

This is a “preproof” accepted article for *Parasitology*.

This version may be subject to change during the production process.

10.1017/S0031182025000587

Trypanosoma tertium n. sp.: Prevalences in natural hosts, development
in the mosquito vector

Magdaléna Kulich Fialová^{1*}, Anna Kapustová¹, Ivan Čepička², and Milena Svobodová^{1*}

¹Department of Parasitology, Faculty of Science, Charles University, 128 00 Prague, Czech Republic

²Department of Zoology, Faculty of Science, Charles University, 128 00 Prague, Czech Republic

*Corresponding authors: fialomag@natur.cuni.cz (M.K.F.), milena@natur.cuni.cz (M.S.)

This is an Open Access article, distributed under the terms of the Creative Commons Attribution licence (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted re- use, distribution and reproduction, provided the original article is properly cited

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. Several families of blood-sucking dipteran insects, including mosquitoes, have been identified as vectors of avian trypanosomes. Mosquitoes have been experimentally confirmed as vectors of *T. culicavium* and *T. thomasbancrofti*. In this study, we describe a third species of avian trypanosomes occurring in mosquitoes, designated as *Trypanosoma tertium* n. sp. This species can be distinguished from related trypanosome species based on morphology and SSU rRNA gene sequence. Two isolates of *T. tertium* n. sp. obtained from a mosquito and a bird host were able to infect two subspecies of laboratory *Culex 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 diuretic liquid of infected mosquitoes, suggesting, besides transmission through ingestion of the infected vector, a possible transconjunctival infection. Among wild mosquitoes, avian trypanosomes were detected exclusively in *Cx. pipiens* with 3.3% total prevalence while *T. tertium* n. sp. prevalence was only 0.08% among 1128 dissected *Cx. pipiens* individuals. In birds, *T. tertium* n. sp. was detected in eight species within which the prevalence was 1.3% (686 birds) while it was 0.3% in total (3084 birds). We discuss the relationship of the newly described *T. tertium* n. sp. with other mosquito-transmitted trypanosomes.

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

Introduction

Protists of the genus *Trypanosoma* (Trypanosomatidae, Kinetoplastea) are dioxenous blood parasites transmitted by bloodsucking invertebrates. They are known for causing illnesses in humans and animals such as Chagas disease, sleeping sickness, nagana, etc. Trypanosomes also readily infect birds, with a relatively high prevalence (Bennett *et al.*, 1974; Greiner *et al.*, 1975; Sebaio *et al.*, 2012; Zamora-Vilchis *et al.*, 2012; Galen *et al.*, 2020; Svobodová *et al.*, 2023; Musa *et al.*, 2024). However, due to their low health and economic impact, avian trypanosomes remain largely neglected.

Little attention is paid to the vectors of avian trypanosomes as well, despite their essential role in maintaining the parasites in the host populations. Various insect species have been identified as vectors of avian trypanosomes, including black flies (Simuliidae, Bennett, 1961; Votýpka *et al.*, 2002; Votýpka and Svobodová, 2004), hippoboscids (Hippoboscidae, Baker, 1956; Votýpka *et al.*, 2002; Santolíkova *et al.*, 2022), mosquitoes (Culicidae, Bennett, 1961; 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). Avian trypanosome mature infections are localized in the intestine (midgut, hindgut) of their vectors, and the most common transmission mode to birds is peroral, by ingestion of an infected vector (Baker, 1961; Votýpka and Svobodová, 2004; Votýpka *et al.*, 2012; Svobodová and Rádrová, 2018; Fialová *et al.*, 2021). An alternative way of transmission is through the conjunctiva, when metacyclic stages from vector hindgut are expelled with the prediuretic/diuretic liquid while the vector feeds (Votýpka and Svobodová, 2004; Fialová *et al.*, 2021).

Mosquitoes are notoriously associated with the transmission of the protozoan of the genus *Plasmodium*, the causative agents of malaria in reptiles, amphibians, mammals, and

birds. In contrast, their role in the life cycle of trypanosomes is less explored. However, as early as in 1843, mosquitoes were identified as vectors of *Trypanosoma rotatorium*, the species from anurans (Mayer, 1843 in Desser *et al.*, 1973) and they were also among the first suspected vectors of avian trypanosomes (Novy *et al.*, 1907; Bennett, 1961, 1970; Chatterjee, 1977). Presently, three species/lineages of avian trypanosomes infecting mosquitoes are recognized, namely: *Trypanosoma culicavium*, *Trypanosoma thomasbancrofti*, and *Trypanosoma* sp. lineage III (Votýpka *et al.*, 2012; Zídková *et al.*, 2012; Šlapeta *et al.*, 2016; Fialová *et al.*, 2021; Kulich Fialová, 2024). While *T. culicavium* and *T. thomasbancrofti* life cycles have been experimentally confirmed and the species have been formally described, the life cycle of the *Trypanosoma* sp. lineage III remains unknown as well as its characteristics have not been studied in detail.

Our objective was to characterize the least known avian trypanosome found in mosquitoes – *Trypanosoma* sp. lineage III. Together with its sister species *T. thomasbancrofti*, this trypanosome belongs to the group C of avian trypanosomes, which includes other *T. avium* s. l. lineages as well (Zídková *et al.*, 2012). This study investigated the experimental life cycle and prevalence of an uncharacterized avian trypanosome in both avian hosts and mosquitoes. It provides new insights into its life cycle and morphological features, which were previously unknown, further expanding our understanding of mosquito-transmitted avian trypanosomes.

Materials and methods

Prevalence in naturally infected mosquitoes

Mosquitoes were trapped in July 2018-2021 in Milovice forest, South Moravia, Czechia (48.8213 N, 16.6932 E), using CDC traps (JW Hock Company, Gainesville, FL, USA) with CO₂ as an attractant. Mosquitoes were collected into nylon nets connected to the traps,

anesthetized on ice, and sorted according to species (Kramář, 1958). Individual mosquitoes were killed in 70% ethanol, rinsed twice in sterile saline, and then dissected using tweezers (Dumont tweezers, Ted Pella, California, USA), which were sterilized in a flame between each dissection to prevent cross contamination. Dissected guts were examined under the light microscope for the presence of kinetoplastids. A part of each positive gut was used for the cultivation of kinetoplastids, and the rest was stored in ethanol for barcoding of the parasites (see below).

Prevalence in wild birds

Bird sampling was done at several Czech localities in 2014-2016 between May and July. Adults and yearlings were mist-netted by the authors or in cooperation with other registered ringers contributing to the program Constant Effort Site (CES), organized by the Prague Ringing Centre. Trapping and sampling were done by licensed workers according to national law and experimental guidelines. Blood was collected as described in Fialová *et al.* (2021); a part was used for trypanosome cultivation; a part was stored in ethanol at -20 °C prior to further use. Blood smears on glass slides were prepared from each bird as well. After air-drying of blood film, the smears were fixed in absolute methanol and stained with Giemsa for 30 minutes.

Parasite strains and cultures

Both trypanosome strains used in this study originated from our own collection and were acquired in Milovice forest: CUL5 was isolated from a *Culex pipiens* female (ICUL/CZ/2000/CUL5) and PAS416 from a willow warbler (*Phylloscopus trochilus*) (APHY/CZ/2016/PAS416). The isolate CUL5 was assigned to the lineage III of group C previously (Zídková *et al.*, 2012; GenBank Acc. Nr. JN006838), PAS416 was barcoded by

nested PCR as described below. The SSU rRNA sequence comparison of PAS416 with CUL5 using BLAST algorithm revealed 99.7% identity while there were 2 differences in a 635 bases-long segment.

Trypanosomes were cultivated in flat tubes or PEN tubes (diagnostic cultivation) on blood agar (SNB-9; Diamond and Herman, 1954) prepared from rabbit (Bioveta, Ivanovice na Hané, Czech Republic) or sheep (LabMediaServis, Jaroměř, Czech Republic) blood, overlaid with liquid medium. Liquid medium was prepared by mixing 1:1 RPMI 1640 and Schneider's *Drosophila* Medium (both Sigma-Aldrich, St. Louis, MO, USA), 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). Media for isolation used in field studies were moreover supplemented with penicillin (10 000 IU/mL, BB Pharma a.s., Prague, Czechia) and fluorocytosine (1500 µg/mL, TCI, Tokyo, Japan). Thriving cultures from PEN tubes or flat tubes were subcultured into new flat tubes with fresh blood agar and overlay. Cultures were held at 23 °C.

Experimental infections of mosquitoes

Two subspecies of *Cx. pipiens* were used in this study; both were bred in our laboratory; *Culex pipiens quinquefasciatus* (*Cx. p. quinquefasciatus* henceforth) originating from India, kept in our laboratory for more than 30 years, and *Culex pipiens molestus* (*Cx. p. molestus* henceforth) colonized from individuals caught in Czechia in 2016. A colony of *Aedes aegypti* was established temporarily from mosquitoes obtained from the National Institute of Public Health, Prague.

Mosquito females (8-11days old) were infected by feeding through a chick skin membrane on a glass feeder containing inactivated (30 min in 56 °C water bath) sheep or rabbit blood mixed with 12–18-day-old cultures of trypanosomes ($2-6 \times 10^8$ parasite

cells/mL). After each blood feeding, the blood fed to mosquitoes was controlled under the microscope for the presence of live trypanosomes. Fed females were separated and kept in nets in an incubator with stable conditions (21 °C, convenient humidity), and provided with a 50% sucrose solution on a cotton pad. Mosquitoes were dissected 10-45 days post-infection. Dissected guts were examined under the light microscope for infection status, intensity of infection, parasite localization and its changes during the course of infection. Infection intensities were defined as low, 1-100 parasites; medium, 100-1000 parasites; and heavy, >1000 parasites per gut.

In the experiment testing *Ae. aegypti* susceptibility, *Cx. p. quinquefasciatus* and *Ae. aegypti* mosquitoes were fed in parallel on the same blood meal with isolate CUL5. Five mosquitoes in each group were dissected three days after inoculation to control whether trypanosomes were transmitted during experimental feeding and were present in the ingested blood. The rest of mosquitoes was dissected after blood ingestion as described above.

Trypanosomes in diuretic liquid

Mosquitoes, membrane-fed on blood with strain PAS416, were provided with a bowl of water four days after feeding to allow oviposition. After oviposition, 10 days after first feeding, the mosquitoes were provided with an anesthetized laboratory mouse BALB/c. The feeding was monitored, and females that finished blood feeding were transferred immediately into plastic *Drosophila* tubes with a coverslip placed on the bottom, which served to catch droplets of diuretic liquid. Air-dried droplets were fixed with methanol, stained with Giemsa, and examined for the presence of trypanosomes. Mosquito females were dissected to assess their infection status.

Experimental inoculation of birds

All experimental birds, originally purchased at a pet store, were screened for the presence of trypanosomes prior to inoculation. Screening was done with blood obtained from vein articulation (vena metatarsalis plantaris superficialis media), using cultivation (see above) and PCR screening (see below). All birds were negative.

Birds were inoculated with 7-10 *Cx. p. molestus* or *Cx. p. quinquefasciatus* infected guts homogenized in saline, applied orally, subcutaneously, or by placing on the conjunctiva i.e. transconjunctivally. Birds were tested for the presence of trypanosomes by blood cultivation in weekly intervals (four weeks), then monthly (five months); obtained cultures were checked microscopically three times in weekly intervals.

PCR diagnostic

High Pure PCR Template Preparation Kit (Roche Diagnostic, Mannheim, Germany) was used for DNA extraction. Obtained DNA was subsequently stored at -20 °C until further use. Amplification of trypanosome SSU rRNA gene from bird blood and dissected guts of mosquitoes was done by specific nested PCR with primers S762/S763 for the first step (Maslov *et al.*, 1996) and TR-F2/TR-R2 for the second step (Votýpka *et al.*, 2015), cultivated trypanosomes established from mosquito guts were barcoded by single step PCR with primers Medlin A/Medlin B (Medlin *et al.*, 1988) as described in Fialová *et al.* (2021). Obtained PCR products were purified using the enzymatic solution ExoSap (Thermo Scientific, Waltham, MA, USA), then sequenced at the core facility of the Faculty of Science (Laboratory OMICS – Genomics, Biocev). Sequences were examined in the BioEdit software and analysed using the BLAST algorithm and nucleotide database NCBI.

Scanning electron microscopy

Guts of mosquitoes positive for trypanosomes after experimental infection 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 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 Bal-Tec SCD050 (Bal-Tec Ag, Balzers, Liechtenstein), and examined by the authors using a JEOL 6380LV (JEOL, Tokyo, Japan) scanning electron microscope.

Light microscopy and measurement of trypanosomes

Positive guts of mosquitoes from experimental infections were fixed on slides with methanol, stained with Giemsa (Sigma-Aldrich, St. Louis, MO, USA) and photographed at 1000x magnification with a CDC camera (DP70) using an Olympus BX51 microscope. Measurement of the cells was done using ImageJ software. Data from the cell measurements were processed using R software – the mean values of trypanosomes in mosquito gut and cultures (body length, body width, flagellum length) and their standard deviation were calculated as described in Crawley (2013).

Blood smears (fixed with methanol and stained with Giemsa) of birds positive by PCR for *Trypanosoma* sp. lineage III were inspected under the microscope at magnification x1000 for 10 min, and the whole smear area at x200 for unlimited time.

Results

Prevalence of Trypanosoma tertium n. sp. in wild-caught mosquitoes

In total 2246 wild-caught mosquitoes were dissected; the majority of them was identified as *Cx. pipiens* (1128), the rest were mosquitoes of the genera *Aedes* (*Ae. annulipes*, *Ae. cantans*, *Ae. caspius*, *Ae. cinereus*, *Ae. excrucians*, *Ae. punctor*, *Ae. rusticus*, *Ae. sticticus*, *Ae. vexans*), *Anopheles* (*An. claviger*, *An. maculipennis*, *An. plumbeus*), *Culiseta annulata*, and *Mansonia richiardii*. All species of mosquitoes infected with kinetoplastids were investigated by PCR, however, mosquito-specific avian trypanosomes (*T. culicavium*, *T. tertium* n. sp., *T. thomasbancrofti*) were found exclusively in *Cx. pipiens*, with the prevalence of 3.3% from 1128 dissected individuals. Furthermore, *Culex* mosquitoes harboured mammalian trypanosomes belonging to *T. theileri* s. l. as well as monoxenous kinetoplastids of the genus *Crithidia* and *Paratrypanosoma* (for parasite species and prevalences, see **Table 1**). *Trypanosoma theileri* s. l. was identified in *Aedes*, *Anopheles*, *Culiseta* and *Mansonia* spp., monoxenous kinetoplastids in *Aedes* and *Mansonia* spp. as well.

Prevalence of Trypanosoma tertium n. sp. in wild-caught passerines

Trypanosoma tertium n. sp. was detected in 9 (0.3%) of the 3084 sampled individuals. Infected species included barn swallow (*Hirundo rustica*, 1 infected), chiffchaff (*Phylloscopus collybita*, 1), eurasian blackcap (*Sylvia atricapilla*, 2), eurasian reed warbler (*Acrocephalus scirpaceus*, 1), lesser whitethroat (*Sylvia curruca*, 1), sand martin (*Riparia riparia*, 1), spotted flycatcher (*Muscicapa striata*, 1) and willow warbler (*Phylloscopus trochilus*, 1).

If considering only these species, the prevalence was 1.3% (n = 686), and if considering all screened individuals belonging to these genera, the prevalence was 0.8% (n = 1037). The samples were either positive by PCR (two samples) or by cultivation (seven samples); in

three cases, the alternative diagnostic method used in parallel revealed coinfection with another trypanosome lineage (lineages from *T. everetti/bennetti* group twice, *T. culicavium* once). Only a single trypomastigote was found on all the inspected slides obtained from positive birds (**Figure 3I**).

Experimental infections of mosquitoes

Two subspecies of *Culex pipiens* - *Cx. p. quinquefasciatus* and *Cx. p. molestus* - were fed on blood with two different strains of *T. tertium* n. sp. originating from two different hosts: CUL5 (mosquito) and PAS416 (bird). The CUL5 isolate was able to develop infection in 45% of *Cx. p. molestus* and 55% of *Cx. p. quinquefasciatus*, with 100% prevalence of heavy infection among all infected *Cx. p. molestus* and 90% of heavy infection among all infected *Cx. p. quinquefasciatus*. Mosquitoes were also highly susceptible to the avian isolate, with approximately 65% prevalence in both *Culex pipiens* subspecies, and with 89% and 75% of heavy infections in *Cx. p. molestus* and *Cx. p. quinquefasciatus*, respectively (**Figure 1**).

To assess the vector specificity of *Culex* mosquitoes, 40 females of *Ae. aegypti* fed on blood with CUL5 were dissected. None of the females was found to be positive. Control mosquitoes *Cx. p. quinquefasciatus* fed on the same inoculum were infected in 60% of cases.

Trypanosomes in guts dissected 12 days post infection (dpi) were localized mainly in midgut as unattached long epimastigotes along with rosettes in hindgut, or exclusively in midgut as unattached epimastigotes (**Figure 2**). From 15 dpi onwards, the ratio of individuals with trypanosomes present only in the hindgut as rosettes increased slowly, while the proportion of infections in the hindgut and midgut or exclusively in the midgut decreased. At days 43-45, only rosettes were present, exclusively in the hindgut. Infections with rosettes in the midgut were not detected.

Trypanosomes in diuretic liquid

Stages of *T. tertium* n. sp. were observed on three out of seven Giemsa-stained droplets of diuretic liquid of examined mosquitoes. The expelled cell types were short trypanosomes/epimastigotes (**Figure 3k**).

Light microscopy

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

We used the analysis of variance to compare *T. tertium* n. sp. cell lengths based on the isolate (CUL5, PAS416), origin (gut, culture), and cell form (long or short epimastigote, rosette, diuresis form). The isolate as a factor had no significant impact ($F=3.86$; $P>0.05$) while both the origin and the cell form were significant ($F=101.19$ and 315.40 , respectively; $P<0.001$). Therefore, the measurements of the two isolates were merged for Tukey post hoc comparison test, which revealed that both gut and culture forms of short epimastigotes and rosettes are not significantly different from the diuresis form, while both long epimastigotes from the gut and culture differ from all remaining groups (see **Table 2**). Interestingly, cell lengths of long epimastigotes from cultures and guts are also significantly different.

Inoculation of experimental birds

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

The isolate CUL5 was used for peroral inoculation of one zebra finch and five canaries. The isolate PAS416 was used for eight peroral and eight subcutaneous inoculation of canaries, two subcutaneous inoculation of zebra finches, and two transconjunctival inoculation of canaries. All birds remained negative till the end of experiment (six months).

Discussion

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

Based on phylogenetic studies (SSU rRNA), it is evident that *T. tertium* n. sp. is a unique and well supported monophyletic lineage of avian trypanosomes. It is closely related to *T. thomasbancrofti*, and both fall into the *T. avium* s. l. group C of avian trypanosomes. Moreover, the existence of these lineages was supported also by RAPD analysis (Zídková *et al.*, 2012). These findings reinforce their distinct phylogenetic position and underscore its evolutionary divergence from related taxa. The SSU rRNA sequence comparison of *T. tertium* n. sp. (CUL5) with its closest relative, *T. thomasbancrofti*, using BLAST algorithm revealed 99.4% identity with first hit of *T. thomasbancrofti* (CUL15) while there were 13 differences in a 2163 bases-long segment.

Trypanosoma thomasbancrofti and *T. tertium* n.sp. even share some characteristics, e.g., mature infections are localized in the vector's hindgut, similarly to other members of the group whose proven vectors are blackflies (group C lineages X and XI). On the contrary, *T. culicavium* lineage belongs to group B of avian trypanosomes and is localized on the

stomodaeal valve of the vector (Votýpka *et al.*, 2012; Zídková *et al.*, 2012). However, *T. corvi*, a species transmitted by hippoboscids and belonging to the same group B as *T. culicavium*, has infectious stages localized in the hindgut. That points out to the fact that although the localization is important for the ways of transmission, it is not related to the phylogenetic position of the parasite.

Apart from *T. culicavium* and *T. thomasbancrofti*, two recently described avian trypanosomes infecting *Culex* mosquitoes (Votýpka *et al.*, 2012; Zídková *et al.*, 2012; Fialová *et al.*, 2021), other kinetoplastids in *Culex* mosquitoes have been reported as early as the beginning of the 20th century and it is crucial to distinguish *T. tertium* n. sp. from these previously described species to confirm that it represents a truly new species. *Trypanosoma noctuae*, described by Schaudinn (1904), was reported to infect *Culex* mosquitoes after feeding on an infected little owl (*Athene noctua*). However, it is likely that Schaudinn worked with mosquitoes naturally infected with monoxenous kinetoplastids from the genus *Crithidia* (see Novy *et al.*, 1907) rather than with any avian trypanosome, including *T. tertium* n. sp. A second species reported from *Cx. pipiens* mosquitoes is *T. (Herpetomonas) culicis* (Novy *et al.*, 1907). These authors described this trypanosome from the intestines of naturally infected mosquitoes and reported two morphological forms. “Very long forms” measured 30-35 µm while “short forms” ranged between 12-20 µm. The long epimastigotes of *T. tertium* n. sp. documented in this study measured 55 µm on average, almost twice as much; such a difference is substantial. Novy *et al.*, 1907 also described fast movement of long forms from gut, which made them difficult to follow. We did not observe such a fast movement of long epimastigotes of *T. tertium* n. sp. The size of short epimastigotes (average 9-11, maximum 14 µm) overlaps with that of *T. culicis* short epimastigotes, however, short forms in the intestine overlapping in length can be found in many other trypanosome species belonging to all three groups (Zídková *et al.*, 2012). In addition, Novy *et al.* (1907) do not mention any stages

similar to the rosette forms found in the intestines of mosquitoes infected by *T. tertium* n. sp. For these reasons, we consider *T. culicis* as a separate species from *T. tertium* n. sp. In 1961, *T. culicis* was transferred to the genus *Blastocrithidia* by Wallace and Johnson. However, we consider this inappropriate since the authors did not work with the original isolate; on the contrary, they used their own isolate originating from a different mosquito species and genus, *Aedes vexans*. During our studies, we failed to find any *Aedes* mosquitoes infected by *T. tertium* n. sp. despite a substantial number of examined specimens (959 individuals in this study, 1398 individuals in Brotánková *et al.*, 2022). Furthermore, there is a considerable degree of host specificity of mosquito trypanosomes, since neither *T. culicivium* nor *T. thomasbancrofti* were ever detected in *Aedes* mosquitoes (Fialová *et al.*, 2012; Brotánková *et al.*, 2022). Moreover, *Aedes* mosquitoes were not susceptible to the infection by *T. tertium* n. sp. in our experiments, nor to *T. culicivium* (Votýpka *et al.*, 2012). It is therefore unlikely that different mosquito genera would share trypanosomes including *T. tertium* n. sp. This applies not only to avian trypanosomes transmitted by mosquitoes but also to hippoboscid/avian lineages as well as to mammalian *T. theileri* trypanosomes (Brotánková *et al.*, 2022; Santolíkova *et al.*, 2022).

Mosquitoes naturally infected with trypanosomes that molecularly match *T. tertium* n. sp. have been found in Czechia for the first time in 1999/2000 (Zídková *et al.*, 2012); from 28 strains established from 898 examined *Cx. pipiens* guts, two belonged to *T. tertium* n. sp. (prevalence 0.22%, Svobodová *et al.*, 2015). In our study, only one individual out of 1128 dissected *Cx. pipiens* mosquitoes harboured *T. tertium* n. sp. (prevalence 0.08%). The prevalence of *T. tertium* n. sp. was comparable to that of *T. thomasbancrofti*, but notably lower than the prevalence of *T. culicivium* (3.1 %), which was similar to previous findings (Svobodová *et al.*, 2015). Given its low prevalence and the historical underrepresentation of avian trypanosomes in research, it is more likely that the absence of earlier records reflects a

lack of detection rather than a recent introduction. Moreover, the prevalence of *T. tertium* n. sp. between wild mosquitoes is so low, that studies with less investigated mosquitoes can fail to detect it at all, like study from Lithuania with 561 dissected *Cx. pipiens* females (Valavičiūtė-Pocienė *et al.*, 2024)

The prevalence among avian hosts was likewise low, with only 9 (0.3%) out of the 3084 screened passerines found to be infected by *T. tertium* n. sp. Since the mode of transmission of *T. tertium* n. sp. is probably similar to *T. culicavium* and *T. thomasbancrofti*, i.e., after ingestion of the infectious vector, it is appropriate to focus on insectivorous avian species when assessing prevalence, in which the prevalence of *T. tertium* n. sp. rises to 1.3%. In our previous studies in the years 2002-2007, using cultivation as a diagnostic method, 207 out of 722 passerines were positive for trypanosomes; 105 isolated were established in culture, and just four belonged to *T. tertium* n. sp. Besides the predominantly insectivorous species, the collared flycatcher (*Ficedula albicollis*, 1 infected), the chiffchaff (*Phylloscopus collybita*, 2 infected) and one reed warbler (*Acrocephalus scirpaceus*; Musa *et al.*, 2024) *T. tertium* n. sp. was found in one nuthatch (*Sitta europaea*; Černý 2006, Szabová 2008; Zídková *et al.*, 2012).

On all the inspected slides originating from positive birds only a single trypomastigote of *T. tertium* n. sp. was found. The morphological differentiation of avian trypomastigotes is challenging and requires an experienced observer. However, certain morphological traits of trypomastigotes can provide clues for classification - usually not at the species level, but rather at the group level - such as the presence of longitudinal striations (Kostygov *et al.*, 2021). Moreover, avian trypanosomes occur in the peripheral blood of birds at low prevalence, making it difficult to detect trypomastigotes microscopically as was the case for *T. tertium* n. sp. in our study (one trypomastigote found). The *T. tertium* n. sp. trypomastigote from the naturally infected barn swallow had evident longitudinal striations, comparable to

T. thomasbancrofti and characteristic for trypanosomes belonging to the group C (Šlapeta *et al.*, 2016; Fialová *et al.*, 2021; Kostygov *et al.*, 2021).

Despite low prevalence of avian trypanosomes transmitted by mosquitoes, their occurrence is considered transcontinental since *T. thomasbancrofti* was found in mosquitoes and birds in Europe as well as in regent honeyeaters (*Anthochaera phrygia*) in Australia (Zídková *et al.*, 2012; Šlapeta *et al.*, 2016). The same applies to *T. tertium* n. sp., sequences from this parasite were detected in Europe as well as in the Australian mudlark (*Grallina cyanoleuca*) in Australia (Zídková *et al.*, 2012; Cooper *et al.*, 2017; Musa *et al.*, 2024). It is possible that the distribution of all avian trypanosome species is worldwide, and the lack of reports of the mosquito-transmitted species from other continents is caused by insufficient sampling. Although *T. avium* s. s. and *T. bennetti*/*T. everetti* were found in Africa and America (Valkiūnas *et al.*, 2016; Galen *et al.*, 2020, Magaña Vázquez *et al.*, 2022), the mosquito species remained undetected.

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

The localization of *T. tertium* n. sp. in the gut after blood digestion changes with time. Initially, the majority of mosquitoes harboured long epimastigotes in the midgut and rosettes in the hindgut. In the course of infection, midgut stages disappeared, resulting in rosettes localized exclusively in the hindgut, which is typical for group C of avian trypanosomes and

is consistent across multiple vectors (Votýpka and Svobodová, 2004; Svobodová and Rádrová, 2018; Fialová *et al.*, 2021). The localization of trypanosomes within the gut of infected mosquitoes exhibits notable differences among species transmitted by mosquitoes. Mature *T. culicavium* infections are localized exclusively on the stomodeal valve (Votýpka *et al.*, 2012). In contrast, *T. thomasbancrofti* typically forms rosettes in the hindgut but can be observed in the midgut at later stages of the infection as short epimastigotes (Fialová *et al.*, 2021). However, *T. thomasbancrofti* epimastigotes body measure only 8.7 μm on average and are therefore many times shorter than midgut epimastigotes of *T. tertium* n. sp. (55.5 μm), which are moreover present in the midgut from the very beginning of infection. Some studies also described midgut localization of mosquito trypanosomatids, but these observations were from initial phases of infections, when the peritrophic matrix was still present (Bennett 1961, 1970; Chatterjee 1977). Moreover, trypanosomes transmitted by mosquitoes can be distinguished not only by their localization in the gut but also by their dimensions and the thickness of the kinetoplast (Zídková *et al.*, 2012). For comparison of dimensions of *T. tertium* n. sp. with other trypanosome species transmitted by mosquitoes see **Table 3**. However, identification of avian trypanosomes from vectors based solely on morphology—without knowledge of the vector and without information on localization within the gut—is challenging. The morphological features, as cell size ranges can overlap (Zídková *et al.*, 2012), and multiple developmental forms can be found in the gut of vectors. Molecular techniques are key to accurately identifying the species, as they provide the necessary resolution that morphology alone cannot achieve.

To achieve transmission, several birds were inoculated. Based on the localization of *T. tertium* n. sp. in the midgut and hindgut of mosquitoes along with its presence in the diuretic liquid, three distinct inoculation methods were tested based on previous studies: peroral, transconjunctival, and subcutaneous (Votýpka *et al.*, 2004; Svobodová *et al.*, 2017;

Svobodová and Rádrová 2018; Fialová *et al.*, 2021). Transmission by mosquito bite is unlikely, as even *T. culicavium*, which is localized on stomodeal valve, is not transmitted this way (Votýpka *et al.*, 2012). Despite substantial effort, transmission to experimental birds was not successful. However, *T. tertium* n. sp. has not been identified in bloodsucking Diptera other than *Culex* mosquitoes (Zídková *et al.*, 2012, Svobodová *et al.* 2015, Brotánková *et al.*, 2022, and unpublished data) and is highly infective to laboratory mosquitoes, which strongly suggests mosquitoes as natural vectors of *T. tertium* n. sp. Canaries and zebra finches used in this study might not represent the best model hosts; indeed, previous transmission experiments also yielded somewhat different outcome based on the parasite species used. In most cases, transmission was achieved through oral or conjunctival inoculation (Votýpka and Svobodová, 2004; Votýpka *et al.*, 2012; Svobodová and Rádrová, 2018, Fialová *et al.*, 2021). However, in the case of *T. everetti/bennetti*, transmission only succeeded after subcutaneous inoculation. It seems thus probable that trypanosomes differ in their avian host specificity, resulting in different prevalences in wildlife.

In conclusion, we describe a new species of avian trypanosome, *T. tertium* n. sp., whose probable vector is *Cx. pipiens*. Trypanosomes isolated from both the vector and the avian host developed high prevalences and parasitaemias in experimentally infected mosquitoes, and putative infectious stages were observed in the hindgut as well as in the diuretic liquid, suggesting transconjunctival transmission in addition to vector ingestion. Prevalences of *T. tertium* n. sp. in nature are low both in vectors and birds when compared to other avian trypanosomes including those with mosquito vectors. This might be related to a narrower range of hosts since only insectivorous birds are potentially exposed to the mosquito-dwelling parasite.

Taxonomic Summary

ZooBank registration of this work: urn:lsid:zoobank.org:pub:995C568B-8479-44CE-978D-11B21CE148C1.

Taxonomic assignment: Discoba: Discicristata: Euglenozoa: Kinetoplastea:

Trypanosomatida: Trypanosomatidae: *Trypanosoma*

Trypanosoma tertium n. sp.

Diagnosis: *Trypanosoma* with three different morphotypes in mosquito host: long epimastigotes, 55.5 ± 5.5 μm long and 2.1 ± 0.3 μm wide with free flagellum 4.1 ± 1.5 μm long; short epimastigotes, 9.1 ± 0.9 long, 1.5 ± 0.2 wide and free flagellum 6.6 ± 0.8 μm long, rosette forms 9.0 ± 2.0 μm long 1.9 ± 0.5 μm wide with flagellum 5.4 ± 1.9 μm long.

Kinetoplast is 0.310 ± 0.031 μm thick **Figure 3c** (Zídková *et al.* 2012, Table 2).

Two different morphotypes are present in culture: long epimastigotes, 29.9 ± 3.1 long, 1.8 ± 0.4 μm wide with 7.1 ± 2.5 μm long flagellum; short epimastigotes, 10.7 ± 1.2 μm long, 1.5 ± 0.2 μm wide, with flagellum 7.5 ± 1.8 μm long.

Trypanosomes expelled during diuresis are in a form of trypano-/epimastigote, 9.5 ± 0.5 μm long, 1.3 ± 0.1 μm wide and with flagellum 6.6 ± 0.6 μm long.

Trypomastigote narrow, with a tapering posterior end and visible longitudinal striation. An undulating membrane is located on the external side of the curvature. The cell body is approximately 32 μm long and 6 μm wide.

Type locality: Czechia, South Moravia, Milovice forest (48.8213 N, 16.6932 E).

Type isolate: CUL5 was isolated by Milena Svobodová and is deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czechia.

Type material: 11 permanent slides with Giemsa stained syntypes of *T. tertium* n. sp. deposited in the collection of the National Museum of the Czech Republic, inventory

numbers P6E 5584-P6E 5595 (Table 4). All cells on the slides are syntypes. Some syntypes are shown in **Fig.3** (f, g, h, i, j).

Type host (vector): common house mosquito *Culex pipiens* (Insecta: Diptera, Culicidae)

Additional hosts (avian): Insectivorous songbirds (Passeriformes): barn swallow *Hirundo rustica* (Linnaeus, 1758), collared flycatcher *Ficedula albicollis* (Temminck, 1815), common chiffchaff *P. collybita* (Vieillot, 1817), Eurasian blackcap *Sylvia atricapilla* (Linnaeus, 1758), eurasian nuthatch *Sitta europaea* (Linnaeus, 1758), Eurasian reed warbler *Acrocephalus scirpaceus* (Hermann, 1804), lesser whitethroat *Sylvia curruca* (Linnaeus, 1758), sand martin *Riparia riparia* (Linnaeus, 1758), spotted flycatcher *Muscicapa striata* (Pallas, 1764) and willow warbler *Phylloscopus trochilus* (Linnaeus, 1758)

Gene sequence: An SSU rRNA gene sequence is deposited in GenBank with accession number JN006838.

Etymology: epithet. L. n. adj. *tertium* (third). The name relates to the fact that this is the third *Trypanosoma* species, which is described from mosquitoes and characterized molecularly. Besides, the organism was originally designated as lineage III in group C of avian trypanosomes (Zídková *et al.*, 2012).

Acknowledgements. Multiple people helped us with bird trapping, with the most important contribution of František Novák and Oldřich Myška. The authors acknowledge the Viničná Microscopy Core Facility (VMCF of the Faculty of Science, Charles University), an institution supported by the MEYS CR (LM2023050 Czech-BioImaging), for their support and assistance in this work. Karolína Volfová for *Culex pipiens molestus* breeding. Tomáš Bělka for providing the photography of willow used in the graphical abstract.

Author's contribution. Conceptualization, M.S.; methodology, M.S. and M.K.F.; investigation, M.K.F., A.K., MS; taxonomy and nomenclature, I.Č.; writing - original draft preparation, M.K.F.; writing - review and editing, M.S., IČ; supervision, M.S.; funding acquisition, M.K.F. and M.S.

Financial support. This research was funded by Grant Agency of Charles University, grant number 194121. Bird sampling was done within the project supported by Czech Science Foundation, 14-02482S.

Competing interests. The authors declare there are no conflicts of interest.

Ethical standards. Animals were maintained and handled in the animal facility of Charles University in Prague following institutional guidelines. 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 MSMT-31949/2019-5, MSMT-31949/2019-6, of the Ministry of Education and 50982/ENV/14-2961/630/14 of the Ministry of Environment. Investigators are certified for experimentation on animals by the Czech Ministry of Agriculture. MS has a ringing licence allowing bird catching.

References

- Baker, J. R.** (1956). Studies on *Trypanosoma avium* Danilewsky 1885 II. Transmission by *Ornithomyia avicularia* L. *Parasitology*, 46, 321-334.
- Bennett, G. F.** (1961). On the specificity and transmission of some avian trypanosomes. *Canadian Journal of Zoology*, 39(1), 17-33.
- Bennett, G. F.** (1970). *Trypanosoma avium* Danilewsky in the avian host. *Canadian Journal of Zoology*, 48, 803-807.
- Bennett, G. F., Okia, N. O., & Cameron, M. F.** (1974). Avian hematozoa of some Ugandan birds. *Journal of Wildlife Diseases*, 10(4), 458-465.
- Bernotienė, R., Iezhova, T. A., Bukauskaitė, D., Chagas, C. R. F., Kazak, M., & Valkiūnas, G.** (2020). Development of *Trypanosoma everetti* in *Culicoides* biting midges. *Acta Tropica*, 105555.
- 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.
- Chatterjee, D. K.** (1977). Development of *T. avium bakeri* Chatterjee and Ray, 1971 in *Aedes albopictus* and its subsequent transmission to birds. *Indian Journal of Parasitology*, 1, 97-100.
- Cooper, C., Thompson, R. A., Botero, A., Kristancic, A., Peacock, C., Kirilak, Y., & Clode, P. L.** (2017). A comparative molecular and 3-dimensional structural investigation into cross-continental and novel avian *Trypanosoma* spp. in Australia. *Parasites & vectors*, 10(1), 234.
- Černý, O.** (2006). Hostitelé a vektorů trypanosom pčevců. Master's Thesis, Charles university, Prague, Czech Republic
- Crawley, M. J.** (2013). The R book. Chichester, West Sussex, United Kingdom: Wiley

- Desser, S. S., McIver, S. B., & Ryckman, A.** (1973). *Culex territans* as a potential vector of *Trypanosoma rotatorium*. I. Development of the flagellate in the mosquito. *The Journal of Parasitology*, 353-358.
- Diamond, L. S., & Herman, C. M.** (1954). Incidence of trypanosomes in the Canada goose as revealed by bone marrow culture. *The Journal of Parasitology*, 40(2), 195-202.
- Fialová, M., Santolíkova, 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.
- Galen SC, Borner J, Perkins SL, Weckstein JD** (2020). Phylogenomics from transcriptomic “bycatch” clarify the origins and diversity of avian trypanosomes in North America. *PLoS ONE* 15(10): e0240062.
- Greiner, E. C., Bennett, G. F., White, E. M., & Coombs, R. F.** (1975). Distribution of the avian hematozoa of North America. *Canadian journal of zoology*, 53(12), 1762-1787.
- Kato, H., Gomez, E. A., Cáceres, A. G., Vargas, F., Mimori, T., Yamamoto, K., Iwata, H., Korenaga, M., Velez, L. & Hashiguchi, Y.** (2011). Natural infections of man-biting sand flies by *Leishmania* and *Trypanosoma* species in the northern Peruvian Andes. *Vector-Borne and Zoonotic Diseases*, 11(5), 515-521.
- Kostygov, A. Y., Karnkowska, A., Votýpka, J., Tashyreva, D., Maciszewski, K., Yurchenko, V., & Lukeš, J.** (2021). Euglenozoa: taxonomy, diversity and ecology, symbioses and viruses. *Open Biology*, 11(3), 200407.
- Kramář, J.** (1958) *Fauna ČSR-Komáři Bodaví—Culicinae*; Academia: Prague, Czech Republic,
- Kulich Fialová, M.** (2024). Trypanosomes transmitted by mosquitoes: Occurrence in hosts, transmission and specificity. PhD Thesis: Charles University in Prague.

- Magaña Vázquez, R., Woog, F., Dinkel, A., Mackenstedt, U., & Musa, S.** (2022). Blood Parasites of Vangas and Other Corvoidea on Madagascar. *Diversity*, 14(10), 888.
- Maslov, D.A., Lukeš, J., Jirků, M., & Simpson, L.** (1996). Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: Implications for the evolution of parasitism in the trypanosomatid protozoa. *Molecular and Biochemical Parasitology* 75, 197–205.
- Medlin, L., Elwood, H.J., Stickel, S., & Sogin, M.L.** (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, 71, 491–499.
- Miltgen, F., & Landau, I.** (1982). *Culicoides nubeculosus*, an experimental vector of a new trypanosome from psittaciforms: *Trypanosoma barkeri* n. sp. *Annales de Parasitologie Humaine et Comparee*, 57, 423–428
- Musa, S., Altenburger, C., Vázquez, R. M., Woog, F., & Mackenstedt, U.** (2024). Parasite airlines: mapping the distribution and transmission of avian blood parasites in migratory birds. *International Journal for Parasitology*.
- Novy, F. G., MacNeal, W. J. & Torrey, H. N.** (1907). The trypanosomes of mosquitoes and other insects. *J Infect Dis* 4, 223–276
- R Core Team.** *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2018; Available online: <https://www.R-project.org/>
- Santolíkuvá, A., Brzoňuvá, J., Čepička, I., & Svoboduvá, M.** (2022). Avian louse flies and their trypanosomes: new vectors, new lineages and host–parasite associations. *Microorganisms*, 10(3), 584.
- Schaudinn, F.** (1904). Generation und Wirtswechsel bei *Trypanosoma* und *Spirochaeta*. *Arb K Gesundheitsamte* 20, 387–439 (in German).

- Sebaio, F., Braga, É. M., Branquinho, F., Fecchio, A., & Marini, M. Â.** (2012). Blood parasites in passerine birds from the Brazilian Atlantic Forest. *Revista Brasileira de Parasitologia Veterinária*, *21*, 7-15.
- Šlapeta, J., Morin-Adeline, V., Thompson, P., Mcdonell, D., Shiels, M., Gilchrist, K., Votýpka, J. & Vogelnest, L.** (2016). Intercontinental distribution of a new trypanosome species from Australian endemic Regent Honeyeater (*Anthochaera phrygia*). *Parasitology*, *143*(8), 1012-1025.
- Svobodová, M., Volf, P., & Votýpka, J.** (2015). Trypanosomatids in ornithophilic bloodsucking Diptera. *Medical and veterinary entomology*, *29*(4), 444-447.
- Svobodová, M., & Rádrová, J.** (2018). Phlebotomine sandflies-potential vectors of avian trypanosomes. *Acta Protozoologica*, *2018*(1), 53-59.
- Svobodová, M., Čepička, I., Zídková, L., Kassahun, A., Votýpka, J., Peške, L., Hrazdilová, K., Brzoňová, J., Voříčsk, P., & Weidinger, K.** (2023). Blood parasites (*Trypanosoma*, *Leucocytozoon*, *Haemoproteus*) in the Eurasian sparrowhawk (*Accipiter nisus*): Diversity, incidence and persistence of infection at the individual level. *Parasites & Vectors*, *16*(1), 1-16.
- Svobodová, M., Dolnik, O. V., Čepička, I., & Rádrová, J.** (2017). Biting midges (Ceratopogonidae) as vectors of avian trypanosomes. *Parasites & Vectors*, *10*(1), 224.
- Szabová, J.** (2008). Kinetoplastida komárov: prenos a prirodzení hostitelia. Hostitelé a vektorů trypanosom pěvců. Master's Thesis, Charles university, Prague, Czech Republic
- Valavičiūtė-Pocienė, K., Kazak, M., Jezhova, T., Kalinauskaitė, G., & Bernotienė, R.** (2024). Blood Parasites (Haemosporida, Trypanosomatida) in *Culex pipiens*: A Study and Review of Hibernating and Active Mosquitoes. *Microbiology Research*, *15*(4), 2184-2198.

- Valkiūnas, G., Iezhova, T. A., & Sehgal, R. N.** (2016). Deforestation does not affect the prevalence of a common trypanosome in African birds. *Acta tropica*, 162, 222-228.
- Votýpka, J., & Svobodová, M.** (2004). *Trypanosoma avium*: experimental transmission from black flies to canaries. *Parasitology Research*, 92(2), 147-151.
- Votýpka, J., Oborník, M., Volf, P., Svobodová, M., & Lukeš, J.** (2002). *Trypanosoma avium* of raptors (Falconiformes): phylogeny and identification of vectors. *Parasitology*, 125, 253-263.
- Votýpka, J., Rádřová, J., Skalický, T., Jirků, M., Jirsová, D., Mihalca, A.D., Amico, G., Petrželková, K.J., Modrý, D., & Lukeš, J.** (2015). A tsetse and tabanid fly survey of African great apes habitats reveals the presence of a novel trypanosome lineage but the absence of *Trypanosoma brucei*. *International Journal of Parasitology*, 45, 741–748.
- Votýpka, J., Szabová, J., Rádřová, J., Zídková, L., & Svobodová, M.** (2012). *Trypanosoma culicavium* sp. nov., an avian trypanosome transmitted by *Culex* mosquitoes. *International Journal of Systematic and Evolutionary Microbiology*, 62(3), 745-754.
- Wallace, F. G. & Johnson, A.** (1961). The infectivity of old cultured strains of mosquito flagellates. *J Insect Pathol* 3, 75–80.
- Zamora-Vilchis I, Williams SE, Johnson CN** (2012). Environmental temperature affects prevalence of blood parasites of birds on an elevation gradient: Implications for Disease in a Warming Climate. *PLOS ONE*
- Zídková, L., Čepicka, I., Szabová, J., & Svobodová, M.** (2012). Biodiversity of avian trypanosomes infection. *Genetics and Evolution*, 12(1), 102-112.

Table 1. Prevalences of trypanosomatids in dissected mosquito species and/or genera.

Mosquito Genus/species	Nr. dissected	<i>T. culicavium</i>	<i>T. tertium</i> n. sp.	<i>T. thomasbancrofti</i>	<i>T. theileri</i> *	<i>C. brevicula</i> **	<i>C. dedva</i> ***	<i>C. dobrovoskii</i> ****	<i>P. confusum</i>
<i>Aedes</i> spp.	959				157 (16.4%)	1 (0.1%)	1 (0.1 %)	3 (0.3 %)	
<i>Anopheles</i> spp.	33				1 (3%)				
<i>Cx. pipiens</i>	1128	36 (3.1%)	1 (0.08%)	1 (0.08%)	4 (0.3%)		1 (0.08 %)	2 (0.17 %)	8 (0.7%)
<i>Cs. annulata</i>	43				2 (4.6%)				
<i>Ms. richiardii</i>	83				1 (1.2%)	1 (1.2%)			

Found in the following species:

* *Ae. annulipes* (2/7), *Ae. cantans* (3/14), *Ae. excrucians* (3/19), *Ae. punctor* (11/27), *Ae. sticticus* (1/4), *Ae. vexans* (137/846), *An. plumbeus* (1/30)

** *Ae. annulipes* (1/7)

*** *Ae. vexans* (1/846)

**** *Ae. caspius* (1/14), *Ae. vexans* (2/846)

Table 2. Morphometry of trypanosomes in mosquito gut and in culture. Values in micrometres, SD, standard deviation. Asterisks in the same column indicate no significant differences in the cell length

Cell type	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)			*
diuresis	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	no free flagellum			

Table 3. Dimensions of whole cells of trypanosomes known to infect *Culex* mosquitoes (average, (range)).

Species	Long epimastigote gut (μm)	Long epimastigote culture (μm)	Short epimastigote gut (μm)	Short epimastigote culture (μm)	Kinetoplast highth (nm)	Blood trypomastigote (μm)	References
<i>T. tertium</i> n. sp.	59.6 (47.6-76.8)	37 (24.9-40.7)	15.7 (14-20.2)	17.6 (9.9-23.9)	749-859 * (579-966)	32.4	This study *Zidková <i>et al.</i> , 2012
<i>T. thomasbancrofti</i>	n. p.	17.8 – 20.4 (13.0-29.9)	16.5 (7.5-27.8)	10.1 – 10.5 (7.3-13.9)	527 – 546 (446-668)	38.3	Zidková <i>et al.</i> , 2012 Šlapeta <i>et al.</i> , 2016 Fialová <i>et al.</i> , 2021
<i>T. culicavium</i>	24.3	27.8 – 30.8 (18.5-50.6)	12.0	13.1 – 13.7 (9.8-16.7)	295- 341 (219-423)	40.7, 40.9	Zidková <i>et al.</i> , 2012 Votýpka <i>et al.</i> 2012
<i>T. culicis</i>	30-45	35-40	10-23	12-15	n. a.	n. a.	Novy <i>et al.</i> , 1907

(n. p. – not present, n. a. - not analysed)

Table 4. Overview of deposited samples containing specimens of *T. tertium* n. sp.

Sample	Inventory No.	Strain	Origin of the sample
1	P6E5584	Pas416	Exp. infection – <i>Cx. p. quinquefasciatus</i> gut
2	P6E5585	Pas416	Exp. infection – <i>Cx. p. quinquefasciatus</i> gut
3	P6E5586	Pas416	Exp. infection – <i>Cx. p. quinquefasciatus</i> gut
4	P6E5587	Pas416	Culture
5	P6E5588	Pas416	Culture
6	P6E5589	Pas416	Culture
7	P6E5590	Cul5	Exp. infection – <i>Cx. p. quinquefasciatus</i> gut
8	P6E5591	Cul5	Exp. infection – <i>Cx. p. quinquefasciatus</i> gut
9	P6E5592	Cul5	Exp. infection – <i>Cx. p. quinquefasciatus</i> gut
10	P6E5593	Cul5	Culture
11	P6E5594	Cul5	Culture
12	P6E5595	Cul5	Culture

Figure 1. Infection rates and intensities in mosquitoes *Cx. p. molestus* (mol) and *Cx. p. quinquefasciatus* (qui) experimentally infected with *T. tertium* n. sp. strains CUL5 and PAS416. Infection intensities: low 1-100 parasites; medium 100-1000 parasites; heavy > 1000 parasites per gut. Numbers in columns represents the number of dissected individuals from relevant infection category (heavy, medium, low, negative).

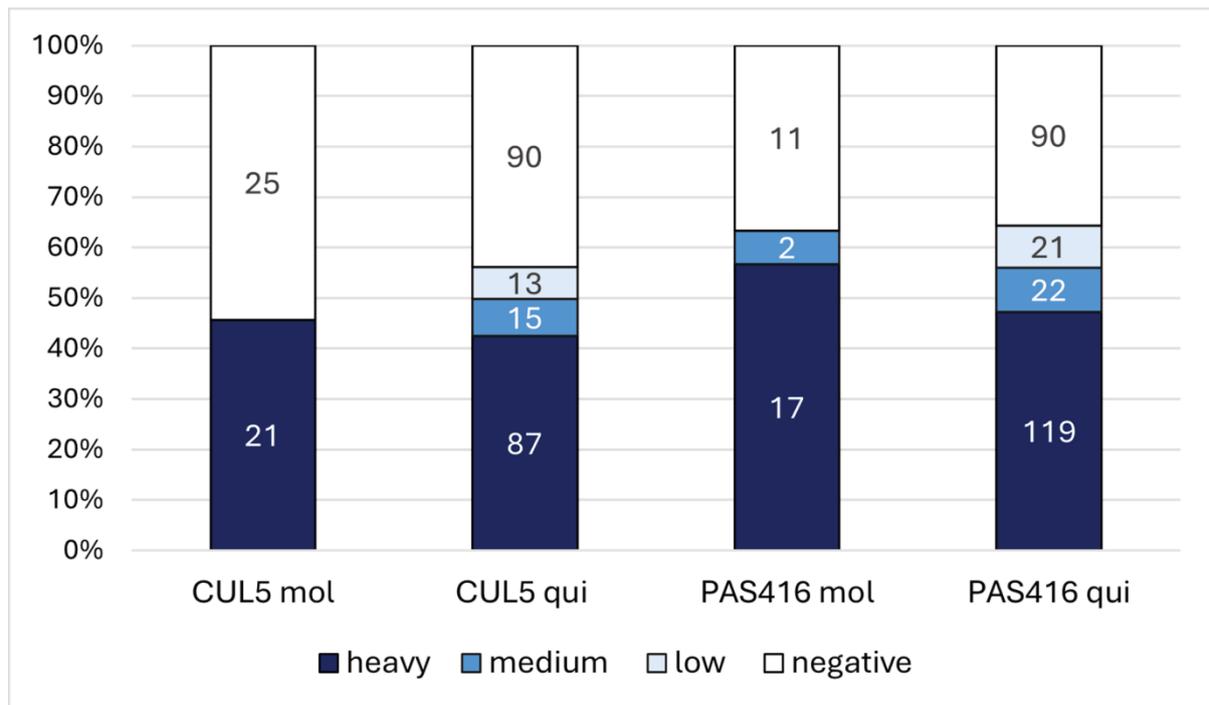


Figure 2. Temporal dynamics of trypanosomes localization in *C. p. quinquefasciatus* guts experimentally infected by trypanosome isolates CUL5 and PAS416. Numbers of dissected females are shown in the columns. Mg, midgut; Hg, hindgut.

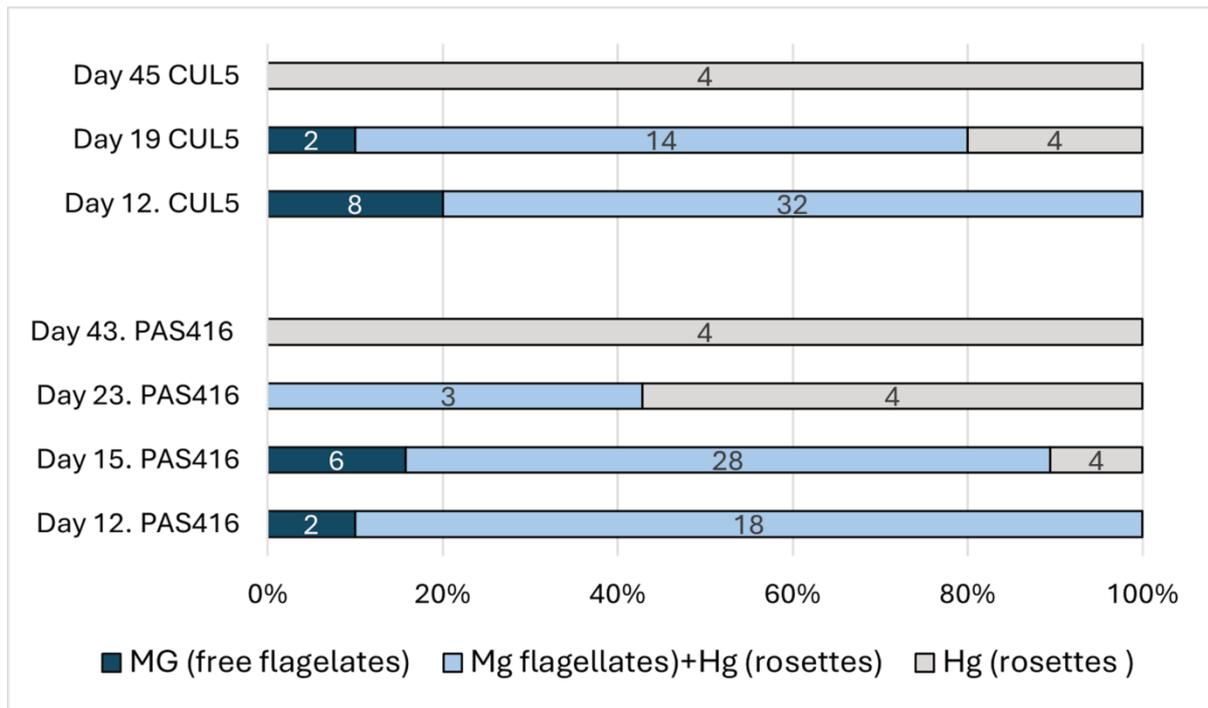
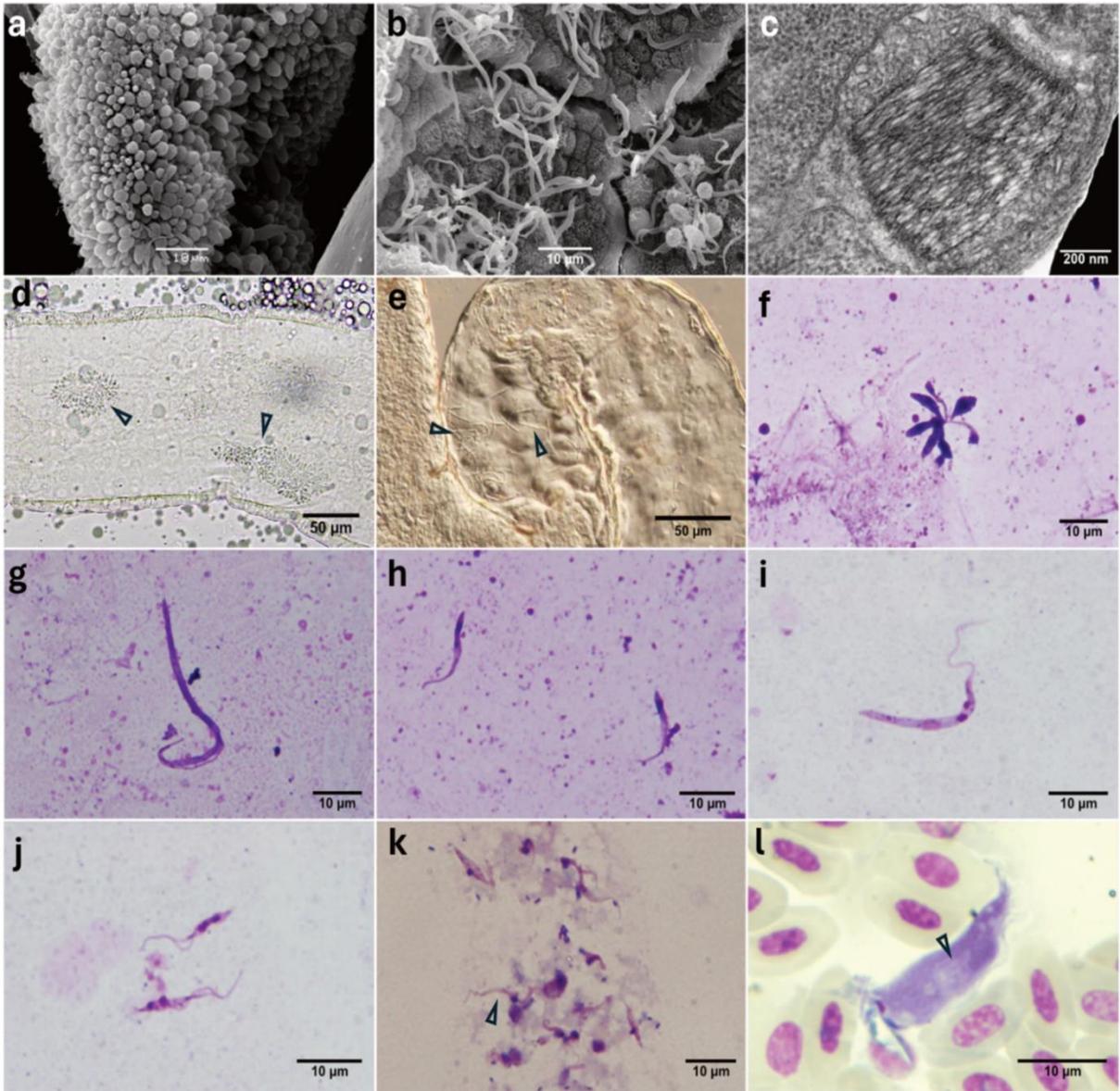


Figure 3. Scanning electron microscopy of *T. tertium* n. sp. isolate CUL5 after experimental infection of *C. p. quinquefasciatus*: rosette in hindgut (a), long epimastigotes in midgut (b). Transmission electron microscopy of kinetoplast of *T. tertium* n. sp. from culture (c) (photo by L. Zídková). 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* n. sp. morphotypes in *C. quinquefasciatus* gut: rosette (f), long epimastigotes (g), short epimastigote (h); and morphotypes from culture: long epimastigote (i) and short epimastigotes (j). Short trypan/epimastigotes from diuretic 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.



Graphical Abstract:

