic liquid is unlikely.

In nature, *T. culicavium* has been isolated from two subspecies of *Culex* mosquitoes – *Culex* (*Culex*) *pipiens* and *Culex* (*Barradius*) *modestus*. In mosquitoes screened by PCR the prevalence was only 0.3% and 0.05% in *Cx. pipiens* and *Cx. modestus*, resp. (Votýpka et al., 2012). However, in a different study, where mosquitoes were dissected, the prevalence was 5.4% in *Cx. pipiens* and 1.4% in *Cx. modestus* (Svobodová et al., 2015). Given its mode of transmission, it is not surprising that *T. culicavium* is mainly found among insectivorous birds, such as the collared flycatcher (*Ficedula albicollis*, Zídková et al., 2012; Votýpka et al., 2012).

1.3.2 *Trypanosoma thomasbancrofti*

T. thomasbancrofti was described based on its discovery in the blood of Australian regent honeyeaters (*Anthochaera phrygia*, Šlapeta et al., 2016). However, the newly described species has been found previously in the mosquito *Cx. pipiens* and the chiffchaff (*Phylloscopus collybita*) in Czechia (Zídková et al., 2012). Due to almost 100% similarity of Czech and Australian isolates, *T. thomasbancrofti* can be considered intercontinental. Mosquitoes were suggested as vectors of *T. thomasbancrofti,* based on naturally infected mosquitoes; however, experimental confirmation was lacking (Šlapeta et al., 2016).

It is interesting to note that *T. thomasbancrofti* has been also isolated from the hippoboscid fly *Ornithomya fringillina* by our team in 2016 (Santolíková et al., 2022). Hippoboscid flies are notoriously associated with transmission of avian *T. corvi* (Baker, 1956; Votýpka et al., 2004). However, their role in the life cycle of *T. thomasbancrofti* is a question, due to challenges associated with the laboratory handling of hippoboscid flies. Thus experiments testing the development of infection are difficult to maintain. Moreover, the association of avian hippoboscid flies with the avian host and their permanent presence of blood in guts potentially aids the survival of nonspecific parasites, which can be later isolated from them.

1.3.3 *Trypanosoma* sp. lineage III

Trypanosoma sp. (lineage III) is formed by isolates from the mosquito *Cx. pipiens*, the collared flycatcher (*Ficedula albicolis*), the chiffchaff (*Phylloscopus collybita*), the Eurasian nuthatch (*Sitta europaea*) and, as well as sister lineage *T. thomasbancrofti*, belongs to the group C (*T. avium* s. l., Zídková et al., 2012). Considering the isolation of *Trypanosoma* sp. from wild *Cx. pipiens* mosquitoes and insectivorous bird species, it is probable that mosquitoes serve as vectors for this lineage, transmitting trypanosomes through ingestion of infected vectors as has been described for other avian trypanosomes (Bennett, 1961; Votýpka and Svobodová, 2004; Votýpka et al., 2012; Svobodová and Rádrová, 2018). However, there is a lack of experimental confirmation regarding the vector role of mosquitoes in this trypanosome life cycle.

The prevalence of this lineage among wild living mosquitoes is low as it was identified in only 2 out of 898 *Cx. pipiens* (Svobodová et al., 2015). Furthermore, similarly to *T. thomasbancrfti*, it can be considered intercontinental, given its presence in the blood of Australian birds (Cooper et al., 2017).

1.4 Mammalian trypanosomes of *T. theileri* group

Trypanosomes of the *T. theileri* group are common blood parasites in ungulates, belonging to the subgenus *Megatrypanum*, which was recently indicated as a sister group to avian trypanosomes (Galen et al., 2020). Isolates of *T. theileri* were obtained from cervids, bovids, and insects all over the world (Rodrigues et al., 2006; Garcia et al., 2011; Yokoyama et al., 2015; Pacheco et al., 2015; Brotánková et al., 2022). In comparison to notoriously known mammalian trypanosomes such as *T. cruzi, T. brucei, T. evansi* and *T. equiperdum*, *T. theileri* is usually not pathogenic, therefore, neglected in studies.

Trypanosomes belonging to *T. theileri* group have been described under various names since the early 20th century (Hoare, 1972). Similar to avian trypanosomes, trypanosomes from *T. theileri* group have faced numerous misidentifications over the years based on findings in new hosts or on seemingly different morphologies. According to phylogenetic analyses, the *T. theileri* group consist of several characterized species (*T. theileri*, *T. melophagium*, *T. cervi*) and various genotypes which form two main lineages, TthI and TthII. However, the relationship between these genotypes is not clearly resolved, as they are based on various genes (Rodrigues et al., 2003; Rodriguez et al., 2006; Rodriguez et al., 2010; Pacheco et al., 2015; Yokoyama et al., 2015). Moreover, Garcia et al. (2020) obtained 2 isolates from elk which significantly diverged from TthI and TthII, and suggested the existence of new lineage inside *T. theileri* group. The study also described a new species, *T. trinaperronei* (Garcia et al., 2020).

The life cycle of *T. theileri* is not completely understood. Following infection, trypomastigotes and dividing epimastigotes have been observed in the peripheral blood (Hoare 1972; Friedhoff et al., 1984). The prepatent period varied depending on the infectious dose, ranging from four to twenty days, with higher parasitaemia at the beginning of infection. The chronic phase of infection is characterized by the presence of trypomastigotes in the peripheral blood, which are stages infective to vectors (Hoare, 1972). The level of parasitaemia is also affected by seasonal prevalences of vectors (Jaimes-Dueñez et al., 2017; Suganuma et al., 2022; Hong et al., 2023). In addition to blood, *T. theileri* trypanosomes have been detected in bone marrow, wide variety of organs, and even in peritoneal fluid (Braun et al., 2002; Sood et al., 2011; Amato et al., 2019; Sharma et al., 2021). *T. theileri* infections are mostly asymptomatic without significant threats to the infected individuals. However, clinical symptoms have been described in some cases,

usually as a result of coinfections or stress (Doherty et al., 1993; Villa et al. 2008, Braun et al., 2002; Sood et al., 2011; Bittner et al., 2021; Sharma et al., 2022; Suganuma et al., 2022).

Similarly to avian trypanosomes, the reported prevalence of *T. theileri* depends on the chosen diagnostic method. The prevalence in peripheral blood is low, so blood smears are the least reliable method for detection. For accurate detection, and to avoid false-negative results, it is advisable to use blood cultivation or PCR diagnostics (Rodrigues et al., 2003; Rodrigues et al., 2011; Garcia et al., 2011; Suganuma et al., 2019; Garcia et al., 2020). In some cases, indirect detection from engorged insects can be used, such as detection of ovine trypanosomes in hippoboscid flies *Melophagus ovinus* gathered from sheep (Gibson et al., 2010; Martinković et al., 2012).

Specificity within the *T. theileri* group varies, with some genotypes being host-specific, such as those found in sheep or water buffaloes (Rodrigues et al., 2003; Rodriguez et al., 2006; Gibson et al., 2010; Martinković et al., 2012), in contrast with unspecific genotypes isolated from cattle and deer (Rodrigues et al., 2003; Rodriguez et al., 2006; Garcia et al., 2011; Fisher et al., 2013).

1.5 Vectors and transmission of *T. theileri* trypanosomes

T. theileri trypanosomes have been detected in various blood-sucking dipterans, including tabanids (Hoare, 1972; Böse et al., 1987; Ganyukova et al., 2018), deer and ovine keds (Hoare, 1972; Böse and Petersen, 1991; Garcia et al., 2020; Werszko et al., 2020), mosquitoes (Shoener et al., 2018), sand flies (Calzolari et al., 2018), tse-tse flies (Votýpka et al., 2015; Ngomtcho et al., 2017) and even in several tick species (Krinsky et al., 1976; Shastri and Deshpande, 1981; Latif et al., 2004; Martins et al., 2008). Nevertheless, the significance of these discoveries remains uncertain as the life cycle was not verified, and metacyclic stages were exclusively documented in tabanids and sheep keds (Hoare, 1972; Molyneux, 1975; Molyneux et al., 1978; Böse et al., 1987; Böse and Heister 1991)

As ungulates are the hosts of *T. theileri* trypanosomes, conducting experimental transmission is logistically challenging. Some studies attempted to infect laboratory animals (mice, rats, guinea pigs), but none of them succeeded in establishing infection (Hoare, 1972; Dire et al., 1990).

Trypanosomes of *T. theileri* group develop in the gut of infected vectors with final localization in hindgut. As a result of this localization, *T. theileri* trypanosomes occur in vector faeces. Besides transmission by vector ingestion, transmission can thus also occur through skin abrasions (Hoare, 1972; Bose et al., 1987). Moreover, transplacental transmission has been described in cattle and deer (Kingston et al., 1981; Lanevschi-Pietersma et al., 2004). Another potential mode of transmission could be via conjunctiva by infective stages following prediuresis, similarly to avian trypanosomes (Votýpka and Svobodová, 2004). Prediuresis was described in tsetse flies, mosquitoes and sand flies, all of which are potential vectors of *T. theileri* (Gee, 1975; Jones and Brandt, 1981; Nijhout and Grant, 1987; Sádlová et al., 1998; Votýpka et al., 2015; Ngomtcho et al., 2017; Shoener et al., 2018). However this hypothesis needs confirmation.

Tabanids are among the experimentally confirmed vectors. Böse et al. (1987) were able to orally infect cattle and deer with positive tabanid guts. Trypanosomes begin to develop in the midgut, subsequently moving to the hindgut, where the infection persists even after blood digestion. Metacyclic stages are also excreted with the faeces (Böse and Heister 1993). It has been molecularly confirmed that tabanid flies can host various genotypes of *T. theileri* as well as *T. cervi* (Ganyukova et al., 2018; Werszko et al., 2019; Kostygov et al., 2022). The prevalence of *T. theileri* in wild tabanids ranges from 13 to 34% and suggests a significant role of tabanids in transmission (Dire et al., 1990; Ganyukova et al., 2018; Werszko et al., 2019). Among vertebrates, *T. theileri* isolates were documented in domestic cattle, buffaloes, antelopes, deer, and moose. (Rodrigues et al., 2006; Filip-Hutsch et al., 2022).

Other confirmed vectors are sheep keds (*M. ovinus*) transmitting *T. melophagium* (Hoare, 1972). These keds do not have wings, so they are permanently living in the wool of sheep and spread within a herd through close contact of individuals. Infection in keds occurs despite low parasitaemia of *T. melophagium* in the sheep blood. The development of trypanosomes occurs within the midgut and hindgut of the vector. Metacyclic trypomastigotes are produced in the hindgut and excreted along with faeces. Transmission is either by accidental ingestion of keds during grooming or by licking keds faeces from the wool (Hoare, 1972; Molyneux, 1975; Molyneux et al., 1978). *T. melophagium* has evolved as a host- and vector-specific species inside *T. theileri* group, cycling exclusively between sheep and its ked (Gibson et al., 2010, Fisher et al., 2013). No clinical symptoms are recorded

in sheep. Due to the low blood parasitaemia, the best method for detection is through cultivation or indirect identification from keds (Hoare, 1972).

Lipoptena cervi keds are common parasites of deer, possibly functioning as another vector of *T. theileri.* Compared to *M. ovinus, L. cervi* undergoes part of the development apart from the host. The prepupa falls to the ground, pupate, and than hatch into a winged adult which look for a host. Once the adult finds its host, it sheds its wings and remain with that host thereafter. The reported prevalence of *T. theileri* among wild *L. cervi* varies around 25% (Böse and Petersen, 1991, Werszko et al., 2020). Trypanosomes were detected also in five winged individuals, that means in individuals which did not feed, leading to speculation about vertical transmission through feeding glands (Werszko et al., 2020). Beside *L. cervi*, *T. theileri* was described from *Lipoptena fortisetosa* (Werszko et al., 2020). Additionally, the newly described species *T. trinaperronei* was detected in another ked species, *Lipoptena mazamae.* The role of hippoboscid flies as vectors of trypanosomes is established, as it has been confirmed for avian *T. corvi* (Baker, 1956; Votýpka et al., 2004). However, since keds feed on their host frequently, and the blood is permanently present in their gut, PCR positivity does not necessarily prove keds as specific vectors of *T. theileri*.

Mosquitoes were not considered as important vectors. However, high prevalence of *T. theileri* in wild mosquitoes was found recently, suggesting their potential role in transmission (Schoener et al., 2018). The highest prevalence was reported in *Aedes* mosquitoes which are considered opportunistic or mammalophilic (Börstler et al., 2016; Schönenberger et al., 2016). On the contrary, *Culex* mosquitoes were infected only rarely (Schoener et al., 2018), what is not surprising considering their feeding preferences tending to birds (Lura et al., 2012; Rádrová et al., 2013; González et al., 2020; Tiron et al., 2021). Mosquitoes have been already confirmed as vectors of avian trypanosomes and their role in transmitting other trypanosomes should be considered (Votýpka et al., 2012).

T. theileri has also been found in *P. perfiliewi* in Italy. The localization of *T. theileri* in the hindgut is consistent with development of infection in tabanid flies or keds (Calzorali et al., 2018). Sandflies are known vectors of *Leishmania*; however, they are competent vectors of avian trypanosomes as well (Svobodová and Rádrová, 2018). It is thus probable that they are capable to serve as additional vectors for *T. theileri.*

There is also speculation about the role of ticks in the life cycle of *T. theileri*, as it has been found in the haemolymph of various tick species (Krinsky et al., 1976; Shastri and Deshpande, 1981; Latif et al., 2004; Martins et al., 2008). Transmission of trypanosomes to ticks from infected calves and even oral transmission from ticks to calves was described (Shastri and Deshpande, 1981; Morzaria et al., 1986). However, to confirm the vector role of ticks, experiments are necessary to demonstrate, that *T. theileri* can develop in these potential vectors.

2 Aims of the thesis

My research focuses on avian and mammalian trypanosomes, examining their relationships with mosquitoes as their potential vectors.

The main objectives were to elucidate:

1. Trypanosomes life cycle including vectorial capacity of putative vectors
2. Occurrence of trypanosomes in wild vector and host populations
3. Host specificity of trypanosomes in both vertebrate and invertebrate hosts

These specific questions should help us to understand a wider context of host-parasite-vector interactions.

3 List of publications

Fialová, M., Santolíková, A., Brotánková, A., Brzoňová, J., & Svobodová, M. (2021). Complete life cycle of *Trypanosoma thomasbancrofti*, an avian trypanosome transmitted by culicine mosquitoes. *Microorganisms*, *9*(10), 2101.

Brotánková, A., **Fialová, M.**, Čepička, I., Brzoňová, J., & Svobodová, M. (2022). Trypanosomes of the *Trypanosoma theileri* group: Phylogeny and new potential vectors. *Microorganisms*, *10*(2), 294.

Kulich Fialová, **M.**, Kapustová, A., Čepička, I., & Svobodová, M. *Trypanosoma tertium* n. sp.: How many trypanosome species are transmitted by mosquitoes? (in preparation for *Parasitology*)

Fialová, M., Santolíková, A., Brotánková, A., Brzoňová, J., & Svobodová, M. (2021)

Complete life cycle of *Trypanosoma thomasbancrofti*, an avian trypanosome transmitted by culicine mosquitoes.

Microorganisms,

9(10), 2101.

microorganisms

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Article

Complete Life Cycle of *Trypanosoma thomasbancrofti*, an Avian Trypanosome Transmitted by Culicine Mosquitoes

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Abstract: Avian trypanosomes are cosmopolitan and common protozoan parasites of birds; nevertheless, knowledge of their life cycles and vectors remains incomplete. Mosquitoes have been confirmed as vectors of *Trypanosoma culicavium* and suggested as vectors of *T. thomasbancrofti*; however, transmission has been experimentally confirmed only for the former species. This study aims to confirm the experimental transmission of *T. thomasbancrofti* to birds and its localization in vectors. *Culex pipiens* were fed on blood using four strains of *T. thomasbancrofti*, isolated from vectors and avian hosts; all strains established infections, and three of them were able to develop high infection rates in mosquitoes. The infection rate of the culicine isolates was 5–28% for CUL15 and 48–81% for CUL98, 67–92% for isolate OF19 from hippoboscid fly, while the avian isolate PAS343 ranged between 48% and 92%, and heavy infections were detected in 90% of positive females. Contrary to *T. culicavium*, trypanosomes were localized in the hindgut, where they formed rosettes with the occurrence of free epimastigotes in the hindgut and midgut during late infections. Parasites occurred in urine droplets produced during mosquito prediuresis. Transmission to birds was achieved by the ingestion of mosquito guts containing trypanosomes and via the conjunctiva. Bird infection was proven by blood cultivation and xenodiagnosis; mature infections were present in the dissected guts of 24–26% of mosquitoes fed on infected birds. The prevalence of *T. thomasbancrofti* in vectors in nature and in avian populations is discussed in this paper. This study confirms the vectorial capacity of culicine mosquitoes for *T. thomasbancrofti*, a trypanosome related to *T. avium*, and suggests that prediuresis might be an effective mode of trypanosome transmission.

Keywords: avian parasite; *Trypanosoma thomasbancrofti*; Culex; mosquito; life cycle; transmission; prediuresis

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1. Introduction

Digenetic protozoa of the genus *Trypanosoma* (Euglenozoa; Kinetoplastea; Trypanosomatida) [1] are blood parasites transmitted by bloodsucking invertebrates, notorious for the illnesses they cause in humans and animals (Chagas disease, sleeping sickness, nagana, etc.). Trypanosomes were found in birds more than 120 years ago by Danilewsky [2]. Recently, they were divided into three paraphyletic groups named after principal species: *Trypanosoma avium*, *Trypanosoma culicavium*/*Trypanosoma corvi*, and *Trypanosoma bennetti* [3]. Even though avian trypanosomes are widespread and their prevalence in birds can be high [4–9], they are neglected due to their low pathogenicity and economic importance.

Knowledge of avian trypanosomes' vectors remains incomplete, despite their importance in parasite life cycles. The diversity of trypanosome dipteran vectors is high; namely, *T. avium* s. s. is transmitted by blackflies (Simuliidae) [10–12] and sandflies (Psychodidae) [13,14], *T. corvi* by hippoboscid flies (Hippoboscidae) [15], *T. bennetti* group by biting midges (Ceratopogonidae) [16–18], and *T. culicavium* by mosquitoes (Culicidae) [19]. The mode of transmission into birds is by ingestion of the infected vector [12,15,19] or via the conjunctiva [12].

Mosquitoes were among the first studied vectors of avian trypanosomes in times when molecular barcoding was not available [10,20–22]. Until recently, *T. culicavium*

was the only species of avian trypanosome transmitted by mosquitoes whose life cycle had been experimentally confirmed. Mature *T. culicavium* infections are localized strictly on the stomodeal valve [19], while other mosquito infections are found in the hindgut or midgut [10,21,23]. Recently, *T. thomasbancrofti* was described from the regent honeyeater (*Anthochaera phrygia*), a passerine endemic to Australia [24]. This species is identical to *T. avium* s. l. lineage II, which contained isolates originating from chiffchaffs (*Phylloscopus collybita*) and the mosquito *Culex pipiens* from Czechia [3]. Based on the high similarity of SSU rRNA sequences from Australian and Czech trypanosomes, *T. thomasbancrofti* was described as cosmopolitan, with the mosquito *Culex pipiens* being the suspected vector [24]. *Trypanosoma thomasbancrofti* was localized in the hindgut of the mosquito [3,22]. Since we possessed several isolates belonging to *T. thomasbancrofti*, we were interested in the life cycle, and transmission mechanism of a mosquito trypanosome species, which was probably among the first found in vectors [10,21]. Data on the natural prevalences in mosquitoes and birds are given as well.

2. Materials and Methods

2.1. Parasite Strains and Cultures

All *T. thomasbancrofti* strains used in this study were our own isolates originating from Czechia: CUL15 was isolated from a *Culex pipiens* female from Prague, Central Bohemia (ICUL/CZ/2000/CUL15); CUL98 from a *Culex pipiens* from Milovice forest, South Moravia (ICUL/CZ/2018/CUL98); OF19 from a hippoboscid fly *Ornithomya fringillina* from Neuměřice, Central Bohemia (IORN/CZ/2016/OF19); and PAS343 from a wood warbler (*Phylloscopus sibilatrix*) from Milovice forest, South Moravia (APHY/CZ/2016/PAS343).

Trypanosomes were cultivated on rabbit (Bioveta, Ivanovice na Hané, Czech republic) or sheep (LabMediaServis, Jaroměř, Czech republic) blood agar (SNB-9), in flat tubes with liquid medium containing RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) and Schneider's Drosophila Medium (Sigma-Aldrich) mixed 1:1, supplemented with 10% FCS (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2% sterile human urine, and 50 μg/mL amikacin (Medochemie, Prague, Czech republic).

2.2. Experimental Infections of Mosquitoes

Mosquitoes were bred in our laboratory: *Culex pipiens quinquefasciatus* (*Cx. quinquefasciatus* henceforth) originating from India, kept in our laboratory for more than 30 years, and *Culex pipiens molestus* (*Cx. molestus* henceforth) that were colonized recently (2016). Mosquito females were infected by feeding through a chick skin membrane on heat-inactivated (30 min in 56 °C) rabbit or sheep blood with 12–18 day-old culture of 2–6 × 10^8 parasite cells/mL. Fed females were separated after blood feeding, kept at 21 °C, 60% humidity, and with access to 50% sucrose solution on a cotton pad. Mosquitoes were dissected 10–27 days post-infection, and their guts were examined under the light microscope for infection status, intensity, parasite localization, and its dynamics. Infection intensities were defined as: light, 1–100 parasites; medium, 100–1000 parasites; and heavy, >1000 parasites per gut.

Experimental infections were always performed in pairs, both subspecies of *Culex* mosquitoes feeding at the same time on the same blood-parasite cocktail to minimize the influence of experimental conditions. Experiments were repeated three times with strain PAS343, twice with strains CUL15 and OF19, while strain CUL98 was tested only once.

2.3. Experimental Inoculation of Birds

Four canaries (*Serinus canaria*) and two zebra finches (*Taeniopygia guttata*) were screened before the experiment by blood cultivation as described in [14] from the metatarsus vein articulation (*vena metatarsalis plantaris superficialis media*) for trypanosome infections; all were negative. Birds were inoculated with 7–8 *Cx. molestus* or *Cx. quinquefasciatus* guts infected with different strains of *T. thomasbancrofti* (CUL98, OF19, PAS343), which were homogenized in saline, applied orally or placed on the conjunctiva. Infection status was checked by

blood cultivation at 7–14 day intervals; cultures were checked 3 times in weekly intervals. In positive cases, parasite identity was confirmed by sequencing of the SSU rRNA.

2.4. Transmission of Trypanosomes from Canaries to Mosquitoes

Mosquitoes were allowed to feed on trypanosome-positive birds to test if trypanosomes could establish infection in mosquitoes after natural exposure. Positive birds were kept for 60 min in a small cage placed into the net with 50 mosquito females in complete darkness. Blood-fed mosquitoes were separated, kept at 21 °C, 60% humidity, with access to 50% sucrose solution on a cotton pad, and dissected 10–15 days after feeding. Guts were examined under the light microscope for infection status.

2.5. Prediuresis

Mosquitoes previously fed on blood with trypanosomes (CUL98) were allowed to defecate and lay their eggs. Subsequently, they were allowed to feed on an anesthetized laboratory mouse. The feeding process was monitored, and fully fed mosquitoes were transferred into plastic tubes. Urine droplets were caught on the coverslip placed on the bottom of the plastic tube. Air-dried droplets were fixed by methanol, stained with Giemsa, and examined for the presence of trypanosomes.

2.6. Wild Mosquito Collection and Identification of Parasites

Mosquitoes were trapped using CDC light traps (JW, Hock Company, Gainesville, FL, USA) without a bulb and baited with dry ice in 2018–2020 in Milovice forest, South Moravia, Czechia. Insects were collected in nylon nets connected to the traps, sorted according to species [25], and dissected. Dissected guts were examined under the light microscope for the presence of trypanosomes (infection status, intensity, and parasite localization). A part of the positive guts was used for the cultivation of kinetoplastids, and the rest was stored in ethanol for the barcoding of parasites (see below).

2.7. Wild Bird Studies

Bird sampling was done at three localities between May and July, as described in [14]. Adults and yearlings were mist-netted at watering places in a game reserve (Milovice forest, South Moravia, 48.821274, 16.693175) or in cooperation with registered ringers contributing to the program Constant Effort Site (CES), organized by the Prague Ringing Centre (Zeměchy, 50.231783, 14.272371 and Choteč, 49.999069, 14.280239 in Central Bohemia). Trapping and sampling were done by licensed workers according to national law and experimental guidelines.

2.8. Diagnostic PCR in Mosquitoes and Birds

DNA from mosquitoes' guts and wild birds' blood was extracted using a High Pure PCR Template Preparation Kit (Roche Diagnostic, Manheim, Germany) according to the manufacturer's instructions and kept at −20 °C until further use. For parasite identification, trypanosome SSU rRNA was amplified using a specific nested PCR with S762 and S763 primers [26] for the first step and TR-F2 and TR-R2 [27] primers for the second step. For the identification of trypanosomes from cultures, single-step PCR with primers MedlinA and MedlinB [28] were used. All positive PCR products were purified with the enzymatic solution ExoSap (Thermo Scientific, Waltham, MA, USA), then sequenced at the core facility of the Faculty of Science. Sequences were examined in the program BioEdit and analyzed using the BLAST algorithm and nucleotide database NCBI.

2.9. Scanning Electron Microscopy

Guts positive for trypanosomes after experimental infection of mosquitoes were torn by insulin syringe then fixed in 2.5% glutaraldehyde in 5 mM HCl, 0.1 M cacodylate buffer for 24 h at 4 °C. Thereafter, they were processed at our core facility, the Laboratory of Electron Microscopy (https://www.natur.cuni.cz/biology/service/lem?set_language=en

(accessed on 20 September 2021)), as follows: samples were post-fixed in 2% osmium tetroxide in the same buffer for 2 h at room temperature. After dehydration in a graded ethanol series, the guts were critical-point air-dried, sputter-coated with gold in a Polaron coater, and examined by the authors using a JEOL 6380LV scanning electron microscope.

2.10. Light Microscopy and Measurement of Trypanosomes

After the experimental infections, dissected mosquito guts were fixed with methanol on slides and stained with Giemsa stain, photographed at 1000x magnification with a CDC camera (DP70) using an Olympus BX51 microscope. Measurement of the cells was done using ImageJ software, and the data were processed using R software [29]

2.11. Animal Experimentation Guidelines

Animals were maintained and handled in the animal facility of Charles University in Prague following institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All the experiments were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University in Prague and were performed under permission of no. MSMT-31949/2019-5, MSMT-31949/2019-6, of the Ministry of Education, Youth and Sports and 50982/ENV/14-2961/630/14 of the Ministry of Environment. Investigators are certified for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

3. Results

3.1. Experimental Infection of Mosquitoes

Two subspecies of *Cx. pipiens* were fed on blood with four strains of *T. thomasbancrofti* originating from three different hosts: CUL15, CUL98 (mosquito); OF19 (hippoboscid fly); and PAS343 (bird). Over 80% of *Cx. quinquefasciatus* and 70% of *Cx. molestus* were infected with isolate CUL98, with nearly 100% of them being heavy infections (Figure 1). Mosquitoes were also susceptible to isolate OF19 from the hippoboscid fly with more than 90% of *Cx. quinquefasciatus* and almost 70% of *Cx. molestus* being infected, and heavy infections were found in 98 and 89% of infected mosquito guts, respectively. In the case of isolate PAS343, more than 80% of *Cx. quinquefasciatus* and almost 50% of *Cx. molestus* were infected, with heavy infections prevailing as well. On the other hand, the susceptibility of mosquitoes to strain CUL15 was lower (28% of *Cx. molestus* and 5% of *Cx. quinquefasciatus* infected), and no heavy infections were detected except for one *Cx. quinquefasciatus*. With the exception of the CUL15 strain, infection rates were higher in *Cx. quinquefasciatus* (81–92%) compared to *Cx. molestus* (47–72%).

3.2. Localization of Trypanosomes in Mosquitoes

Trypanosomes in guts dissected 11- and 14-days post-infection (dpi) were localized in the hindgut and mainly formed rosettes (Figures 2 and 3c). Starting from 20 dpi, changes in parasite localization and the appearance of unattached epimastigotes were observed (Figures 2 and 3d). Trypanosomes were localized in the hindgut (Figure 3a–c); however, in some cases, they extended to the midgut. Only epimastigotes were found in the midgut. These free-swimming stages were also detected in the hindgut. Pure midgut infections or rosettes in midgut were not detected. For the dimensions of trypanosomes in mosquito guts, see Table 1.

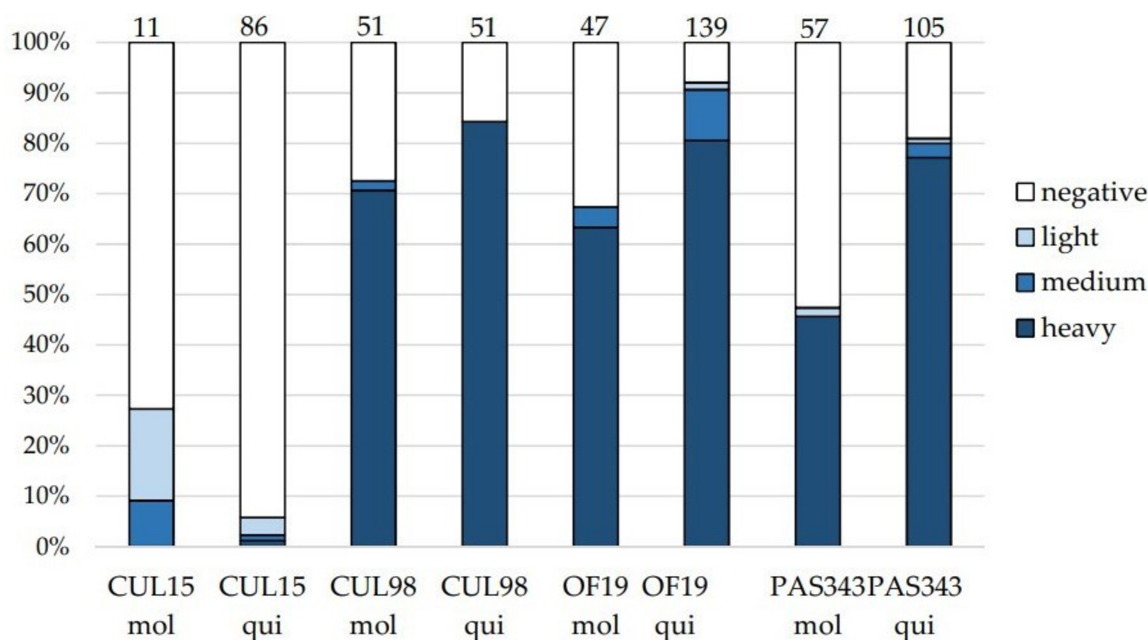

Figure 1. Infection rates and intensities of infection in mosquitoes *Culex molestus* (mol) and *Culex quinquefasciatus* (qui) experimentally infected with *Trypanosoma thomasbancrofti* strains CUL15, CUL98, OF19, and PAS343. Infection intensities: light, 1–100 parasites; medium, 100–1000 parasites; heavy, >1000 parasites per gut. Numbers of dissected females are presented above the columns.

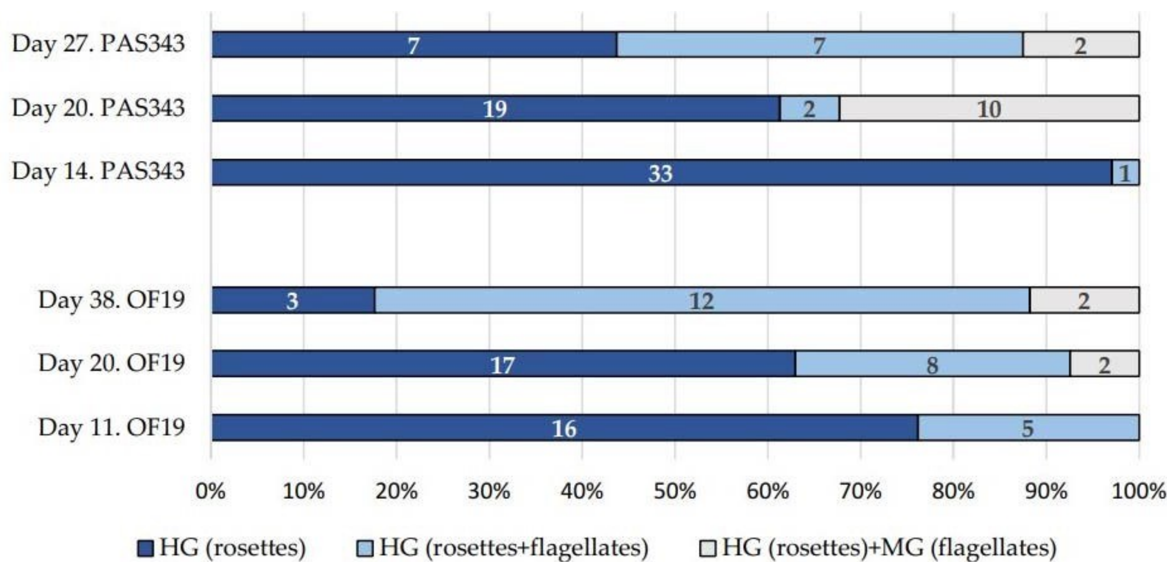

Figure 2. Changes of trypanosome localization in *Cx. quinquefasciatus* guts experimentally infected with isolate OF19 and PAS343. The numbers of dissected females are shown in the columns. HG, hindgut; MG, midgut.

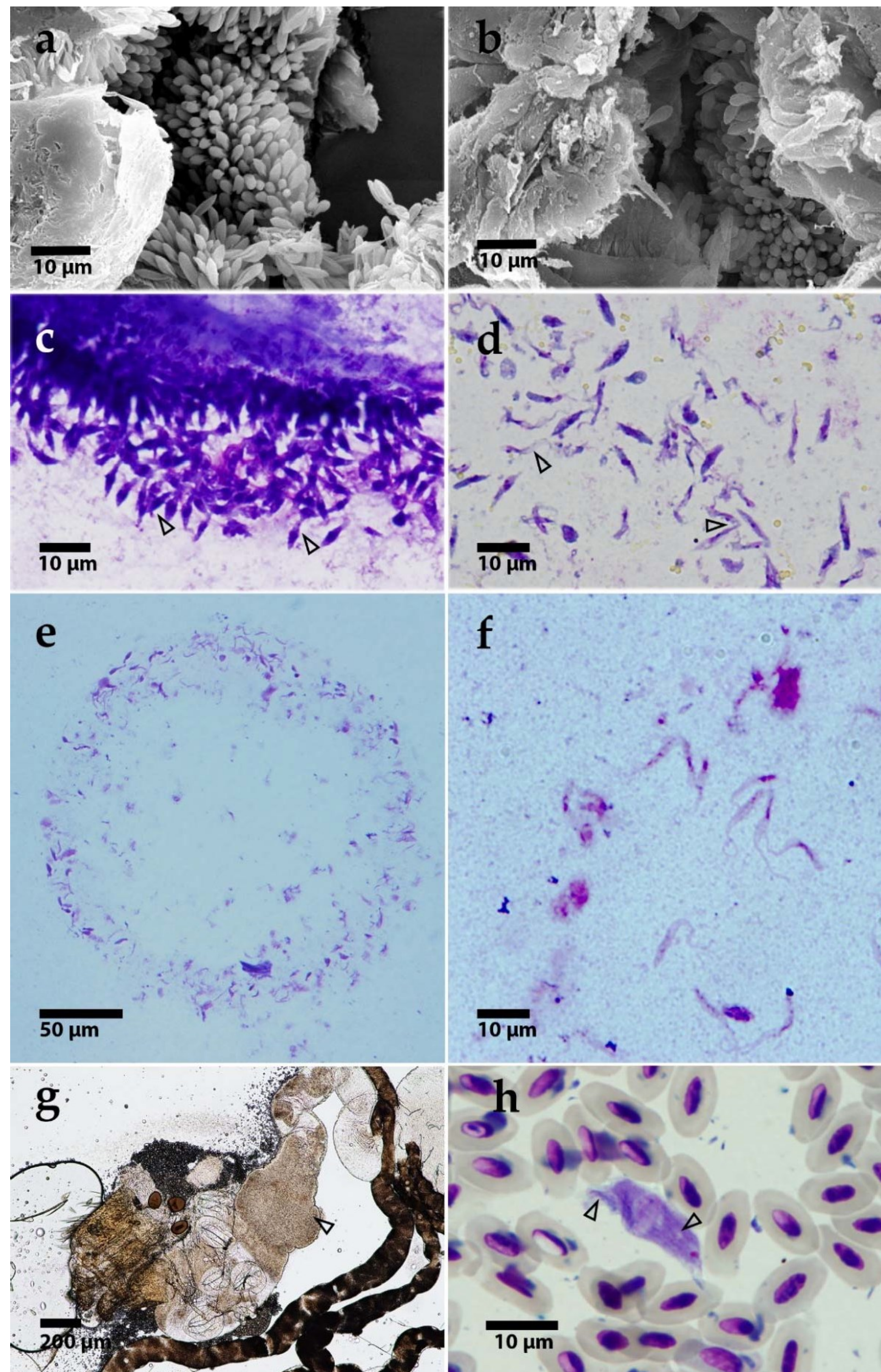

Figure 3. (**a**,**b**) Scanning electron microscopy of *T. thomasbancrofti* OF19 (**a**) and PAS343 (**b**) in the gut of *Culex quinquefasciatus* after experimental infection (**c**,**d**). Light microscopy of trypanosome morphotypes in *Culex quinquefasciatus* gut: rosettes (**c**) and epimastigotes (**d**). Prediuresis droplet with numerous trypanosomes (CUL98) (**e**). A detail of epimastigotes from prediuresis droplet (**f**). Dissected *Culex* mosquito gut with trypanosomes after xenodiagnosis; arrowhead pointing to the mass of parasites (**g**). *T. thomasbancrofti* trypomastigote from barn swallow (*Hirundo rustica*) blood with visible striation (see arrows for striation) (**h**).

Table 1. Morphometry of trypanosomes in mosquito gut. Values in micrometers. SE, standard error. At least 30 cells were measured for each strain/host combination.

Cell Type	Body Length Mean ± SE (Range)	Body Width Mean ± SE (Range)	Flagellum Length Mean ± SE (Range)
Epimastigote	8.7 ± 0.2 (4.5–14.6)	1.2 ± 0.0 (0.6–2.0)	7.8 ± 0.2 (3.0–13.2)
Rosette	7.2 ± 0.1 (3.5–11.3)	2.2 ± 0.0 (1.1–3.6)	1.6 ± 0.1 (0–8.8)

3.3. Experimental Transmission of Trypanosomes to Birds

Trypanosome strains PAS343, CUL98, and OF19—which were able to develop heavy infections in the mosquitoes' guts—were used for the inoculation of laboratory birds. Birds were inoculated perorally or transconjunctivally with guts heavily infected by *T. thomasbancrofti*. All three strains of trypanosomes were infective for birds (Table 2).

Table 2. Results of bird inoculations. Birds were inoculated perorally (po) or transconjunctivally (tc) with *T. thomasbancrofti* from the laboratory-reared mosquitoes *Cx. molestus* (mol) and *Cx. quinquefasciatus* (qui).

Bird	Strain	Dose	Mosquito Strain	Infection Route	Result	Day First Positive	Day Last Positive	Day Last Checked
Canary 1	PAS343	8 guts	qui	po	positive	59	665	790
Canary 2	OF19	7 guts	qui	po	positive	70	141	720
Canary 3	CUL98	7 guts	qui	tc	positive	2	2	160
Canary 4	CUL98	7 guts	qui	tc	negative			160
Zebra finch 5	PAS343	8 guts	mol	po	positive	29	29	620
Zebra finch 6	PAS343	8 guts	qui	po	negative			520

Canary 1 was positive between the 59th and 120th days after peroral inoculation (PAS343), then remained negative until a relapse of infection on days 580 and 665. Canary 2 was first positive on day 70 and last positive 141 days after peroral inoculation (OF19). Canary 3 was first positive two days after transconjunctival inoculation (CUL98) then remained negative until the last day of sampling. Zebra finch 5 was positive only on a single occasion on day 29 after inoculation. The identity of the parasites was confirmed by sequencing the SSU rRNA. All obtained sequences were identical with strains used for the experimental infection of mosquitoes.

3.4. Transmission from Birds to Mosquitoes

Mosquitoes were allowed to feed on birds infected with *T. thomasbancrofti*. After dissection, 13% of the 23 *Cx. molestus* and 27% of the 47 *Cx. quinquefasciatus* fed on canary 1 were infected; similarly, 31% of the 29 *Cx. Molestus*, and 9% of the 11 *Cx. quinquefasciatus* fed on canary 2 were infected. Mosquitoes were allowed to feed on birds several times between the 60th and 120th days after inoculation. The first positive mosquitoes were detected on day 95 and the last positive on day 120 after inoculation. Trypanosomes were able to develop heavy infections localized in the hindgut, comparable to experimental infections as well as to infections from wild-caught mosquitoes (Figure 3g). The identity of the parasites was confirmed by sequencing SSU rRNA. Twenty-three mosquitoes fed on zebra finch 5 (35, 47, and 52 dpi) remained negative.

3.5. Prediuresis

Stages of *T. thomasbancrofti* were observed on Giemsa-stained droplets of urine from prediuresis. Trypanosomes were observed as epimastigote forms in 5 out of 12 examined droplets (Figure 3e,f).

3.6. Prevalence of *T. thomasbancrofti* in Wild-Caught Mosquitoes

Between 2018 and 2020, 1367 wild-caught mosquitoes were dissected, of which 771 belonged to the genus *Culex*. The rest were mosquitoes of the genera *Aedes* (n = 592), *Anopheles* (n = 15), *Culiseta* (n = 28), and *Mansonia* (n = 24). Out of the 771 dissected *Culex* mosquitoes, 49 individuals (6.2%) were infected with kinetoplastids. *T. culicavium* had the highest prevalence, with 35 positive individuals (4.5%). Twelve mosquitoes were infected

with monoxenous kinetoplastids, and a single individual with *T. thomasbancrofti* (0.13%). One *Cx. pipiens* had a mixed infection, and one harbored the mammalian species *T. theileri.* All species of mosquitoes infected by kinetoplastids were tested, but avian trypanosomes were identified exclusively in *Culex* mosquitoes.

3.7. Prevalence of *T. thomasbancrofti* in Wild Passerines

In passerines screened between 2014 and 2019, *T. thomasbancrofti* was detected using blood cultivation in 13 (0.44%) of the 2943 sampled individuals. Infected species included Eurasian reed warbler (*Acrocephalus scirpaceus*), common chiffchaff (*Phylloscopus collybita*), wood warbler (*P. sibilatrix*), barn swallow (*Hirundo rustica*), sand martin (*Riparia riparia*), and Eurasian blackcap (*Sylvia atricapilla*). Prevalence in these species was 2.9% (n = 446), and generic prevalence was 1.7% (n = 762). The negative sampled bird species/genera included *Parus* spp., *Coccothraustes coccothraustes*, *Emberiza* spp., *Turdus* spp., *Carduelis* spp., *Ficedula albicollis*, *Fringilla coelebs*, *Sitta europaea*, *Passer montanus*, *Sturnus vulgaris*, *Erithacus rubecula*, and *Certhia* spp. (in descending order according to numbers; only those with at least 15 sampled individuals were included). Blood smears of birds positive for *T. thomasbancrofti* were inspected under the microscope at magnification x1000 for 10 min and the whole smear area at x200; a single trypomastigote was found (Figure 3h).

4. Discussion

Mosquitoes are notorious vectors of multiple pathogens (viruses, bacteria, protozoa, nematodes); however, their importance as trypanosome vectors is relatively unexplored, perhaps overshadowed by the transmission of *Plasmodium*. A trypanosome known to be transmitted by mosquitoes is *Trypanosoma rotatorium* from frogs [30]; mosquitoes were included with certainty among the vectors of avian trypanosomes much later [3,19]. Avian trypanosomes found in mosquitoes in our earlier studies were usually localized on the stomodeal valve, resembling the suprapylarian *Leishmania* in sandflies; their transmission, however, occurred by vector ingestion and not by bite as in sandflies. These trypanosomes belong to a species described as *T. culicavium* [19]. In this paper, we described the experimental life cycle of *T. thomasbancrofti* [24], which belongs to a different trypanosome group related to *T. avium* [3], and for which experimental evidence of transmission and a description of development in mosquitoes was lacking. Unlike *T. culicavium*, infections by *T. thomasbancrofti* are localized in the hindgut, similar to parasites found in *Aedes aegypti* [10,21].

For our experimental work, we used several isolates of *T. thomasbancrofti* to test their potential to develop infections in two *Cx. pipiens* subspecies, *Cx. quinquefasciatus* and *Cx. molestus*. These isolates originated from mosquitoes, a bird, and a hippoboscid fly. Surprisingly, the isolate from the hippoboscid fly was able to develop high infection rates, while one of the isolates from mosquitoes produced low infection rates and intensities. Strains differ in their performance; therefore, conclusions concerning vectorial competence should not be based on a single strain–vector combination. Moreover, strains with higher developmental plasticity (lower vector specificity) may have the potential to bridge different vertebrate hosts or even set up new vector–parasite combinations. Nevertheless, the role of hippoboscid flies as vectors of *T. avium* group in nature remains unclear. Due to the difficulty of the laboratory handling of hippoboscid flies, the development of infective stages in hippoboscids could not be tested. Hippoboscids spend most of their active lives on the host, and the blood is permanently present in their intestine, enabling nonspecific parasites to thrive and be isolated [3].

The localization of *T. thomasbancrofti* was in the hindgut, typical for trypanosomes belonging to *T. avium* s. l. (group C) [3,14,17]. Similar to other species from this group, after the rupture of the peritrophic matrix, trypanosomes migrate to the hindgut and rectum, where they stay attached by hemidesmosomes to the hindgut chitinous lining, as seen in *Simulium* spp. and *Ae. aegypti* [12,21]. However, in the course of infection, free and

unattached epimastigotes appeared in the hindgut, and in some cases also in the midgut. We assume that these free stages are metacyclic forms infective for birds; guts with these forms were therefore used for the experimental inoculation of birds.

To test mosquitoes' potential as vectors of *T. thomasbancrofti*, we experimentally inoculated laboratory birds. Despite different localization of *T. culicavium* and *T. thomasbancrofti* in mosquito guts, we showed that the route of transmission was identical (i.e., peroral). *T. thomasbancrofti* localization in hindgut however opens another potential way of transmission: transconjunctival. Bloodsucking Nematocera excrete excessive fluids during feeding on the host (prediuresis) [31], and metacyclic kinetoplastids can be present in these droplets [32]. Because mosquitoes feed readily around the eyes, prediuresis can play an important role in the life cycle of *T. thomasbancrofti* as well as in *T. avium* transmitted by black flies [12]. The number of trypanosomes in the droplets was significant (approximately 400 cells), and the morphology corresponded to putative metacyclic forms.

Inoculated canaries repeatedly tested positive, either using blood cultures or xenodiagnosis. This natural way of mosquito infection further confirms the vectorial role of mosquitoes in nature. A zebra finch was positive only once, similar to the single canary from the study of Svobodová et al. [17] inoculated with *T. bennetti*. The sudden appearance of parasites in the peripheral blood can be associated with prolonged photoperiod, stress caused by breeding, or experimental manipulation, as demonstrated for trypanosomes [33]. A relapse after two years from inoculation confirms that infection by avian trypanosomes is chronic with an intermittent appearance in peripheral blood [15,21,34].

The prevalence of *T. thomasbancrofti* in wild-caught mosquitoes in Central Europe is low [23,35]. Only 0.9% of *Culex pipiens* trapped at raptor nests in Czechia harbored infections in the hindgut compared to 8% localized on the stomodeal valve; out of the 28 trypanosomatid strains established, only one belonged to *T. thomasbancrofti* [23]. Mosquitoes caught using CO_2 in our study showed a low prevalence of *T. thomasbancrofti* as well. The prevalence of *T. culicavium* was 3.6%, while that of *T. thomasbancrofti* was 0.1%. In avian hosts, the prevalence of *T. thomasbancrofti* was low as well: only 0.4% of screened passerines were positive. Since the trypanosome is transmitted by ingestion, it is appropriate to focus on insectivorous avian species; then, the prevalence increases to nearly 3%. Therefore, it can be seen that parasites circulate in specific hosts, and the prevalence obtained by screening for unspecific hosts may be misleading.

5. Conclusions

Our study confirms that (1) *Culex* mosquitoes are highly susceptible to *T. thomasbancrofti* infections; (2) trypanosomes can develop metacyclic stages in mosquito guts that are transmissible to birds perorally or transconjunctivally; (3) the prevalence of *T. thomasbancrofti* among wild mosquitoes and birds was lower than the prevalence of *T. culicavium*; and (4) the complete life cycle of the *T. thomasbancrofti* was achieved, and mosquitoes can thus be considered as confirmed vectors.

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Trypanosomes of the *Trypanosoma theileri* Group: Phylogeny and New Potential Vectors

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Abstract: Trypanosomes belonging to *Trypanosoma theileri* group are mammalian blood parasites with keds and horse fly vectors. Our aim is to study to vector specificity of *T. theileri* trypanosomes. During our bloodsucking Diptera survey, we found a surprisingly high prevalence of *T. theileri* trypanosomes in mosquitoes (154/4051). Using PCR and gut dissections, we detected trypanosomes of *T. theileri* group mainly in *Aedes* mosquitoes, with the highest prevalence in *Ae. excrucians* (22%), *Ae. punctor* (21%), and *Ae. cantans/annulipes* (10%). Moreover, *T. theileri* group were found in keds and blackflies, which were reported as potential vectors for the first time. The vectorial capacity was confirmed by experimental infections of *Ae. aegypti* using our isolates from mosquitoes; sand fly *Phlebotomus perniciosus* supported the development of trypanosomes as well. Infection rates were high in both vectors (47–91% in mosquitoes, 65% in sandflies). Furthermore, metacyclic stages of *T. theileri* trypanosomes were observed in the gut of infected vectors; these putative infectious forms were found in the urine of *Ae. aegypti* after a second bloodmeal. On the contrary, *Culex pipiens quinquefasciatus* was refractory to experimental infections. According to a phylogenetic analysis of the 18S rRNA gene, our trypanosomes belong into three lineages, TthI, ThII, and a lineage referred to as here a putative lineage TthIII. The TthI lineage is transmitted by Brachycera, while TthII and ThIII include trypanosomes from Nematocera. In conclusion, we show that *T. theileri* trypanosomes have a wide range of potential dipteran vectors, and mosquitoes and, possibly, sandflies serve as important vectors.

Keywords: *Trypanosoma theileri*; *Trypanosoma melophagium*; mosquito; *Phlebotomus*; tabanid; ked; vector; phylogeny; prediuresis; transmission

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1. Introduction

Trypanosomes (Euglenozoa; Kinetoplastea; Trypanosomatida) [1] belong among the most important and widespread parasites worldwide, causing important diseases in humans and livestock. They are digenetic blood parasites transmitted mainly by various bloodsucking insects. Trypanosomes of the *Trypanosoma theileri* group (*T. theileri* henceforth) have been reported from various ungulates in cattle, buffaloes, sheep, antelopes, and deer [2–7]. Although widespread, the *T. theileri* group is largely neglected due to its low economic importance and causing no pathology [2,3]. Infections by *T. theileri* are mostly cryptic; however, pathologies might have resulted from coinfections or stress when fever, anorexia, and anemia were reported as symptoms in several bovid infections [8–15].

The *Trypanosoma theileri* group consists of several species (*Trypanosoma theileri*, *T. melophagium*, *T. cervi*, and *T. trinaperronei*) and various trypanosome genotypes reported from cervids, bovids, and insects [6,16–19]. Some genotypes are specific for a single host species, such as sheep or water buffalo [3,16,20,21], while others, belonging to both TthI and TthII lineages, have been reported from cattle and deer [3,16,17,22].

Mammals are infected by ingesting the vector with metacyclic trypomastigotes or by contamination of skin abrasion or mucous membrane by feces of the vector [2,23,24]. A possible transplacental transmission was considered in bovids and cervids [25,26]. Prediuresis (i.e., removing excess water to concentrate the bloodmeal) of the bloodfeeding vectors represents another potential transmission mode [27,28]. Prediuresis was described in various bloodsucking insects, such as kissing bugs, tsetse flies, sand flies, and mosquitoes [27,29–34]. Putative infectious stages of kinetoplastids were found in the urine of kissing bugs, sand flies, and mosquitoes [33–35]. Putative infectious stages of avian trypanosomes, probably belonging to the same *Megatrypanum* subgenus as *T. theileri* [36], were observed in mosquito urine [33].

Trypanosomes of *Trypanosoma theileri* group were detected in different groups of Diptera, such as tabanids [2,23,37,38], deer keds [6,39,40], mosquitoes [41], *Phlebotomus perfiliewi* [42], and tsetse flies [43,44]; in addition, they were also reported from several species of ticks: *Hyalomma anatolicum*, *Amblyomma americanum*, *Boophilus microplus,* and *Ornithodoros moubata* [45–48]. Despite deer keds having been assumed to be vectors of *T. theileri* trypanosomes [6,39], tabanids are the vectors confirmed by experimental infections [23], and development of *T. theileri* trypanosomes, including metacyclic stages, was described in the tabanid gut [37,38]. Furthermore, sheep ked *Melophagus ovinus* was confirmed as a vector of *T. melophagium*, a species belonging to the *T. theileri* group but occurring exclusively in sheep [2,49,50].

Mosquitoes are not considered important vectors of trypanosomes, and only a few studies have focused on them. *Culex* mosquitoes are confirmed vectors of the bird species *Trypanosoma culicavium* and *T. thomasbancrofti* [33,51]. The role of mosquitoes in the lifecycle of *T. theileri* is unclear; this trypanosome was detected in seven mosquito species but only using PCR [41].

The two main lineages of the *T. theileri* group, TthI and TthII, were previously defined based on analyses of ITS (internal transcribed spacer), and SL (spliced leader) genes [16,52]. Subsequently, several trypanosome strains were placed into TthI or TthII based on 18S rRNA gene phylogenies [17,38,53,54]. Recently, an additional lineage was distinguished based on 18S rRNA gene analysis [6].

During studies of trypanosome vectors, we have found a surprisingly high prevalence of *T. theileri* trypanosomes in examined mosquitoes by dissections and PCR. The infections in dissected mosquitoes suggested possible vectorial capacity. Therefore, we decided to focus on mosquitoes as possible vectors of *T. theileri* by examining wild mosquitoes and using *T. theileri* isolates for experimental infections of possible vectors. We also carried out a phylogenetic analysis of *T. theileri* based on the 18S rRNA gene sequences to assess the associations of *T. theileri* with various vectors.

2. Materials and Methods

2.1. Insect Trapping and Processing, and Sampling of Deer Blood

Mosquitoes were trapped monthly from May to August in 2017–2019 in three localities in the Czech Republic, namely Choteč (49.9991 N, 14.2802 E), Zeměchy (50.2318 N, 14.272 E), and Milovice forest (48.8213 N, 16.6932 E). Six CDC light traps (JW, Hock Company, Gainesville, FL, USA) baited with dry ice were used at each trapping event. Traps were installed between 4:00 p.m. and 6:00 p.m. and removed between 8:00 and 10:00 a.m. the next day. Collected insects were killed in a −80 °C freezer or a box with dry ice and were sorted by families. Mosquitoes were stored in Petri dishes at −20 °C for species determination.

Tabanids were collected in the Milovice forest in 2019, mainly as bycatch in mistnets; some were caught in CDC traps (see above) or by hand inside a car.

In 2017–2018, sheep keds *Melophagus ovinus* were collected from sheep at six localities: Vlkov (49.1512 N, 14.7252 E), Ratíškovice (48.9200 N, 17.1656 E), Hořice (50.3661 N, 15.6318 E), Statenice (50.1426 N, 14.3185 E), Valašská Senice (49.2253 N, 18.1169 E), and Přerov Předmostí (49.4675 N, 17.4374 E). Deer keds were collected by hunters in 2017–

2019 directly from shot fallow deer (*Dama dama*), red deer (*Cervus elaphus*), and roe deer (*Capreolus capreolus*) at Boršov nad Vltavou (48.9218 N, 14.4340 E), Milovice forest (48.8213 N, 16.6932 E), Blíževedly (50.6084 N, 14.3965 E), Planá (49.8682 N, 12.7438 E), Bystřice (49.7321 N, 14.6674 E), Neveklov (49.7537 N, 14.5329 E), Nové Strašecí (50.1527 N, 13.9004 E), Obecnice (49.7162 N, 13.9473 E), and Vonoklasy (49.9501 N, 14.2767 E). Blood samples were collected by hunters from the shot game in Blíževedly (50.6084 N, 14.3965 E), Doupov (50.2572 N, 13.1432 E), Hvězda (50.6023 N, 14.4396 E), Kublov (49.9437 N, 13.8767 E), Litice (50.6134 N, 14.4393 E), Milovice forest (48.8213 N, 16.6932 E), Nižbor (49.1000 N, 14.0024 E), Nové Hrady (48.7896 N, 14.7783 E), Nové Strašecí (50.1527 N, 13.9004 E), Skalka (50.5857 N, 14.4118 E), and Vonoklasy (49.9501 N, 14.2767 E). Keds for dissection were stored alive in zip-lock bags; dead keds were stored in ethanol. A sample of game blood was fixed in ethanol for PCR detection, and another was used for cultivation (see below).

The insects were identified under a stereomicroscope using determination keys [55,56]; undetermined insects were barcoded when possible [57]. Insects (except tabanids) were pooled according to the species and locality in pools containing ten or fewer specimens and were examined using nested PCR (see below). Engorged insects with visible blood in the gut were processed individually. Some living keds, tabanids, and mosquitoes were killed and dissected, and their intestines were examined for the presence of trypanosomes.

2.2. Dissection and Cultivation

Insects were killed and washed in 70% ethanol, followed by a sterile saline solution. The gut was dissected in a drop of sterile saline under a stereomicroscope, and infection status was checked under a light microscope. Parasite localization, appearance, and quantity were recorded. Infections were considered to be weak if fewer than 100 parasite cells were visible, moderate with 100–1000 cells present, and heavy with more than 1000 cells per gut. A part of positive guts was used to cultivate kinetoplastids, and the rest was stored in ethanol for PCR detection.

Kinetoplastids from positive guts and deer blood samples were cultivated in 4 mL glass vials on rabbit (Bioveta, Ivanovice na Hané, Czech Republic) or sheep (LabMediaServis, Jaroměř, Czech Republic) blood agar (SNB-9) overlayed with RPMI 1640 (Sigma–Aldrich, St. Louis, MO, USA) and Schneider Drosophila Medium (Sigma–Aldrich, St. Louis, MO, USA) in a 1:1 ratio supplemented with 20% FCS (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2% sterile human urine, 100 μg/mL amikacin (Medochemie, Prague, Czech Republic), 5000 U/mL penicillin, and 1.5 mg/mL 5-fluorocytosine (Sigma–Aldrich, St. Louis, MO, USA) at 23 °C. The presence of kinetoplastids was checked weekly. Thriving cultures were subcultured into flat tubes with blood agar and cryopreserved in liquid nitrogen. Trypanosomes for experimental infections were cultivated in the same medium without fluorocytosine and penicillin.

2.3. PCR Detection of Kinetoplastids, Sequencing

DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostic, Manheim, Germany) according to the manufacturer's instructions. EmeraldAmpGT PCR Master Mix (TaKaRa Bio, Kusatsu, Shiga, Japan) was used for PCR reactions. The 18S rRNA gene was amplified using a single-step or nested PCR. MedA (CTGGTTGATC-CTGCCAG) and MedB (TGATCCTTCTGCAGGTCCACCTAC) primers [58] were used to amplify the DNA from cultures obtained from the positive dissected insect guts or deer blood samples. Conditions were as follows: denaturation temperature was 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min 30 s, 72 °C for 1 min 30 s, and final extension at 72 °C for 5 min. Nested PCR was used to detect kinetoplastids in the dead insects, positive guts, and deer blood. Primers S762 (GACTTTTGCTTCCTCTAWTG) and S763 (CATATGCTTGTTTCAAGGAC) [59] were used for the first step with the same cycle condition as single-step PCR. TRnF2 (GARTCTGCGCATGGCTCATTACATCAGA) and TRnR2 (CRCAGTTTGATGAGCTGCGCCT) primers [43] were used for the second

step with the same conditions as the single-step PCR but with an annealing temperature of 64 °C.

PCR products of the positive samples (visualized in gel electrophoresis) were purified by ExoSAP (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Primers 1000R (ATGCCTTCGCTGTAGTTCGTCT) and 1000F (AGACGAACTACAGCGAAGGCAT) [60] and 577F (GCCAGCACCCGCGGT) [61] were used for sequencing.

2.4. Prevalence of *T. Theileri*

The prevalence was calculated as the Minimal Infection Rate (MIR) as follows:

$$\mathrm{MIR}(\%) = \frac{\text{n of } \textit{T. theileri} \text{ positive pools}}{\text{n of examined pools}} \cdot 100$$

Prevalence was counted when at least 15 individuals were examined.

2.5. Experimental Infections and Prediuresis Experiments

Mosquitoes *Aedes aegypti*, *Culex pipiens molestus, Cx. p. quinquefasciatus,* and the sandfly *Phlebotomus perniciosus* were used for experimental infections. *Culex* and *Phlebotomus* were permanently reared at the Department of Parasitology, Charles University, Prague, Czech Republic. A colony of *Ae. aegypti* was temporarily established; mosquitoes were obtained from The National Institute of Public Health, Czech Republic. Colonies were maintained at 25 °C and 80% relative humidity. About 100 females were exposed to parasites by feeding through a chick skin membrane on heat-inactivated rabbit or sheep blood (30 min at 56 °C) containing 5–7 days old culture of 10^7 parasite cells/mL. Due to autogeny of *Cx. p. molestus*, fed mosquitoes were sorted after feeding. In other species, fed specimens were recognized during dissection by the presence of developing eggs. Ambient humidity and 50% sucrose solution on a cotton pad were provided to blood-fed insects. All trypanosome isolates used in the experiments were our own and are summarized together with temperature conditions in Table 1. Low temperatures were used to mimic the natural conditions, as some kinetoplastids are known to develop better at lower temperatures [62]. After defecation (10–62 post-infection for mosquitoes, 7–17 post-infection for sandflies), guts were dissected at several time points and examined under a light microscope for infection status, infection intensity, and parasite localization.

Aedes aegypti mosquitoes experimentally infected with the *Trypanosoma* isolate CUL46 were used for the prediuresis experiment 22 days after infection. Mosquitoes were blood-fed through a membrane, and, immediately after feeding, they were placed individually in tubes with coverslips at the bottom. After defecation, the coverslips were dried, fixed with methanol, and stained with Giemsa–Romanowski (Sigma–Aldrich, St. Louis, MO, USA).

Table 1. Vector species, trypanosome isolates, and environmental temperatures used in the infectious experiments. 8–11→15: Fed mosquitoes were stored in fluctuating temperatures (8–11 °C), and after the 21st day, they were held at 15 °C.

Vector Species	Trypanosome Isolate	Environmental Temperature (°C)
Culex p. quinquefasciatus	CUL59 (CUL/CZ/2015/CUL59) ex *Culiseta annulata* *	15
		21
Cx. p. quinquefasciatus *Cx. p. molestus*	CUL46 (CUL/CZ/2014/CUL46) ex *Cs. annulata* *	8–11
		15
		8–11→15
		21

Table 1. *Cont.*

Vector Species	Trypanosome Isolate	Environmental Temperature (°C)
Aedes aegypti	CUL46 (CUL/CZ/2014/CUL46) ex *Cs. annulata*	21
	CUL107 (AED/CZ/2018/CUL107) ex *Aedes vexans*	
	CELA1 (CER/CZ/2017/CELA1) ex *Cervus elaphus*	
	TAB1 (HYB/CZ/2019/TAB1) ex *Hybomitra ciureai*	
	MOVI1 (MEL/CZ/2017/MOVI1) ex *Melophagus ovinus*	
Phlebotomus pernicious	CUL46 (CUL/CZ/2014/CUL46) ex *Cs. annulata* *	21

* Strains obtained during studies of trypanosome vectors in previous years.

2.6. Light and Scanning Electron Microscopy

Dissected positive mosquito guts and samples from prediuresis were fixed on slides with methanol and stained with Giemsa–Romanowski. Slides were examined under the light microscope Olympus BX51 TF with a CDC camera (DP70), and cells were photographed with software QuickPHOTO CAMERA 3.2. ImageJ software was used for the measuring of cell length [63]. Positive guts of *Ae. aegypti* and *Ph. perniciosus* from experimental infections were prepared for scanning electron microscopy (JEOL 6380 LV) as described earlier [33].

2.7. Phylogenetic Analysis

A dataset of the 18S rRNA gene sequences consisted of 238 *T. theileri* sequences from mosquitoes, tabanids, black flies, deer keds, sheep keds, and deer blood. *T. avium* (KT728402), *T. grayi* (KF546526), *T. microti* (AJ009158), and *T. conorhini* (AJ012411) were used as an outgroup. The sequences were aligned by MAFFT [64] with the MAFFT server (https://mafft.cbrc.jp/alignment/server/, accessed on 24 January 2022) and the following algorithms and parameters: G-INS-I, 200PAM/κ = 2, the penalty for the first gap 1.53, offset value 0.0 and N does not affect the alignment score. BioEdit 7.2.5 [65] was used for manual alignment masking. The final dataset consisted of 1800 positions. RAxML 8.2.10 [66] with the GTRGAMMAI model was used for a maximum-likelihood analysis, which was conducted with 100 repeated tree searches. The tree was bootstrapped with 1000 replicates.

3. Results

3.1. Prevalence of *T. theileri* in Mosquitoes

A total of 4051 mosquito females belonging to 18 species were caught in 2017–2019; from these, 3282 were tested by PCR in 560 pools, and 769 specimens were examined by dissection of the gut. The most abundant species were *Cx. pipiens*, *Ae. vexans*, and *Mansonia richiardii*. *T. theileri* was detected in 14 mosquito species belonging to five genera (*Aedes*, *Anopheles*, *Culex*, *Culiseta*, and *Mansonia*). The prevalence ranged from 0.05% in *Cx. pipiens* to 21.7% in *Ae. excrucians* (Figure 1). Findings of *T. theileri* in minority species include *Ae. cataphylla* (1/2), *Ae. sticticus* (1/2), *An. claviger* (1/6), and *An. plumbeus* (2/13). Four tested mosquito species were *T. theileri* negative, *Ae. caspius* (n = 40), *Ae. flavescens* (n = 4), *Cx. modestus* (n = 8) and *Cs. morsitans* (n = 2).

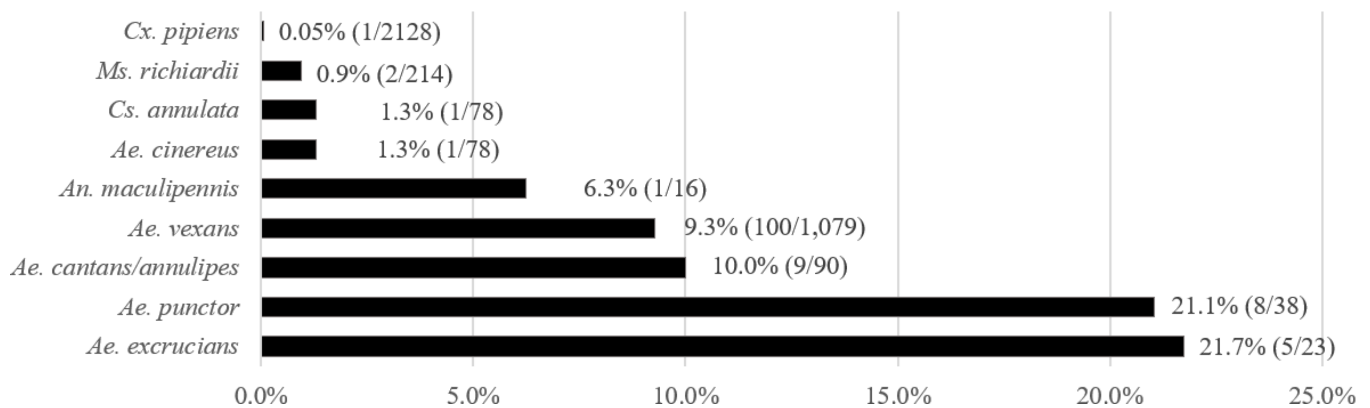

Figure 1. MIR of *T. theileri* for individual species with at least 15 examined individuals. Mosquito species are ordered by prevalence.

3.2. Prevalence of T. theileri in Deer Keds

Three *T. theileri* positive specimens of *Lipoptena fortisetosa* were detected among 224 examined (Table S1). No trypanosomes were detected in *L. cervi* (*n* = 22) and *L.* sp. (*n* = 2). Positive keds originated from two red deer (Milovice forest) and a roe deer (Bystřice). MIR is 1.2% (3/248).

3.3. Prevalence of T. melophagium in Sheep Keds

By PCR, *T. melophagium* was detected in 53 from 79 tested pools (67%, Hořice), and three *T. melophagium* isolates (MOVI1–3) were obtained from sheep keds from Vlkov. A total of 184 sheep keds were examined, and the prevalence of 33% was calculated per site to prevent pseudoreplication when the keds came from the same sheep or herd. For numbers of sheep keds from individual localities, see Table S2.

3.4. Prevalence of T. theileri in Tabanids

Twenty-five tabanids of four species (*Hybomitra ciureai, Tabanus bromius, Haematopota pluvialis*, and *Atylotus leowianus*) were caught in the Milovice forest. Fifteen tabanids (60%) were positive for kinetoplastids by dissection, and subsequent sequencing confirmed *T. theileri* in 11 individuals with the prevalence of 44% (Table 2).

Table 2. *T. theileri* detection in tabanids. *n* specimens: number of tested samples, *n* Kinetoplastid+: number of kinetoplastid-positive specimens, *n T. theileri*+: number of *T. theileri* positive samples confirmed by sequencing.

Species	*n* Specimens	*n* Kinetoplastid + (Prevalence)	*n T. Theileri* + (Prevalence)
Hybomitra ciureai	16	11 (69%)	8 (50%)
Tabanus bromius	6	4	3
Haematopota pluvialis	2	0	0
Atylotus leowianus	1	0	0
Total	25	15 (60%)	11 (44%)

3.5. Comparison of T. theileri Prevalence in Insects

The highest *T. theileri* prevalence of 44% was found in tabanids. *T. theileri* prevalence (18%) in deer keds is counted per mammalian host to prevent pseudoreplication. Overall, in *Aedes* mosquitoes, a prevalence of 7% was counted, and only 1% of blackflies were positive for *T. theileri* (Figure 2).

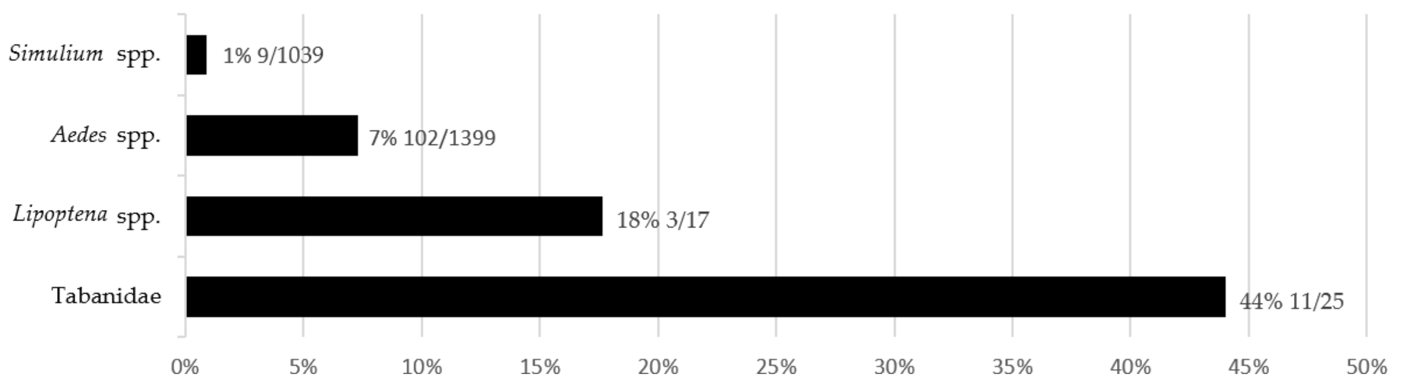

Figure 2. Comparison of *T. theileri* prevalence in bloodsucking insects. The prevalence of mosquitoes, tabanids, and blackflies is counted for insects trapped in the Milovice forest. The prevalence in deer keds was counted for insects collected at multiple localities.

3.6. Detection of *T. theileri* in Deer Blood

We collected 33 deer blood samples from red deer (n = 24), roe deer (n = 7), and fallow deer (n = 2). All samples were PCR negative. However, two out of five samples tested by cultivation were positive for *T. theileri* from red deer in Doupov (CELA1) and Milovice forest (CELA2).

3.7. Experimental Infections of Mosquitoes

3.7.1. Experiments with Cx. *p. quinquefasciatus* and Cx. *p. molestus*

Trypanosomes failed to develop in 95 *Culex* specimens kept at 21 °C (Table S3); weak (n = 2) and moderate (n = 4) infections were detected in *Culex* mosquitoes kept at 15 °C (n = 87) or transferred from initial temperature of 8–11 °C to 15 °C (n = 20). Undigested blood was still observed in the gut of several dissected mosquitoes kept at 8–11 °C after the 21st day. Therefore, only defecated mosquitoes were considered positive. Weak (n = 6) to moderate (n = 4) infections were detected in *Cx. p. quinquefasciatus* (n = 52). In 29 tested *Cx. p. molestus*, moderate (n = 1) and weak (n = 5) infections were found (Figure 3). Moderate infections were localized in the abdominal midgut or hindgut; trypanosomes were in rosettes or present as individual cells. Weak infections were localized in the abdominal midgut.

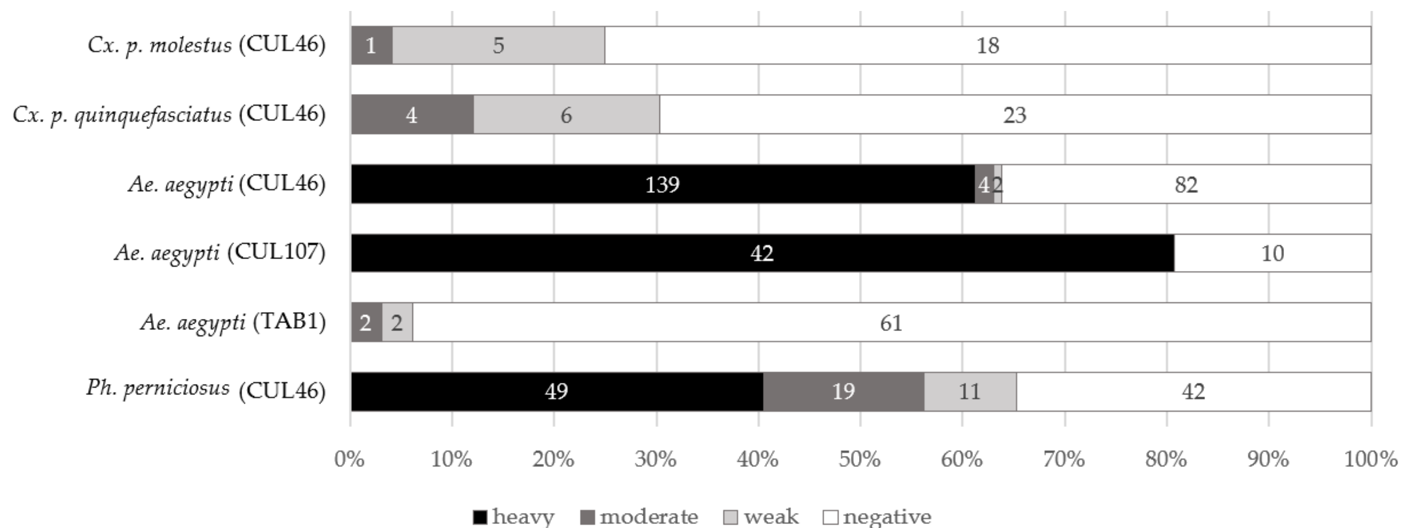

Figure 3. *T. theileri* prevalence in experimentally infected mosquitoes and sandflies. Prevalence for *Culex* spp. is shown for low-temperature experiments only.

3.7.2. Infectious Experiments with *Aedes aegypti*

Aedes aegypti was successfully infected with strains isolated from mosquitoes (*Cs. annulata* and *Ae. vexans*), with prevalence ranging from 47% to 91% (Figure 3). Most of the mosquitoes were heavily infected in both experiments. Trypanosomes formed immotile rosettes localized primarily in the area of the rectal ampulla, but in the case of very heavy infection, they were also found in other parts of the hindgut. Free cells of trypanosomes were round or pear-shaped, not very mobile, and seemed to be aflagellated under the light microscope. Giemsa-stained positive mosquito guts revealed a presence of epimastigotes, sphaeromastigotes, and metacyclic stages. Guts with heavy infection were used for scanning electron microscopy. The parasites were observed with hemidesmosome attached to the intestine wall (Figure 4c,d).

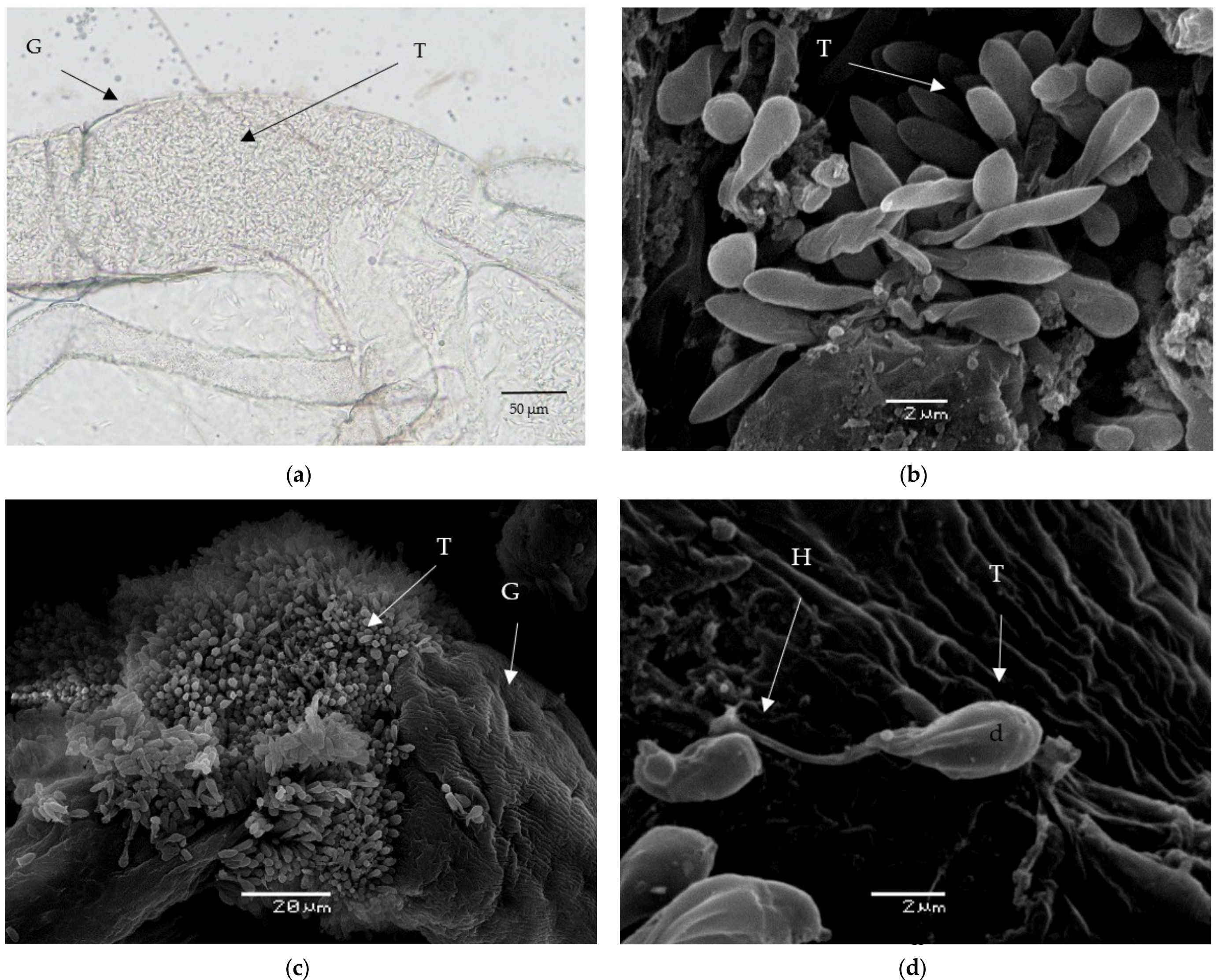

Figure 4. Light and electron microscopy. *T. theileri* in experimentally infected *Ph. perniciosus* (**a**,**b**) and *Ae. aegypti* (**c**,**d**). G—gut, T—trypanosomes in the disrupted gut, H—hemidesmosome.

The susceptibility of mosquitoes was low for the isolate TAB1 (ex *H. ciureai*), with a prevalence of 6%. Two moderate and two weak infections were localized in the rectum. Rosettes were present in one case only. In addition, both motile and immotile unattached parasites were observed.

All mosquitoes experimentally fed on the isolates MOVI1 (ex *M. ovinus*; $n = 36$) and CELA1 (ex *C. elaphus*; $n = 50$ and $n = 38$) were negative.

3.7.3. Experimental Infection of the Sand Fly *Phlebotomus perniciosus*

The prevalence of 65% was detected in *T. theileri*-infected sandflies, and most positive females had heavy infections (Figure 3). Free cells of trypanosomes were noticed in various parts of the gut (rectum, hindgut, abdominal midgut) in weak infections, and rosettes were observed in heavy infections, similar to experiments with *Ae. aegypti.* Moderate and heavy infections were localized in the hindgut, mainly in the rectum. Two types of cells were seen under the light microscope: moving epimastigotes (Figure 4a) and rounded, aflagellated cells with minimal motility. Giemsa-stained positive gut showed a presence of epimastigotes, sphaeromastigotes, and metacyclic stages (Figure 5). Scanning electron microscopy revealed trypanosomes with hemidesmosomes as in *Ae. aegypti* guts (Figure 4b).

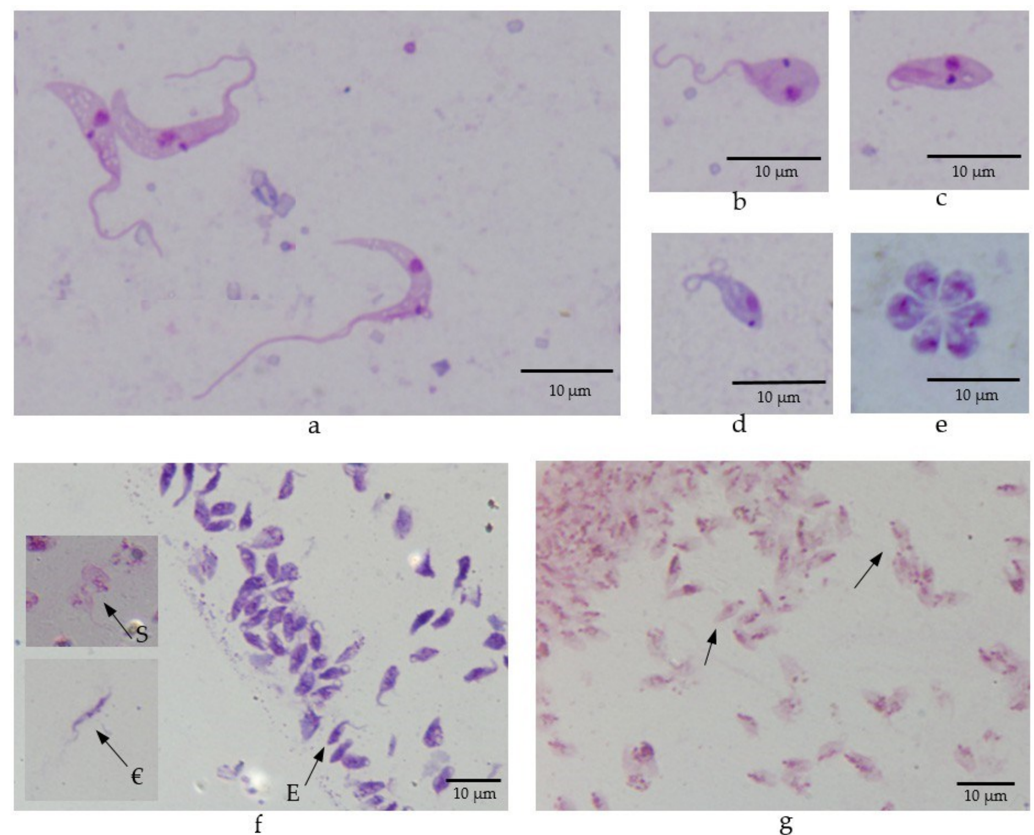

Figure 5. *T. theileri* morphotypes observed after infection of *Ph. perniciosus* (**a**–**d**) or *Ae. aegypti* (**e**) with CUL46 strain (ex *Cs. annulata*): **a**—elongated epimastigotes, **b**—spheromastigote, **c**—droplet-shaped epimastigote, **d**—metacyclic stage, **e**—rosette; (**f**,**g**): *T. theileri* morphotypes in prediuresis experiments with *Ae. aegypti* and CUL46 strain: **f**—elongated epimastigote (inset) (€), droplet-shaped epimastigotes (E), and spheromastigote (inset) (S), **g**—metacyclic stages (trypomastigotes; arrows).

3.8. Morphology of Trypanosomes in Vectors

Epimastigotes and metacyclic stages were observed and measured in the guts of positive tabanids (Table 3). Elongated or droplet-shaped epimastigotes were identified in the abdominal midgut and hindgut of tabanids.

Table 3. Summary table of the measured length of *T. theileri*.

Morphotype	Tabanid Mean (Range) (μm)	Mosquito Mean (Range) (μm)	Sandfly Mean (Range) (μm)	Prediuresis Mean (Range) (μm)
Elongated epimastigote	16.0 (8.9–22.6) *	15.3 (12.6–23.5) *	16.8 (11.4–25.0)	-
Sphaeromastigote	-	8.4 (7.0–10.0) *	7.4 (3.6–13.9)	-
Droplet-shaped epimastigote	7.5 (5.1–11.0)	7.2 (3.3–23.0)	8.8 (5.0–16.5)	5.3 (4.3–7.0)
Metacyclic stages	4.5 (3.9–7.8)	5.0 (3.3–8.0)	5.5 (3.5–7.4)	5.3 (3.8–6.8)

* Less than 15 measured cells.

Trypanosomes originating from the experimental infection of *Ae. aegypti* or *Ph. pernicious* with the strain CUL46 were also measured (Table 3). Elongated and droplet-shaped epimastigotes, spheromastigotes, and infectious stages were observed (Figure 5a–d). The flagella were not always seen in epimastigotes. Some epimastigotes were observed in rosettes (Figure 5e).

Trypanosoma theileri epimastigotes, sphaeromastigotes, and metacyclic stages were observed in six out of 18 coverslips in prediuresis experiments (*Ae. aegypti*, CUL46) (Figure 5f,g). The length of metacyclic stages ranged from 3.1 μm to 6.6 μm, and the average was 5.3 μm.

3.9. Phylogenetic Analysis

The phylogenetic tree of the *Trypanosoma theileri* group as inferred from the 18S rRNA gene is shown in Figure 6. In our 18S rRNA gene tree, TthI was recovered paraphyletic. TthII appeared monophyletic, though with low support (bootstrap support, BS, 51). Sequences AY971802 and AY971803 from tabanids, which were previously placed into the lineage TthII solely based on 18S rRNA gene [6], were not closely related to TthII in our tree (Figure 6, marked with †). We, therefore, do not consider them to belong to TthII. Most of our newly determined sequences (130 out of 170) were placed into the lineage TthII. Some of them were identical or nearly identical to already published sequences, but two novel groups within TthII, mainly consisting of sequences obtained from mosquitoes, were identified (box in Figure 6). Seven new sequences clustered with TthI. The remaining 33 sequences formed a robust clade (BS 99), which was distinct from TthI and TthII; It is here referred to as the putative *T. theileri*-group lineage TthIII. Besides the newly determined sequences from mosquitoes, TthIII contained two previously published sequences from deer [22]. TthIII further split into two clades (BS 86 and 65, respectively).

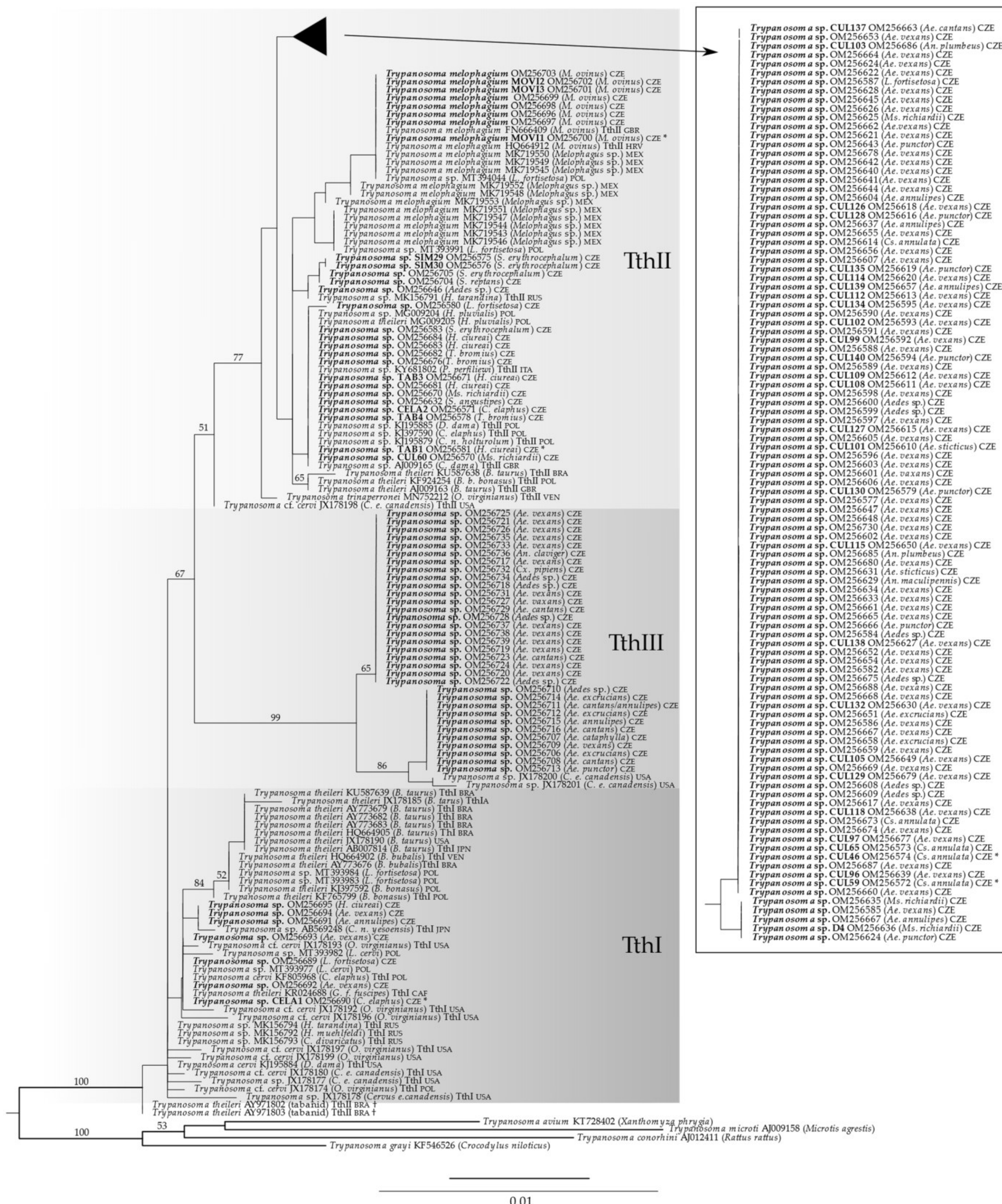

Figure 6. Phylogenetic tree of the *Trypanosoma theileri* group based on the 18S rRNA gene analysis. The tree was constructed using the maximum-likelihood method in RAxML (GTRGAMMAI model). Bootstrap values are shown at nodes. Newly determined sequences in bold. Cultures used in the infectious experiments are marked with asterisks. Daggers mark sequences which we do not consider to belong to TthII. The host species name is given in parentheses, followed by reported genotypes and abbreviations of the country of origin.

4. Discussion

Trypanosoma theileri has been recently detected in several potential vector groups (keds, mosquitoes, and a sandfly). However, the relevance of these findings is still unclear since the lifecycle was not confirmed, and metacyclic stages were reported only in tabanids (*T. theileri*) [23,67] and sheep keds (*T. melophagium*) [2,49,50]. Our study focused not only on the molecular detection of *T. theileri* in bloodfeeding insects and mammalian hosts but also on its development and infectious stages occurrence in the intestine of potential vectors to assess the lifecycle and host/vector range of this species. We detected *T. theileri* in various bloodsucking Diptera, and it is obvious that it is not only tabanids and keds that can transmit these trypanosomes.

Similar to previous studies, we have detected *T. theileri* lineage TthI and TthII; in addition, a third lineage, whose existence was revealed previously [6], was here designated as TthIII. Some mosquito *T. theileri* sequences created separate groups in the TthII and TthIII lineages. Furthermore, we revealed different vectorial specificity of lineages since Brachycera transmit the TthI while other lineages are transmitted by both Brachycera and Nematocera.

Several mosquito species were infected with *T. theileri,* some of which had a high prevalence (22% in *Aedes excrucians*). *Aedes* mosquitoes are considered opportunistic or mammalophilic [68,69]; the abundance of mammals is high in some of the studied localities (game reserve Milovice forest), enabling intensive circulation of the parasite. Contrary to the genus *Aedes*, *Culex* mosquitoes are considered ornithophilic [70]; the low prevalence of *T. theileri* in this genus is thus not surprising. Nevertheless, a single positive specimen confirms the willingness of *Culex* to feed on mammals reported previously [41,70]. The detected prevalence of 7% in *Aedes* mosquitoes (including nulliparous females) and heavy, mature infections in naturally infected mosquitoes suggests that they are effective vectors of *T. theileri*.

Trypanosoma melophagium was isolated from sheep keds with a prevalence of 67% in Hořice, similar to a previous study from Scotland [20]. The high prevalence is influenced by the fact that sheep keds do not leave their host and suck blood daily [71], so the probability of being positive is high when a sheep is infected [21].

The keds *Lipoptena cervi* and *L. fortisetosa* were collected from the cervids. In *L. cervi,* we did not detect *T. theileri* trypanosomes, possibly due to a small number of tested keds. However, *T. theileri* was found in *L. fortisetosa*. *T. theileri* was detected by PCR in both deer ked species in Poland [40]. However, PCR alone is not sufficient to confirm the transmission potential of a positive vector [72], and we did not find any infection in dissected keds. Therefore, it remains unclear if *L. fortisetosa* is a vector. Böse and Petersen [39] described rosettes and epimastigotes in the intestine of *L. cervi* but did not report any metacyclic stages; they did not perform transmission experiments either. Similarly, *L. mazamae* was predicted as a possible vector of the recently described *Trypanosoma trinaperronei* [6], which was detected in this ked species by PCR, but neither development in the intestine nor metacyclic stages were described in the study. After finding a host, deer keds drop their wings, limiting their potential to switch hosts. However, the exchange of keds among animals in a herd by direct contact is possible, although not to the same extent as in sheep herds and *M. ovinus*, where the animals are in close contact [71]. Moreover, a recent study has detected trypanosomes in unfed, winged deer keds [40]. This observation begs a hypothesis of *T. theileri* transmission from adult females to larvae through feeding glands in keds [40]. *Lipoptena* spp. could have a role as additional vectors. Since keds feed on their host frequently, and blood is present in their gut permanently [56], PCR positivity does not necessarily prove keds as specific vectors of *Trypanosoma theileri* [21,72].

Tabanids such as *Tabanus bromius* have been previously confirmed as *T. theileri* vectors of cattle and deer trypanosomes [23]. The high prevalence detected during our study (44%) is slightly higher than those detected in Poland (34%) or Russia (31%) [38,73] and suggests a significant role of tabanids in *T. theileri* transmission at these study sites. We also record *T. theileri* in *H. ciureai* for the first time.

This is the first record of *T. theileri* in blackflies where the bloodmeal was detected in only one specimen out of nine positive. Black flies thus could have an additive role in transmission, but the prevalence was low (1%).

In the case of vertebrate host blood, *T. theileri* was detected using cultivation in two samples, while PCR gave negative results in all 33 specimens of blood. Blood cultivation seems to be a more sensitive diagnostic method; however, it is prone to contamination with yeast and bacteria [74,75], especially when using blood from shot animals.

Experiments with laboratory-bred vectors correspond with field observations. *Ae. aegypti* was highly sensitive to *T. theileri* clade II infection with 67% infected specimens and 97% of heavy infections, and the occurrence of metacyclic stages, identical to those previously described from tabanids and sheep keds [2,37,38,49,50]. Furthermore, in the infected gut, the length of epimastigotes (12.7–23.5 μm) corresponded to the earlier observation of epimastigotes that proliferate into shorter cells [2,37]. Both the observed infection intensity and cell morphology, therefore, support our conclusion that *Aedes* mosquitoes are competent vectors. Most importantly, *T. theileri* metacyclic stages were detected in the urine of infected mosquitoes during our prediuresis experiments. It has been confirmed experimentally that mammalian trypanosomes can be transmitted through conjunctiva [34]. In conclusion, *Aedes* mosquitoes can be considered vectors of *T. theileri* clade II trypanosomes.

On the other hand, the role of *Culex* mosquitoes as vectors is likely negligible. In the infectious experiments, *Culex* mosquitoes were not infected with *T. theileri* at 21 °C or 15 °C, but a few moderate infections developed at 8–11 °C, which slowed down blood digestion, causing delayed defecation. In wild mosquitoes, only one out of 2128 tested *Culex* mosquitoes harbored *T. theileri*. Nevertheless, this infection was mature, without blood in the dissected gut, opening the potential of *Culex* mosquitoes as bridging vectors. Moreover, experiments with different environmental temperatures, which affect pathogen development, attachment, or invasion of the gut wall and influence transmission to a new host [62,76–78], showed a few weak and moderate infections only at a lower temperature.

The infection experiments confirmed some extent of vector specificity among different *T. theileri* genotypes. Infections of laboratory-bred *Aedes aegypti* have been successful only using mosquito isolates (CUL46 *Culiseta annulata*, CUL107 *Aedes vexans*). Experimental infections with a sheep ked isolate were unsuccessful, agreeing with sheep keds as specific vectors of *T. melophagium*, and only a few moderate/weak infections were observed using a tabanid isolate. Negative results were obtained with the deer isolate CELA1, which, unlike the cultures obtained from the insects, belongs to the TthI clade.

Interestingly, a sandfly species, *Ph. perniciosus,* was successfully infected by our mosquito isolate belonging to the *T. theileri* TthII lineage. Heavy infections were observed in the hindgut, similar to the finding of a single naturally infected specimen of *Ph. perfiliewi,* which belongs to the same subgenus, *Larroussius* [42]. In addition, short epimastigotes, sphaeromastigotes, and metacyclic stages were observed. These results suggest that phlebotomine sandflies could serve as additional vectors of the *T. theileri* TthII lineage.

Analysis showed that our sequences belong to both the lineages, and a part of them belong to the putative TthIII lineage. Most of our sequences differed from the sequences available in GenBank. The TthI lineage mainly consists of sequences originating from bovids, cervids, and tabanids; these putative vectors were sampled in Brazil and Russia [16,38]. Besides finding the TthI lineage in one specimen of a horse fly *H. ciureai*, we found it in one deer ked *L. fortisetosa* and four *Aedes* mosquitoes. Mosquitoes harboring *T. theileri* TthI lineage contained undigested blood, which suggests that they are likely not the specific vectors. Based on the available data, the *T. theileri* TthI lineage is probably transmitted by Brachycera, since most sequences of vectors originate from tabanids.

On the other hand, the TthII lineage included all tested mammalophilic Diptera, including the sequence obtained from a sand fly and sequences from blackflies, and newly identified potential vectors. Since only one out of the nine examined blackflies had blood in the intestine, their vectorial role is highly probable. Furthermore, *T. theileri* TthII trypanosome was previously found in five out of 4512 screened specimens of biting midges

(*Culicoides obsoletus, C. pulicaris, C. punctatus*) [79], which supports wide vectorial specificity that has been postulated previously [38]. However, most of the vector sequences were obtained from mosquitoes that belonged to the genera *Anopheles*, *Culiseta*, *Mansonia*, and, most frequently, *Aedes*. Mosquitoes thus represent a substantial part of the vectors.

Experimental infections of mosquitoes further support the results obtained during field sampling. Strains of one TthII genotype, both isolated from mosquitoes, gave high infection rates and intensities in laboratory-bred vectors (mosquitoes and sand flies). Nevertheless, the development of other strains was not supported; namely TthI isolate CELA1 from deer, and TthII isolate MOVI1 from *T. melophagium* and TAB1 from *H. ciureai* were not infectious for mosquitoes. A possibility thus still exists that there is some extent of vectorial specificity among the genotypes of TthII clade.

The results have shown a very high diversity of vectors of the *T. theileri* group. *Aedes* mosquitoes probably play a crucial role in transmitting some genotypes of *T. theileri*-related trypanosomes.

5. Conclusions

We conclude that mosquitoes of the genus *Aedes* are competent vectors of *T. theileri* TthII and putative TthIII trypanosome groups. Infection probably occurs by vector ingestion or prediuresis. Phlebotomine sandflies have the potential to serve as additional vectors. Mosquitoes host diverse *T. theileri* TthII lineages; TthI lineages are transmitted by bloodsucking Brachycera, while *T. theileri* trypanosomes from TthII have a wide variety of bloodsucking vectors in Diptera.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms10020294/s1, Table S1: Results of deer keds screening for trypanosomes, Table S2: Results of *sheep* keds screening for trypanosomes. Table S3: Infection rates of *Culex* mosquitoes kept at different temperatures after feeding.

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Kulich Fialová, **M.**, Kapustová, A., Čepička, I., & Svobodová

Trypanosoma tertium **n. sp.: How many trypanosome species are transmitted by mosquitoes?**

(in preparation for *Parasitology*)

1 # *Trypanosoma tertium* n. sp.: How many trypanosome species are
2 transmitted by mosquitoes?

3

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Abstract

Avian trypanosomes (*Trypanosoma*, Kinetoplastea) are successful blood parasites occurring worldwide. These parasites are usually non-pathogenic to their avian hosts, thus neglected in studies regarding their life cycles and vectors. Various dipteran families of blood sucking insect have been identified as vectors of avian trypanosomes, including mosquitoes, which have been experimentally confirmed as vectors of *T. culicavium* and *T. thomasbancrofti.* In this study, we describe a third avian trypanosome found in mosquitoes, designated as *Trypanosoma tertium* n. sp. This species can be distinguished from related trypanosome species on the basis of morphology and SSU rRNA gene sequence. Two isolates of *T. tertium* obtained from a mosquito and a bird host, respectively, were able to infect two subspecies of laboratory *C. pipiens* mosquitoes with infection rates reaching 60% and heavy infections in 90% of positive females. In infected mosquitoes, trypanosomes occurred as long epimastigotes in midgut and short epimastigotes and rosettes in hindgut. Putative infectious stages were detected in the prediuretic liquid of infected mosquitoes, suggesting transmission through ingestion of the infected vector or transconjunctivally. Wild mosquitoes and birds were investigated to determine prevalences of *T. tertium*. Among mosquitoes, avian trypanosomes were detected exclusively in *C. pipiens*, with 3.3% prevalence (0.08% for *T. tertium*) among 1128 dissected individuals. In birds, *T. tertium* was detected in eight different species within which the prevalence was 1.3% (686 birds) while it was 0.3% in total (3084 birds). This study describes a new species of avian trypanosomes infecting mosquitoes, *T. tertium*, and discusses its relationship with the other mosquito-transmitted trypanosomes.

Keywords: avian blood parasite; *Trypanosoma*; *Culex*; vector; transmission, monoxenous Kinetoplastea

42 ## Introduction

43 Protists of the genus *Trypanosoma* (Trypanosomatidae, Kinetoplastea) are digenetic blood
44 parasites transmitted by bloodsucking invertebrates. They infect virtually all vertebrate groups,
45 and are known for causing illnesses in humans and animals such as Chagas disease, sleeping
46 sickness, nagana, etc. Trypanosomes also readily infect birds, with a relatively high prevalence
47 (Bennett *et al.*, 1974; Greiner *et al.*, 1975; Sebaio *et al.*, 2012; Zamora-Vilchis *et al.*, 2012;
48 Galen *et al*., 2020; Svobodová *et al*., 2023). However, due to their low health and economic
49 impact, among bird parasites, avian trypanosomes remain largely neglected.

50 Little attention is paid to the vectors of avian trypanosomes as well, despite their essential
51 role in maintaining the parasites in the host populations. Various insect species have been
52 identified as vectors of avian trypanosomes, including black flies (Simuliidae, Bennett, 1961;
53 Votýpka *et al*., 2002; Votýpka and Svobodová, 2004), hippoboscid flies (Hippoboscidae,
54 Baker, 1956; Votýpka *et al*., 2002; Santolíková *et al*., 2022), mosquitoes (Culicidae, Bennett,
55 1961; Votýpka *et al*., 2012; Fialová *et al*., 2021), biting midges (Ceratopogonidae, Miltgen and
56 Landau, 1982; Svobodová *et al*., 2017; Bernotienė *et al*., 2020), and sand flies (Psychodidae,
57 Kato *et al*. 2011; Svobodová and Rádrová, 2018). Avian trypanosome mature infections are
58 localized in the intestine (midgut, hindgut) of their vectors and the most common transmission
59 to birds is peroral, by ingestion of an infected vector (Baker, 1961; Votýpka and Svobodová,
60 2004; Votýpka *et al*., 2012; Svobodová and Rádrová, 2018; Fialová *et al*., 2021). An alternative
61 way of transmission is through the conjunctiva, when metacyclic stages from vector hindgut
62 are expelled with the prediuretic liquid while the vector feeds (Votýpka and Svobodová, 2004;
63 Fialová *et al*., 2021).

64 Mosquitoes are notoriously associated with the transmission of the protozoan genus
65 *Plasmodium,* the causative agent of mammalian and avian malaria. In contrast, their role in the
66 life cycle of trypanosomes is less explored. However, as early as in 1843, mosquitoes were

67 identified as vectors of *Trypanosoma rotatorium*, the anuran species (Mayer, 1843 in Desser *et*
68 *al*., 1973) and they were also among the first suspected vectors of avian trypanosomes (Novy
69 *et al*., 1907; Bennett, 1961, 1970; Chatterjee, 1977). Presently, three species/lineages of avian
70 trypanosomes infecting mosquitoes are recognized, namely: *Trypanosoma culicavium*,
71 *Trypanosoma thomasbancrofti* and *Trypanosoma* sp. lineage III (Votýpka *et al*., 2012; Zídková
72 *et al*., 2012; Šlapeta *et al*., 2016; Fialová *et al*., 2021). While *T. culicavium* and
73 *T. thomasbancrofti* life cycles have been experimentally confirmed, the life cycle of the
74 *Trypanosoma* sp. lineage III found in mosquitoes remains unknown.
75 Our objective was to characterize the least known avian trypanosome found in mosquitoes
76 – *Trypanosoma* sp. lineage III. Together with its sister species *T. thomasbancrofti*, this
77 trypanosome belongs to the group C, which includes *T. avium* s. l. lineages as well (Zídková
78 *et al*., 2012). This study focused on experimental life cycle and prevalences of this
79 uncharacterized avian trypanosome in avian hosts and mosquitoes.
80

81 **Materials and methods**

82 *Prevalence in naturally infected mosquitoes*

83 Mosquitoes were trapped in July between years 2018-2021 in Milovice forest, South Moravia,
84 Czechia (48.8213 N, 16.6932 E) using CDC traps (JW, Hock Company, Gainesville, FL, USA)
85 with CO_2 as an attractant. Mosquitoes were collected into nylon nets connected to the traps,
86 anesthetized on ice, and sorted according to species (Kramář, 1958). Individual mosquitoes
87 were washed in ethanol and saline and dissected using tweezers (Dumont tweezers, Ted Pella,
88 California, USA), which were sterilized in flames between each individual to prevent cross
89 contamination. Dissected guts were examined under the light microscope for the presence of
90 kinetoplastids. A part of positive guts was used for the cultivation of kinetoplastids, and the
91 rest was stored in ethanol for barcoding of the parasites (see below).

92 *Prevalence in wild birds*

93 Bird sampling was done at several Czech localities in 2014-2016 between May and July. Adults
94 and yearlings were mist-netted by the authors or in cooperation with other registered ringers
95 contributing to the program Constant Effort Site (CES), organized by the Prague Ringing
96 Centre. Trapping and sampling were done by licensed workers according to national law and
97 experimental guidelines. Blood was collected as described in Fialová *et al*., (2021); a part was
98 used for trypanosome cultivation; a part was stored in ethanol at -20 °C prior to further use.
99 Blood smears were prepared as well.

100

101 *Parasite strains and cultures*

102 Both trypanosome strains used in this study originated from our own collection and were
103 acquired in Milovice forest: CUL5 was isolated from a *Culex pipiens* female
104 (ICUL/CZ/2000/CUL5) and PAS416 from a willow warbler (*Phylloscopus trochilus*)
105 (APHY/CZ/2016/PAS416). The isolate CUL5 was assigned to the lineage III of group C
106 previously (Zídková *et al*., 2012), PAS416 was barcoded as described below.

107 Trypanosomes were cultivated in flat tubes or PEN tubes (diagnostic cultivation) on blood agar
108 (SNB-9) prepared from rabbit (Bioveta, Ivanovice na Hané, Czech Republic) or sheep
109 (LabMediaServis, Jaroměř, Czech Republic) blood, covered with liquid medium. Liquid
110 medium was prepared by mixing 1:1 RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) and
111 Schneider's Drosophila Medium (Sigma-Aldrich), supplemented with 10% FCS (Gibco,
112 Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2% sterile human urine, and 50 µg/mL
113 amikacin (Medochemie, Prague, Czech Republic). Media for isolation used in field studies
114 were supplemented with penicillin (10 000 IU/mL, BB Pharma a.s., Prague, Czechia) and
115 fluorocytosine (1500 µg/mL, TCI, Japan). Cultures were held at 23 °C.

116

117 *Experimental infection of mosquitoes*

118 Two subspecies of *C. pipiens* were used in this study; both were bred in our laboratory; *Culex*
119 *pipiens quinquefasciatus* (*C. p. quinquefasciatus* henceforth) originating from India, kept in
120 our laboratory for more than 30 years and *Culex pipiens molestus* (*C. p. molestus* henceforth)
121 colonized from individuals caught in Czechia recently (2016). A colony of *Ae. aegypti* was
122 temporarily established; mosquitoes were obtained from The National Institute of Public
123 Health, Czech Republic.

124 Mosquito females were infected by feeding through a chick skin membrane on a glass feeder
125 containing inactivated (30 min in 56 °C water bath) sheep or rabbit blood mixed with 12–18-
126 day-old cultures of trypanosomes (2–6 × 10^8 parasite cells/ml). After blood feeding, the blood
127 fed to mosquitoes was always controlled under the microscope for the presence of live
128 trypanosomes. Fed females were separated and kept in nets in an incubator with stable
129 conditions (21 °C, ambient humidity). Fed females were provided with a 50% sucrose solution
130 on a cotton pad. In a single experiment, the same procedure was followed with
131 *C. p. quinquefasciatus* and *Ae. aegypti* mosquitoes as control group, in experiment with isolate
132 CUL5.

133 Mosquitoes were dissected 10-45 days post-infection. Dissected guts were examined under the
134 light microscope for infection status, intensity of infection, parasite localization and its changes
135 during the course of infection. Infection intensities were defined as low, 1–100 parasites;
136 medium, 100–1000 parasites; and strong, >1000 parasites per gut.

137

138 *Prediuresis*

139 Mosquitoes membrane-fed on blood with strain PAS416 were provided with a bowl of water
140 to allow oviposition. After oviposition, mosquitoes were provided with an anesthetized
141 laboratory mouse. The feeding was monitored. Fully fed females were immediately transferred

142 into plastic drosophila tubes with a coverslip placed on the bottom, which served to catch
143 droplets of prediuretic liquid. Air-dried droplets were fixed with methanol, stained with
144 Giemsa, and examined for the presence of trypanosomes. Mosquito females were dissected to
145 assess their infection status.
146
147 *Experimental inoculation of birds*
148 All experimental birds were screened for the presence of trypanosomes prior to inoculation.
149 Screening was done with blood obtained from vein articulation (vena metatarsalis plantaris
150 superficialis media) using cultivation (see above) and PCR screening (see below). All birds
151 were negative.
152 Birds were inoculated with 7-10 *C. p. molestus* or *C. p. quinquefasciatus* infected guts
153 homogenized in saline, applied orally, subcutaneously, or by placing on the conjunctiva. Birds
154 were tested for the presence of trypanosomes by blood cultivation in weekly intervals (four
155 weeks), then monthly (five months); obtained cultures were checked microscopically three
156 times in weekly intervals.
157
158 *PCR diagnostic*
159 High Pure PCR Template Preparation Kit (Roche Diagnostic, Mannheim, Germany) was used
160 for DNA extraction. Obtained DNA was subsequently stored at −20°C until further use.
161 Amplification of trypanosome SSU rRNA gene from bird blood and dissected guts of
162 mosquitoes was done by specific nested PCR, cultures of trypanosomes from mosquito guts
163 were barcoded by single step PCR as described (Fialová *et al.*, 2021). Obtained PCR products
164 were purified using the enzymatic solution ExoSap (Thermo Scientific, Waltham, MA, USA),
165 then sequenced at the core facility of the Faculty of Science. Sequences were examined in the
166 BioEdit software, and analysed using the BLAST algorithm and nucleotide database NCBI.

167 *Scanning electron microscopy*

168 Guts positive for trypanosomes after experimental infection of mosquitoes were torn by insulin
169 syringe then fixed in 2.5% glutaraldehyde in 5 mM HCl, 0.1 M cacodylate buffer for 24 h at
170 4 °C. Thereafter, they were processed at our core facility, the Laboratory of Electron
171 Microscopy as follows: samples were post-fixed in 2% osmium tetroxide in the same buffer
172 for 2 h at room temperature. After dehydration in a graded ethanol series, the guts were critical-
173 point air-dried, sputter-coated with gold in a Polaron coater, and examined by the authors using
174 a JEOL 6380LV scanning electron microscope.

175

176 *Light microscopy and measurement of trypanosomes*

177 Positive guts of mosquitoes from experimental infections were fixed on slides with methanol
178 and stained with Giemsa stain, photographed at 1000x magnification with a CDC camera
179 (DP70) using an Olympus BX51 microscope. Measurement of the cells was done using ImageJ
180 software. Data were processed using the R software as described in (Fialová *et al*., 2021).
181 Blood smears (fixed with methanol and stained with Giemsa stain) of birds positive by PCR
182 for *Trypanosoma* sp. lineage III were inspected under the microscope at magnification x1000
183 for 10 min, and the whole smear area at x200.

184

185 **Results**

186 *Prevalence of Trypanosoma tertium n. sp. in wild caught mosquitoes*

187 Between 2018 and 2021, 2246 wild-caught mosquitoes were dissected; the most of mosquitoes
188 were identified as *C. pipiens,* the rest were mosquitoes of the genera *Aedes* (*Ae. annulipes,*
189 *Ae. cantans, Ae. caspius, Ae. cinereus, Ae. excrucians, Ae. punctor, Ae rusticus, Ae. sticticus,*
190 *Ae. vexans*), *Anopheles* (*An. claviger, An. maculipennis, An. plumbeus*), *Culiseta annulata*,
191 and *Mansonia richiardii*. All species of mosquitoes infected with kinetoplastids were
192 investigated by PCR, however avian trypanosomes transmitted by mosquitoes (*T. culicavium,*
193 *T. tertium, T. thomasbancrofti*) were found exclusively in *C. pipiens* mosquitoes with
194 prevalence 3,3% between 1128 dissected individuals. Furthermore, *Culex* mosquitoes
195 harboured mammalian *T. theileri,* as well as monoxenous kinetoplastids of the genus *Crithidia*
196 and *Paratripanosoma* (for parasite species and prevalences, see **Table 1**). Additionally,
197 mammalian *T. theileri* was identified also in *Aedes, Anopheles, Culiseta* and *Mansonia,*
198 monoxenous kinetoplastids in *Aedes* and *Mansonia.*

199

200 *Prevalence of Trypanosoma tertium in wild caught passerines*

201 The prevalence of *T. tertium* was monitored among passerines caught between 2014 and 2016,
202 *T. tertium*. was detected in 9 (0.3%) of the 3084 sampled individuals. Infected species included
203 eurasian reed warbler (*Acrocephalus scirpaceus*, 1 individual), barn swallow (*Hirundo rustica*,
204 1), spotted flycatcher (*Muscicapa striata*, 1), chiffchaff (*Phylloscopus collybita*, 1), willow
205 warbler (*Phylloscopus trochilus*, 1), sand martin (*Riparia riparia*, 1), Eurasian blackcap (*Sylvia*
206 *atricapilla*, 2) and Lesser whitethroat (*Sylvia curruca*, 1).
207 If considered only in these species, the prevalence was 1.3% (n = 686), and if considered in
208 these genera, the prevalence was 0.8% (n = 1037). The samples were either positive by PCR
209 (two samples) or cultivation (seven samples); in three cases the alternative diagnostic method

210 revealed coinfection with another trypanosome lineage (lineages from *T. everetti/bennetti*
211 group twice, *T. culicavium* once). Only a single trypomastigote was found on all the inspected
212 slides obtained from positive birds (**Figure 3**l)
213
214 *Experimental infections of mosquitoes*
215 Two subspecies of *Culex pipiens* - *C. p. quinquefasciatus* and *C. p. molestus*, were fed on blood
216 with two different strains of *T. tertium* originating from two different hosts: CUL5 (mosquito)
217 and PAS416 (bird). The CUL5 isolate was able to develop infection in 45% of *C. p. molestus*
218 and 55% of *C. p. quinquefasciatus,* with 100% of heavy infection in *C. p. molestus* and 90%
219 of heavy infection in *C. p. quinquefasciatus.* Mosquitoes were also highly susceptible to the
220 avian isolate, with approximately 65% prevalence in both *Culex pipiens* subspecies, and with
221 89% and 75% of heavy infections in *C. p. molestus* and *C. p. quinquefasciatus,* respectively
222 (**Figure 1**).
223 To assess the vector specificity of *Culex* mosquitoes, 40 females of *Aedes aegypti* fed on a
224 blood with CUL5 were dissected. None of the females were found to be positive. Control group
225 of *C. p. quinquefasciatus* fed on the same inoculum were infected in 60% of cases.
226 Trypanosomes in guts dissected 12 days post infection (dpi) were localized mainly in midgut
227 as unattached long epimastigotes along with rosettes in hindgut, or exclusively in midgut as
228 unattached epimastigotes (**Figure 2**). From 15 dpi onwards, the ratio of individuals with
229 trypanosomes present only in hindgut as rosettes increased slowly, while the proportion of
230 infections in hindgut and midgut or exclusively in midgut decreased. At day 43-45, there were
231 only rosettes were present exclusively in the hindgut. Infections with rosettes in midgut were
232 never detected.
233
234

235 *Prediuresis*

236 Stages of *T. tertium* were observed on three out of seven Giemsa-stained droplets of prediuretic
237 liquid of examined mosquitoes. The expelled cell types were short trypo/epimastigotes.
238 (**Figure 3**k)

239

240 *Light microscopy*

241 Three morphologically different types of trypanosomes were found in guts infected with
242 *T. tertium* isolates PAS416 and CUL5: long and short epimastigotes and short promastigotes
243 forming rosettes (**Figure 3**a,b,d-h). In cultures, two different morphotypes were present: long
244 and short epimastigotes (**Figure 3**i,j).

245 To compare *T. tertium* cell lengths based on the isolate (CUL5, PAS416), origin (gut, culture),
246 and cell form (long or short epimastigote, rosette, prediuresis), the analysis of variance was
247 used. The isolate as a factor had no significant impact ($F=3.86$; $P>0.05$) while both the origin
248 and the cell form were significant ($F=101.19$ and 315.40, resp., $P<0.001$). Therefore, the
249 measurements of the two isolates were merged for Tukey post hoc comparison, which revealed
250 that both gut and culture forms of short epimastigotes and rosettes are not significantly different
251 from the prediuresis form, while both long epimastigotes from the gut and culture differ from
252 all remining groups (see **Table 2**). Interestingly, cell lengths of long epimastigotes from
253 cultures and guts are also significantly different.

254

255 *Inoculation of experimental birds*

256 Laboratory birds (canaries and zebra finches) were inoculated with mosquito guts infected with
257 CUL5 or PAS416, respectively. Birds were inoculated perorally, subcutaneously (CUL5,
258 PAS416) or transconjunctivally (PAS416) with heavily infected guts.

259 The isolate CUL5 was used for peroral inoculation of 1 zebra finch and 5 canaries. The isolate
260 PAS416 was used for 8 peroral and 8 subcutaneous inoculation of canaries, 2 subcutaneous
261 inoculation of zebra finches and 2 transconjunctival inoculation of canaries. All birds remained
262 negative till the end of experiment (6 months).
263

264 ## Discussion

265 Despite advances in the last decade, our knowledge of vectors associated with avian
266 trypanosomes and modes of their transmission still has gaps. The spectrum of avian
267 trypanosome vectors includes members of multiple dipteran families; among them, three
268 species are transmitted by mosquitoes: *T. culicavium* (Zídková *et al*., 2012; Votýpka *et al*.,
269 2012), *T. thomasbancrofti* (Zídková *et al*., 2012; Šlapeta *et al*., 2016; Fialová *et al*., 2021),
270 *T. tertium* n. sp., described in this study.

271 Based on phylogenetic studies, it is evident that *T. tertium* is a unique lineage of avian
272 trypanosomes. It is closely related to *T. thomasbancrofti,* and both fall into *T. avium* s. l. group
273 C. They share some characteristics, e. g., mature infections are localized in the vector´s hindgut,
274 similarly to other members of the group whose proven vectors are blackflies (group C linages
275 X+XI). On the contrary, *T. culicavium* lineage (group B) is localized on the stomodeal valve
276 of the vector. However, *T. corvi,* hippoboscid fly species, from the same group, has infectious
277 stages localized in the hindgut, pointing out to the fact that although the localization is
278 important for the ways of transmission, it is not related to the phylogenetic position of the
279 parasite.

280 Findings of mosquito trypanosomes have been reported already in older studies. *T. noctuae,*
281 described by Schaudinn (1904), was reported to infect mosquitoes after feeding on an infected
282 little owl (*Athene noctua*). However, it is likely that Schaudinn worked with monoxenous
283 kinetoplastids from the genus *Crithidia* (see Novy *et al*, 1907). A second species reported from
284 *C. pipiens* mosquitoes is *T.* (*Herpetomonas*) *culicis* (Novy *et al.*, 1907). Novy et al., described
285 this trypanosome from the intestines of naturally infected mosquitoes and reported two
286 morphological forms. "Very long forms" measured 30-35 µm while “short forms” ranged 12-
287 20 µm. The long epimastigotes of *T. tertium* documented in this study measured 55 µm on
288 average, almost twice as much; such a difference is substantial. Novy *et al.* also describe fast

289 movement of long forms from gut, which makes them difficult to follow. We did not observe
290 such movement of long epimastigotes of *T. tertium.* The size of short epimastigotes (average
291 9-11, maximum 14 µm) overlaps with that of *T. culicis* short epimastigotes but is still different;
292 moreover, short forms in the intestine can be found in many other trypanosome lineages
293 belonging to all three groups (Zídková et al. 2012). In addition, Novy *et al*. do not mention any
294 stages similar to the rosette forms found in the intestines of mosquitoes infected by *T. tertium*.
295 For these reasons, we consider *T. culicis* as a separate species. In 1961, *T. culicis* was
296 transferred to the genus *Blastocrithidia* by Wallace and Johnson. However, we consider this
297 inappropriate since the authors did not work with the original isolate; on the contrary, they used
298 their own isolate originating from a different mosquito species and genus, *Aedes vexans*.
299 During our studies, we failed to find any *Aedes* mosquitoes infected by *T. tertium* despite a
300 substantial number of examined specimens (959 individuals in this study, 1398 individuals in
301 Brotánková *et al*., 2022). There is a considerable degree of host specificity of mosquito
302 trypanosomes, since neither *T. culicavium* nor *T. thomasbancrofti* were ever detected in *Aedes*
303 mosquitoes (Fialová et al., 2012; Brotánková *et al*., 2022). Moreover, *Aedes* mosquitoes were
304 not susceptible to the infection by *T. tertium* in our experiments, nor to *T. culicavium* (Votýpka
305 *et al*., 2012). It is thus not probable that different mosquito genera would share their
306 trypanosomes; this extends to the mosquito/mammalian *T. theileri* as well (Brotánková *et al*.,
307 2022) and to hippoboscid/avian trypanosomes as well (Santolíková *et al*., 2022)

308 Mosquitoes naturally infected with *T. tertium* have been found in Czechia for the first time
309 in 1999 and 2000 (Zídková *et al*., 2012); from 28 strains established from 898 examined
310 *C. pipiens* guts, two belonged to *T. tertium* (prevalence 0.22%, Svobodová *et al.*, 2015). Only
311 one individual out of 1128 dissected *C. pipiens* mosquitoes harboured *T. tertium* in our study
312 (prevalence 0.08%). The prevalence of *T. tertium* was comparable to that of

313 *T. thomasbancrofti,* but notably lower than the prevalence of *T. culicavium* (3.1 %), similarly
314 to previous findings (Svobodová et al., 2015).
315 The prevalence among avian hosts was likewise low, with only 9 (0.3%) out of the 3084
316 screened passerines found to be infected by *T. tertium*. Since the mode of transmission of
317 *T. tertium* is probably similar to *T. culicavium* or *T. thomasbancrofti*, i.e. after ingestion of the
318 infectious vector, it is appropriate to focus on insectivorous avian species when assessing
319 prevalence; then, the prevalence of *T. tertium* rises to 1.3%. In our previous studies in the years
320 2002-2007, using cultivation as a diagnostic method, 207 out of 722 passerines were positive
321 for trypanosomes; 105 isolated were established, and just four belonged to *T. tertium*; besides
322 the predominantly insectivorous species, the collared flycatcher (*Ficedula albicollis*,
323 1 infected) and the chiffchaff (2 infected), *T. tertium* was found in one nuthatch (*Sitta*
324 *europaea*, Černý 2006, Szabová 2008, and Svobodová, unpublished data). The prevalence of
325 *T. tertium* was 0.6%. Only one trypomastigote of *T. tertium* was found. The trypomastigote
326 from naturally infected barn swallow had evident longitudinal striations, comparable to
327 *T. thomasbancrofti* and characteristic for trypanosomes belonging to group C (Šlapeta *et al*.,
328 2016; Fialová *et al*., 2021; Kostygov *et al.,* 2021).
329 Despite low prevalence of avian trypanosomes transmitted by mosquitoes, their occurrence
330 is considered transcontinental since *T. thomasbancrofti* was found in mosquitoes and birds in
331 Czechia as well as in Regent honeyeaters (*Anthochaera phrygia*) in Australia (Zídková *et*
332 *al*.,2012; Šlapeta *et al*., 2016). The same applies to *T. tertium* that, besides its occurrence in
333 Czechia, was found in an Australian passerine, the Australian mudlark (*Grallina cyanoleuca,*
334 Cooper *et al.*, 2017). It is possible that the distribution of avian trypanosomes is global, and the
335 lack of reports from other continents is caused by insufficient or poor sampling; however,
336 although *T. avium* s. s. and *T. bennetti/T. everetti* were found in Africa and America (Valkiūnas

337 *et al*., 2016; Galen *et al*., 2020, Magaña Vázquez *et al.,* 2022), the mosquito species remained
338 undetected.

339 For experimental confirmation of vectorial role of mosquitoes in *T. tertium* life cycle,
340 laboratory mosquitoes were infected with two isolates originating from both hosts (mosquito,
341 bird). The mosquito as well as the avian isolates were able not only to infect both *C. p. pipiens*
342 subspecies but developed high prevalences and infection intensities similar to experimental
343 infections with the other two mosquito species (Votýpka *et al*., 2012; Fialová e*t al*., 2021).
344 Experimental mosquitoes were dissected after defaecation, confirming the specificity of the
345 infection. *Culex* mosquitoes thus have a high capacity to sustain the development of *T. tertium*.

346 The localization of *T. tertium* in the gut after blood digestion changes with time. Initially,
347 the majority of mosquitoes harboured long epimastigotes in the midgut, and rosettes in the
348 hindgut. In the course of infection, midgut stages disappeared, resulting in rosettes localized
349 exclusively in the hindgut which is typical for group C of avian trypanosomes and is consistent
350 across multiple vectors (Votýpka and Svobodová, 2004; Svobodová and Rádrová, 2018;
351 Fialová *et al*., 2021). The localization of trypanosomes within the gut of infected mosquitoes
352 exhibits notable differences among species transmitted by mosquitoes. Mature *T. culicavium*
353 infections are localized exclusively on the stomodeal valve (Votýpka *et al*., 2012). In contrast,
354 *T. thomasbancrofti* typically form rosettes in the hindgut but can be observed in the midgut at
355 later stages of the infection as short epimastigotes (Fialová et al., 2021). However,
356 *T. thomasbancrofti* epimastigotes measure only 8.7 µm on average, and are therefore many
357 times shorter than midgut epimastigotes of *T. tertium,* which are moreover present in the
358 midgut from the very beginning of infection. Some studies also described midgut localization
359 of mosquito trypanosomatids, but these observations were from initial phases of infections,
360 when the peritrophic matrix was still present (Bennett 1961, 1970; Chatterjee 1977).

361 To achieve transmission, several birds were inoculated. Based on the localization of
362 *T. tertium* in the midgut and hindgut of mosquitoes along with its presence in the prediuretic
363 liquid, three distinct inoculation methods were tested based on previous studies: peroral,
364 transconjunctival and subcutaneous (Votýpka *et al*., 2004; Svobodová *et al*., 2017; Svobodová
365 and Rádrová 2018; Fialová *et al*., 2021). Transmission by mosquito bite is unlikely, as even
366 *T. culicavium* which is localized on stomodeal valve is not transmitted this way (Votýpka *et*
367 *al*., 2012). Despite substantial effort, transmission to experimental birds was not successful.
368 However, *T. tertium* has not been identified in bloodsucking Diptera other than *Culex*
369 mosquitoes (Zídková *et al*., 2012, Svobodová *et al*. 2015, Brotánková *et al*., 2022 and
370 unpublished data) and is highly infective to laboratory mosquitoes, which strongly suggests
371 mosquitoes as natural vectors of *T. tertium*. Canaries and zebra finches used in this study might
372 not represent the best model hosts; indeed, previous transmission experiments also yielded
373 somewhat different outcome based on the parasite species used. In most cases, transmission
374 was achieved through oral or conjunctival inoculation (Votýpka and Svobodová, 2004;
375 Votýpka *et al.,* 2012; Svobodová and Rádrová, 2018, Fialová *et al*., 2021). However, in the
376 case of *T. bennetti*, transmission only succeeded after subcutaneous inoculation. It seems thus
377 probable that trypanosomes differ in their avian host specificity, resulting in different
378 prevalences in wildlife.
379 In conclusion, we described a new species of avian trypanosome, *T. tertium* n. sp., whose
380 probable vector is *C. pipiens*. Trypanosomes isolated from both the vector and the avian host
381 developed high prevalences and parasitaemias in experimentally infected mosquitoes, and
382 putative infectious stages were observed in the hindgut as well as in the prediuretic liquid,
383 suggesting transconjunctival transmission in addition to vector ingestion. Prevalences of
384 *T. tertium* in nature are low both in vectors and birds, when compared to other avian
385 trypanosomes including those with mosquito vectors. This might be related to a narrower range

386 of hosts since only insectivorous birds are potentially exposed to the mosquito-dwelling
387 parasite.
388
389 **Taxonomic Summary**

390 **Taxonomic assignment**: Discoba: Discicristata: Euglenozoa: Kinetoplastea:
391 Trypanosomatida: Trypanosomatidae: *Trypanosoma*
392 *Trypanosoma tertium* n. sp.
393 **Diagnosis:** *Trypanosoma* with three different morphotypes in mosquito host: long
394 epimastigotes, 55.5±5.5 µm long and 2.1±0.3 µm wide with free flagellum 4.1±1.5 µm long;
395 short epimastigotes, 9.1±0.9 long, 1.5±0.2 wide and free flagellum 6.6±0.8 µm long, rosette
396 forms 9.0±2.0 µm long 1.9±0.5 µm wide with flagellum 5.4±1.9 µm long.
397 Kinetoplast is 0.310±0.031 µm thick **Figure 3**c (Zídková et al. 2012, Table 2).
398 Two different morphotypes, are present in the culture: long epimastigotes, 29.9±3.1 long,
399 1.8±0.4 µm wide with 7.1±2.5 µm long flagellum; short epimastigotes, 10.7±1.2 µm long,
400 1.5±0.2 µm wide, with flagellum 7.5±1.8 µm long.
401 Trypanosomes expelled during prediuresis are in a form of trypo/epimastigote, 9.5±0.5 µm
402 long, 1.3±0.1 µm wide and with flagellum 6.6±0.6 µm long.
403 Trypomastigote narrow, with a tapering posterior end and visible longitudinal striation. An
404 undulating membrane is located on the external side of the curvature. The cell body is 32.4 µm
405 long and 6.0 µm wide.
406 **Type locality**: Czechia, South Moravia, Milovice forest (48.8213 N, 16.6932 E).
407 **Type isolate**: CUL5 was isolated by Milena Svobodová and is deposited in the collection of
408 the Department of Parasitology, Faculty of Science, Charles University, Prague, Czechia.
409 **Type host (vector):** common house mosquito *Culex pipiens* (Insecta: Diptera, Culicidae)

410 **Additional hosts (avian)**: Insectivorous songbirds (Passeriformes), e.g., willow warbler
411 *Phylloscopus trochilus* (Linnaeus, 1758), common chiffchaff *Phylloscopus collybita* (Vieillot,
412 1817), eurasian reed warbler *Acrocephalus scirpaceus* (Hermann, 1804), spotted flycatcher
413 *Muscicapa striata* (Pallas, 1764), barn swallow *Hirundo rustica* Linnaeus, 1758, sand martin
414 *Riparia riparia* (Linnaeus, 1758), eurasian blackcap *Sylvia atricapilla* (Linnaeus, 1758),
415 eurasian nuthatch *Sitta europaea* (Linnaeus, 1758), collared flycatcher *Ficedula albicollis*
416 (Temminck, 1815), and lesser whitethroat *Sylvia curruca* (Linnaeus, 1758).
417 **Gene sequence**: JN006838
418 **Etymology**: epithet. Fem. adj. *tertium* (third). The name relates to the fact that this is the third
419 *Trypanosoma* species, which is described from mosquitoes and characterized molecularly.
420 Besides, the organism was originally designated as lineage III in group C of avian
421 trypanosomes (Zídková et al., 2012).
422
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428
429 **Author's contribution.**
430 Conceptualization, M.S.; methodology, M.S. and M.K.F.; investigation, M.K.F., A.K., MS;
431 nomenclature, I.Č.; writing - original draft preparation, M.K.F.; writing - review and editing,
432 M.S., IČ; supervision, M.S.; funding acquisition, M.K.F. and M.S.
433
434

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549 **Table 1.** Dissected mosquitoes and trypanosomatid prevalences.

Mosquito genus	Dissected	*T. culicavium*	*T. tertium*	*T. thomasb.*	*T. theileri*	*C. brevicula**	*C. dedva**	*C. dobrovol.**	*P. confusum*
Aedes	959				160 (16,7%)	1 (0,1%)	1 (0,1%)	3 (0,3%)	
Anopheles	33				1 (3%)				
Culex	1128	36 (3,1%)	1 (0,08%)	1 (0,08%)	4 (0,3%)		1 (0,08%)	2 (0,17%)	8 (0,7%)
Culiseta	43				2 (4,6%)				
Mansonia	83				1 (1,2%)	1 (1,2%)			

550 *Species infected with *T. theileri* (*Ae. annulipes, Ae. cantans, Ae. excrucians, Ae. punctor,*
551 *Ae. sticticus, Ae. vexans, An. plumbeus, C. pipiens, Cu. annulata, M. richiardi*), *C. brevicula*
552 (*Ae. annulipes, M. richiardi*), *C. dedva* (*Ae. vexans, C. pipiens*), *C. ddobrovolskii* (*Ae. caspius,*
553 *Ae. vexans, C. pipiens*)

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566 **Table 2.** Morphometry of trypanosomes in mosquito gut. Values in micrometers, SD, standard
567 deviation. Asterisks in the same column indicate no significant differences in the cell length
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	n	Body Length Mean ± SD (Range)	Body Width Mean ± SD (Range)	Flagellum Length Mean ± SD (Range)	Tukey test for cell body length		
long epimastigote gut	30	55.5±5.5 (46.0-69.3)	2.1±0.3 (1.2-2.5)	4.1±1.5 (1.6-7.5)	*		
long epimastigote culture	29	29.9±3.1 (23.3-37.5)	1.8±0.4 (1.6-3.2)	7.1±2.5 (1.6-12.4)		*	
short epimastigote gut	23	9.1±0.9 (7.5-11.4)	1.5±0.2 (1.2-1.9)	6.6±0.8 (5.5-8.8)			*
short epimastigote culture	22	10.7±1.2 (8.3-14.1)	1.5±0.2 (1.1-2.0)	7.5±1.8 (1.6-9.8)			*
Rosette gut	44	9.0±2.0 (5.2-13.9)	1.9±0.5 (1.2-3.5)	5.4±1.9 (1.3-8.2)			*
prediuresis	11	9.5±0.5 (9.0-10.4)	1.3±0.1 (1.1-1.6)	6.6±0.6 (5.8-7.9)			*
Trypomastigote	1	32,4	6				

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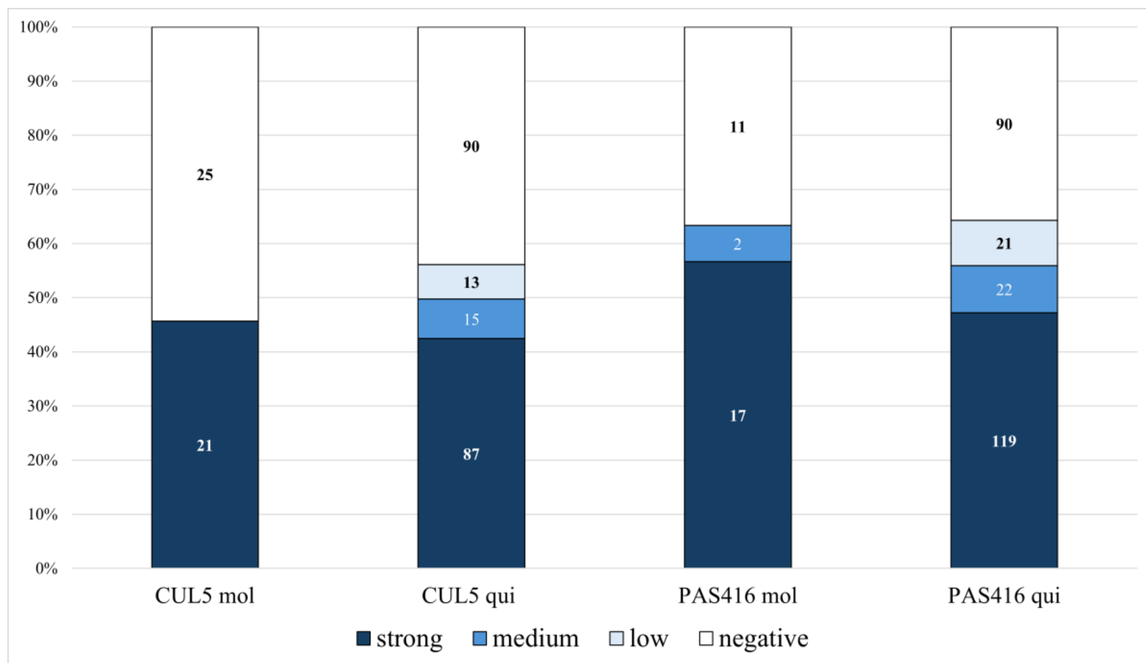

579 **Figure 1.** Infection rates and intensities in mosquitoes *C. p. molestus* (mol) and
580 *C. p. quinquefasciatus* (qui) experimentally infected with *T. tertium* strains CUL5 and PAS416.
581 Infection intensities: low 1-100 parasites; medium 100-1000 parasites; strong > 1000 parasites.
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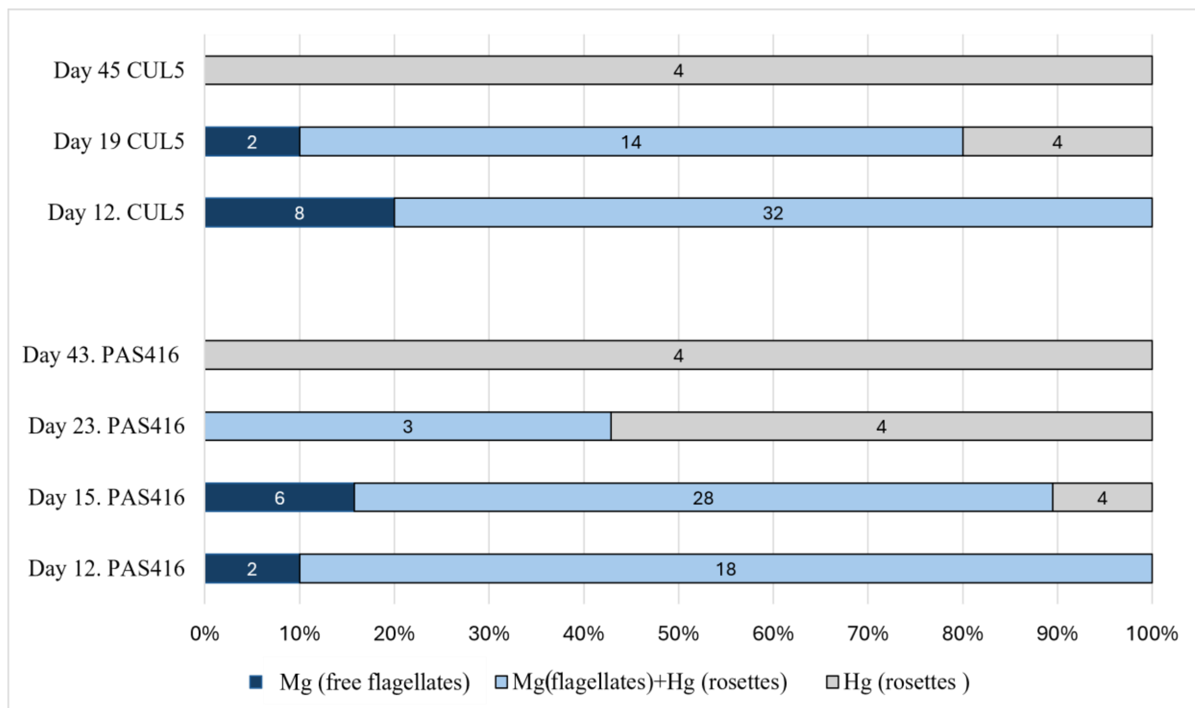

584 **Figure 2.** Temporal dynamics of trypanosomes localization in *C. p. quinquefasciatus* guts
585 experimentally infected by trypanosome isolates CUL5 and PAS416. Numbers of dissected
586 females are shown in the columns. Mg, midgut; Hg, hindgut.

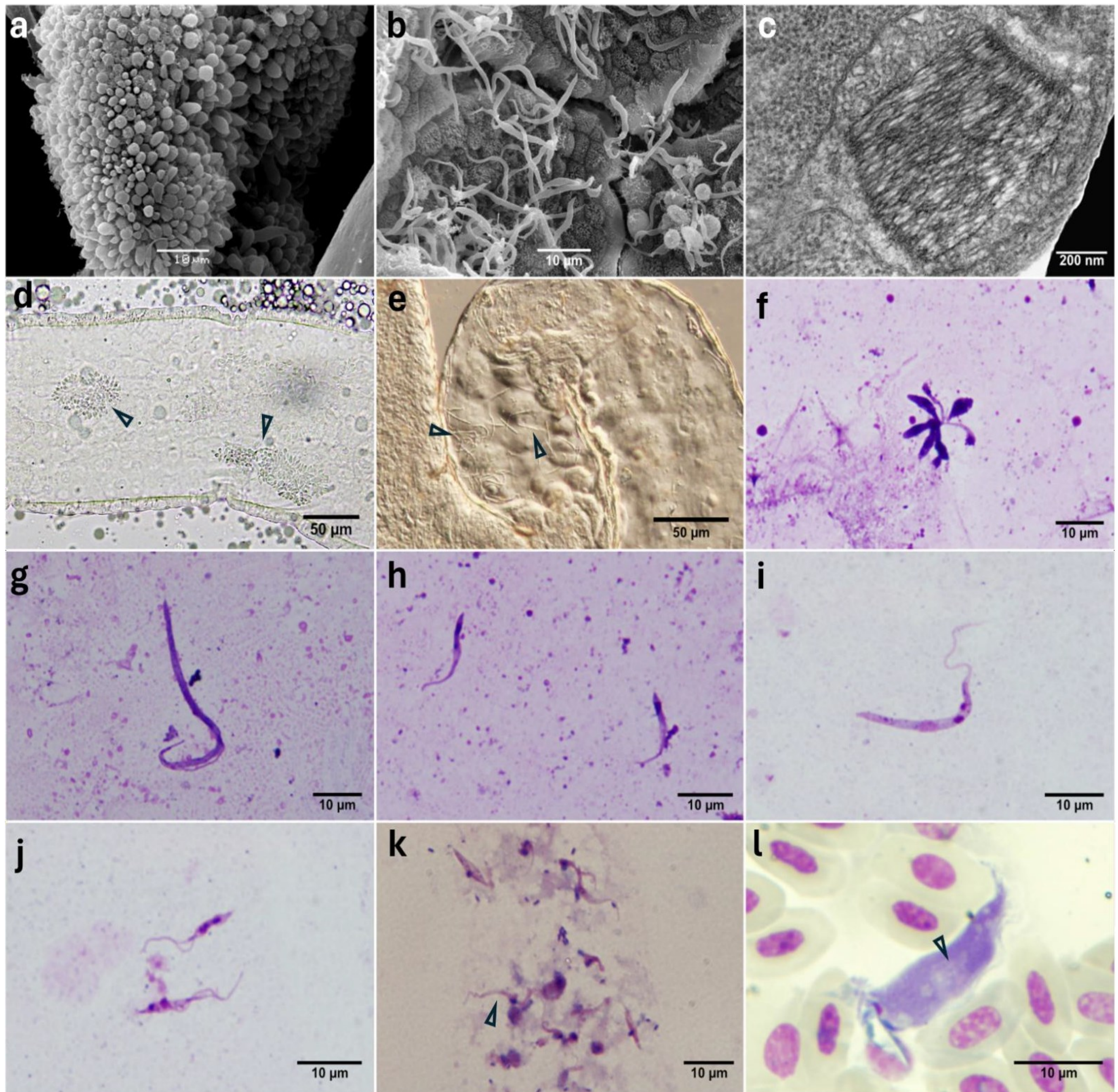

Figure 3. Scanning electron microscopy of *T. tertium* isolate CUL5 after experimental infection: rosette in hindgut (a), long epimastigotes in midgut (b). Transmission electron micrograph of kinetoplast of *T. tritium* from culture (c) (Foto by Zídková). Dissected *Culex* mosquito gut infected by isolate CUL5: arrowheads pointing to the rosettes in hindgut (d) and long epimastigotes in midgut (e). Light microscopy of *T. tertium* morphotypes in *C. quinquefasciatus* gut: rosette (f), long epimastigotes (g), short epimastigote (h); and morphotypes from culture: long epimastigote (i) and short epimastigotes (j). Stages from prediuretic liquid (k). Trypomastigote from barn swallow caught in Neuměřice, Czechia (l) with visible striation (see arrows) the white object partially covering the kinetoplast is an artifact.

4 Summary and conclusions

This study focused on a understudied topic, which is the role of mosquitoes as vectors of avian as well as mammalian trypanosomes. While the role of mosquitoes in the transmission of avian *T. culicavium* had been previously demonstrated (Votýpka et al., 2012), in the case of ruminant *T. theileri*, their role was only speculated (Schoener et al., 2018). Avian and ruminant trypanosomes, parasitising two different classes of vertebrates, but share several common characteristics. One is their localization in the hindgut (except for *T. culicavium*) of infected vectors, thus the transmission route is through the ingestion of infected vectors or via skin or conjunctiva through secretions containing metacyclic stages (Hoare, 1967; Böse et al., 1987; Votýpka and Svobodová, 2014; Svobodová and Rádrová, 2018; Votýpka et al., 2012; Fialová et al., 2021). Second is their close evolutionary relationship which has been suggested, even before the use of molecular methods, based on similar morphology and development (Baker, 1963; Hoare, 1967). This hypothesis was recently confirmed using sequencing of the 18S rRNA fragment (Galen et al., 2020)

Mosquitoes transmit at least three lineages/species of avian trypanosomes: *T. culicavium*, *T. thomasbancrofti*, and *T. tertium* n. sp. (Zídková et al., 2012; Fialová et al., 2021; Kulich Fialová et al., in preparation), however, to date, the vectorial competence of mosquitoes has been experimentally confirmed for *T. culicavium* only (Votýpka et al., 2012). To confirm the susceptibility of mosquitoes to *T. thomasbancrofti* and *T. tertium*, we experimentally infected laboratory mosquitoes with multiple isolates. Both lineages were able to develop infections in mosquitoes with a high prevalence, reaching up to 70% with a high proportion of heavy infections (Fialová et al., 2021; Kulich Fialová et al., in preparation). The typical localization of the closely related *T. avium* is in the hindgut of blackflies, biting midges and sandflies as well (Votýpka and Svobodová, 2024; Svobodová et al., 2017, Svobodová and Rádrová, 2018). *T. thomasbancrofti* also develops primarily in the mosquito hindgut, however, it can appear also in the midgut in the form of short epimastigotes (Fialová et al., 2021). In contrast, *T. tertium* infects both midgut and hindgut at the beginning of infection, and later retracts from the midgut, resulting in infection only in the hindgut (Kulich Fialová et al., in preparation). Both lineages are also excreted in the prediuretic liquid, similarly to *T. avium*. (Votýpka and Svobodová, 2004; Fialová et al., 2021; Kulich Fialová et al., in preparation). Interestingly, in the case of *T. thomasbancrofti,* one isolate from mosquito was able to produce only low infection rates in experimentally

infected mosquitoes, while the isolate from hippoboscid fly developed high infection rates (Fialová et al., 2021). That shows that the assessment of vector competence should not be based on the use of a single isolate. Moreover, isolates with ability to develop infection in multiple vectors have also higher potential to bridge more vertebrate hosts and thus potentially set up new vector-parasite-host combinations.

Given the localization of avian trypanosomes in the vectors' hindgut, it is not surprising that the main mode of transmission is through the ingestion of the infected vector or transconjunctivally by prediuresis of the vector feeding around eyes (Votýpka and Svobodová, 2004; Votýpka et al., 2012; Fialová et al., 2021). In contrast, inoculative transmission of avian trypanosomes does not occur, as shown for *T. culicavium*, which is localized on the stomodeal valve. This localization might suggest transmission through regurgitation similarly to sandflies and *Leishmania*, but does not occur (Votýpka et al., 2012). We completed the life cycle of *T. thomasbancrofti*, which was infectious to birds through peroral and transconjunctival inoculation (Fialová et al., 2021). This allowed us to confirm the role of mosquitoes in transmitting *T. thomasbancrofti*, which had been only speculated previously (Zídková et al., 2012; Šlapeta et al., 2016). Although the inoculation of *T. tertium* to birds was unsuccessful, given the susceptibility of mosquitoes to this species in experiments, and the presence of metacyclic stages in prediuretic liquid, it is probable that *T. tertium* is transmitted similarly to the other two mosquito species , i. e. perorally or transconjunctivally.

T. tertium n. sp. was an unexplored species from group C (Zídková et al., 2012). Thanks to our own isolates, we were able to characterize this newly described species (Kulich Fialová et al., in preparation). Not only it does form a specific clade in the phylogenetic tree (Zídková et al., 2012), but it has a different localization in the infected gut (Votýpka et al., 2012; Fialová et al., 2021; Kulich Fialová et al., in preparation). Its unique character is the presence of very long epimastigotes in the midgut, differentiating *T. tertium* from other species, including the very first described species from mosquitoes as *T. culicis* (Novy et al., 1907)

The prevalence of avian trypanosomes transmitted by mosquitoes among free-living animals is relatively low. Among wild birds, the prevalence is the highest in insectivorous birds, where the prevalence of *T. thomasbancrofti* and *T. tertium* was 3 and 1,5%, respectively (Fialová et al., 2021; Kulich Fialová et al., in preparation). However, despite their

low prevalence, we can consider them as transcontinental species, since besides Europe, these species were isolated from Australian birds as well (Šlapeta et al., 2016; Cooper et al., 2017).

The prevalence among free-living mosquitoes is even lower. *T. culicavium* has consistently the highest prevalence in mosquitoes, while the remaining two species are scarce (Svobodová et al., 2015; Schoener et al., 2018, Schoener et al., 2019). The prevalence of *T. tertium* was the same as of *T. thomasbancrofti,* only 0.08% for both (Fialová et al., 2021; Kulich Fialová et al., in preparation). *Culex* mosquitoes are considered as ornithophilic (Lura et al., 2012; Rádrová et al., 2013; González et al., 2020; Tiron et al., 2021) and were the only genus found to harbour avian trypanosomes in our field studies (Fialová et al., 2021; Kulich Fialová et al., in preparation). Additionally, the inability of *T. culicavium* and *T. tertium* to develop infections in *Ae. aegypti* (Votýpka et al., 2021; Kulich Fialová et al., in preparation) suggests a considerable degree of host specificity, as indicated by both experimental and field studies. In contrast, among mosquitoes of the genus *Aedes*, which are mammalophilic or opportunistic (Börstler et al., 2016; Schönenberger et al., 2016; Cebrián-Camisón et al., 2020), we found trypanosomes from the mammalian, *T. theileri* group (Brotánková et al., 2022). The prevalence reached 22% in *Aedes vexans*, similarly to previous findings (Schoener et al. 2018).

As mentioned earlier, *T. theileri* trypanosomes form a sister group to avian trypanosomes (Galen et al., 2020; Kostygov et al., 2021). Although the phylogenetic relationships within the *T. theileri* group are often unresolved, it is generally accepted that they split into two major lineages, TthI and TthII, which further branch into several genotypes (Rodrigues et al., 2003; Rodriguez et al., 2006; Rodriguez et al., 2010; Pacheco et al., 2015). Our mosquito samples clustered with both lineages; however, to the majority belonged to TthII within which some sequences created a separate group. Moreover, our isolates formed a third lineage, designated as TthIII. This lineage included our isolates obtained from mosquitoes, along with two previously published sequences from deer in USA (Garcia et al., 2020; Brotánková et al., 2022).

Based on the high prevalence of *T. theileri* among free-living mosquitoes (Shoener et al., 2018; Brotánková et al., 2022) and its discovery in sandflies (Calzolari et al., 2018), we decided to experimentally test their vectorial capacity. Infections with isolates of *T. theileri* from group TthII developed well in experimentally infected mosquitoes of the genus *Aedes*

with prevalence reaching 70%. Infections were localized in the hindgut, and metacyclic stages were expelled into prediuretic liquid of mosquitoes similar to infections in tabanids and sheep keds (Hoare, 1967; Molyneux, 1975; Molyneux et al., 1978; Böse et al., 1987; Böse and Heister 1993). On the other hand, *Aedes* mosquitoes were not susceptible to isolates from sheep ked, tabanid fly or to the deer isolate belonging to TthI. *T. theileri* TthII isolate was also able to successfully infect sandflies, while in ornithophilic mosquitoes of the genus *Culex*, the development was only possible at reduced temperatures after blood feeding (Brotánková et al., 2022). These results demonstrate varying degrees of vector specificity among different *T. theileri* genotypes. Based on the high prevalence of *T. theileri* in wild mosquitoes, high susceptibility of *Aedes* mosquitoes to infections and the presence of metacyclic stages in infected guts, we can consider these mosquitoes as vectors *of T. theileri*. Moreover, sandflies are susceptible to *T. theileri* infections as well and have the potential to serve as additional hosts. Infection probably occurs by ingestion or by prediuresis.

Tabanid flies, hippoboscid flies and black flies were screened for presence of *T. theileri* as well. In tabanid flies the prevalence was 44%, slightly higher than in Russia (37%) and Poland (34%) (Ganyukova et al., 2018; Werszko at al., 2019). This high prevalence supports a significant role of tabanids in the life cycle of *T. theileri.* Moreover, *T. theileri* was detected for the first time from a new genus of tabanid fly, *Hybomitra cirueai*. We have identified, for the first time, infections of *T. theileri* among wild black flies indicating their potential as alternative vectors. The prevalence of *T. theileri* among black flies was 1% and all sequences belonged to TthII lineage. Deer ked *L. fortisetosa* harboured *T. theileri* trypanosomes with 18% prevalence per host. No trypanosomes were found in *L. cervi*, probably due to low number of investigated individuals. Sheep ked *M. ovinus* harboured *T. melophagium* with 33% prevalence per site (Brotánková et al., 2022).

In conclusion, this study substantially enhanced our knowledge of avian and mammalian trypanosomes vectors, their modes of transmission, and host specificity. Experiments confirmed that *Culex* mosquitoes are highly susceptible to infections by two avian trypanosomes: *T. thomasbancrofti* and *T. tertium*, with the life cycle successfully completed for *T. thomasbancrofti*. Mammalian *T. theileri* was also able to establish infections, not only in *Aedes* mosquitoes but also in *Phlebotomus* sand flies. Furthermore, all three trypanosome species were found in prediuretic liquid, suggesting that besides peroral transmission by ingestion of infected vector, transconjunctival transmission is also an important mode of

transmission in nature. Phylogenetic analysis of ruminant trypanosomes, which was till now based primarily on isolates from vertebrates and tabanids, revealed a unique, third lineage; genetical diversity of *T. theileri* was previously underestimated. Mosquitoes are known as vectors of many pathogens but their role of trypanosome vectors in nature was previously neglected, overshadowed by the diversity of other parasites they transmit.

5 References

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