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Phlebotomine Sandflies – Potential Vectors of Avian Trypanosomes

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Abstract. Phlebotomine sandflies were tested as potential vectors of avian trypanosomes (Kinetoplastea: Trypanosomatidae). *Lutzomyia longipalpis* and *Phlebotomus arabicus* took bloodmeals with cultured *Trypanosoma avium* parasites; mature infections with stages transmissible to canaries (*Serinus canaria*) developed in the sandflies. The infection rates ranged between 66 and 89%, with heavy infections in 24–78% fed females. *L. longipalpis* that fed on infected birds were also infected, and some developed mature infections (37 and 19%, resp). On the contrary, *Lutzomyia longipalpis* and *Phlebotomus arabicus* were not susceptible to infection with trypanosomes from *T. bennetti* clade. Our results, together with the previous findings of naturally infected *L. caballeroi*, suggest that sandflies could serve as vectors of avian trypanosomes from the *T. avium* clade.

Key words: *Lutzomyia longipalpis*, *Phlebotomus arabicus*, *Trypanosoma avium*, *Trypanosoma bennetti*, host specificity, life cycle, transmission, *Psychodiella chagasi*

Abbreviations
s. s. sensu stricto
s. l. sensu lato
LL *Lutzomyia longipalpis*
AR *Phlebotomus arabicus*

INTRODUCTION

Phlebotomine sandflies (Diptera: Psychodidae) are bloodsucking insects notoriously known as vectors of *Leishmania* spp. Besides transmitting numerous species of *Leishmania* s. s. to humans and other mammals, they also transmit species belonging to the saurian subgenus *Sauroleishmania* as well as other kinetoplastid genera belonging to the subfamily Leishmaniinae (Espinosa *et al*. 2016, Kostygov and Yurchenko 2017).

The relationship between trypanosomes and sandflies is less well understood. A putative trypanosome isolate from *Psychodopygus claustrei* was infective for an opossum (*Didelphis marsupialis*; Naiff *et al*. 1989). Based on SSU rRNA sequence similarities of parasites present in sandflies and vertebrate hosts, sandflies were proposed as vectors of anuran and snake trypanosomes (Ferreira *et al*. 2008, Viola *et al*. 2008, Kato *et al*. 2010). *Phlebotomus duboscqui* were susceptible for reptile trypanosomes after feeding on blood with cultured *Trypanosoma varani* (Minter-Goedbloed *et al*.

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1993). Finally, mature natural infections of *Lutzomyia caballeroi* with *T. avium* have been found (Kato *et al*. 2011). In summary, it seems that sandflies can potentially act as vectors of anuran, saurian, avian as well as mammalian trypanosomes in different regions of the world.

Avian trypanosomes are transmitted by a number of dipteran insects, namely, blackflies, hippoboscids, mosquitoes and biting midges (Simuliidae, Hippoboscidae, Culicidae, Ceratopogonidae). The earlier transmission attempts have been confirmed using molecular methods; confirmed modes of transmission are by vector ingestion and/or transconjunctivally (Baker 1956; Bennett 1961; Miltgen and Landau 1982; Votýpka *et al*. 2002; Votýpka and Svobodová 2004; Votýpka *et al.* 2012; Svobodová *et al.* 2015, 2017). The specificity of avian trypanosomes towards bloodsucking vectors is not well understood but it is supposed that trypanosomes have more specific relationships with vectors than with bird hosts (Apanius 1991). In a study analysing trypanosome strains from passerine, raptor, and insect hosts, three out of 12 lineages are transmitted by mosquitoes, two by hippoboscids, and two by blackflies, while vectors of several avian lineages remain unknown (Zídková *et al.* 2012). The vectors of *T. bennetti* have been recently identified among ornithophilic biting midges (Svobodová *et al.* 2017). Nevertheless, one isolate from a hippoboscid fly clustered with *T. avium* s. s., which might suggest that its vectorial specificity is not restricted to blackflies. Since trypanosomes from *T. avium* clade have been found in naturally infected sandflies (Kato *et al.* 2011), we decided to test the vectorial specificity of *T. avium* s. s., and to examine the potential of phlebotomine sandflies to transmit avian trypanosomes.

MATERIALS AND METHODS

Parasite strains and culture

The trypanosome strains used in this study were our own isolates: BUT15 from a nestling Common Buzzard (*Buteo buteo*; ABUT/CZ/1999/BUT15); SIM3 from a blackfly (*Simulium angustipes*) (IEUS/CZ/1999/SIM3); PAS23 from an adult Yellowhammer (*Emberiza citrinella*) (AEMB/CZ/2002/PAS23); all from Milovice forest, South Moravia, Czechia; APO7 was isolated from a Lesser Spotted Eagle (*Aquila pomarina*) in Eastern Slovakia (AAQU/SK/2000/APO7). These isolates represented two major avian trypanosome clades; SIM3 and BUT15 are *T. avium* s. s. and belong to clade C; APO7 belongs to *T. bennetti* s. s.; PAS23 to *T. bennetti* s. l., both from clade A (for a detailed phylogenetic study see Zídková *et al.* 2012).

Trypanosomes were cultivated on rabbit blood agar (SNB-9) in flat tubes, with the original overlay (*T. avium*) or RPMI 1640/ Schneider Drosophila Medium 1:1, supplemented with 10% FCS/2% sterile human urine/50 µg/ml amikacin (R+S) (*T. bennetti*).

Insect infections by membrane feeding

Sandflies used in the experiments are kept in the laboratory of vector-pathogen interactions at the Department of Parasitology, Charles University, Prague. *L. longipalpis* from Jacobina, Brazil, was a generous gift of Dr. M. Maroli, and kept in our laboratory from 1991. *P. arabicus* from Israel was established by the first author from two foundress females in 1991–1992. Experimental females were exposed to parasites by feeding through a chick skin membrane on heat-inactivated rabbit blood containing 4–10-day-old culture of 1–7 × 10^7 parasite cells/ml. Two independent feeding experiments were performed with *L. longipalpis* and *T. avium* SIM3. Fed females were separated immediately after blood-feeding, kept at 20°C, ambient humidity, and supplemented with 50% sucrose solution on a cotton pad. Eight days after feeding, females were dissected and their guts were examined under a light microscope for infection status, intensity of infection, and parasite localization.

Experimentally infected birds

Canaries (*Serinus canaria*) were bought in a pet shop. Before inoculation, they have been screened for trypanosome infection by blood culture (see further); they were negative. The canaries were inoculated with 6–14 positive guts suspended in 50 µl of saline, perorally or as drops into the eyes, and checked at 14-day intervals for infection status (see Table 1). In positive cases, parasite identity was confirmed by sequencing of the SSU rRNA gene. After three consecutive negative culture results, screening of individual birds was discontinued.

Trypanosome diagnostic sampling in birds

Diagnostic cultivation of trypanosomes was done in glass vials as described previously (Svobodová *et al.* 2015). Briefly, 10–20 µl of blood from the metatarsus vein articulation (*vena metatarsalis plantaris superficialis media*) was diluted in a tuberculine syringe with culture medium R+S (250 µl) and seeded on blood agar.

Transmission of trypanosomes from canaries to sandflies

To test if sandflies can be infected by feeding on the trypanosome-positive bird, *L. longipalpis* females were allowed to feed on canaries experimentally infected with *T. avium* strain SIM3 as described (Svobodová *et al.* 2017). Bodies of females that died prematurely (before defecation) were analysed by PCR, survivors were dissected 7–10 days after feeding and checked under the microscope.

Diagnostic PCR in sandflies and sequencing of the positive cultures were performed as described (Svobodová *et al.* 2017).

Light microscopy

Blood smears and parasites from dissected midge guts were fixed on slides with methanol and stained with Giemsa stain, Fluka.

Cell morphotypes from the guts were photographed at 1000× magnification with CCD camera DP70 using Olympus BX51 microscope and bright-field optics and measured using the Image-J software. ANOVA post-hoc comparisons were performed using Statistica software.

Scanning electron microscopy

Dissected guts positive for parasites were fixed in 2.5% glutaraldehyde in 5 mM HCl, 0.1 M cacodylate buffer for 24 h at 4°C. 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 using a JEOL 6380 LV scanning electron microscope.

RESULTS

Experimental infection of sandflies on membrane feeder

Experimental infections revealed high susceptibility of *L. longipalpis* and *P. arabicus* for *T. avium* s. s. strains. In total, 181 *L. longipalpis* and 36 *P. arabicus* were dissected (Fig. 1). There were minor differencies in the infection intensities between days 8 and 11, and between repeated experiment with *T. avium* SIM3 strain. The infection rate after defaecation on day 8–11 ranged between 66 and 89%. Heavy infections (> 1000 parasites per gut) developed in 24–78% of membrane-fed flies (Fig. 1).

To test the extent of the transmission potential, sandflies were fed on *T. bennetti* strains as well. Out of 33 *L. longipalpis* fed on APO7 strain, five had weak infections in the gut 8 days after feeding, or parasites were in the blood meal remnants in two individuals. At day 10 after feeding, 26 dissected females had no parasites in their gut. In *L. longipalpis* fed on PAS23 strain and dissected at days 8–11 (n = 36), only one had parasites in the bloodmeal remnants, similarly to *P. arabicus* which had very few PAS23 parasites in the gut in 5 out of 35 females but exclusively in the undigested blood.

Transmission of trypanosomes from experimentally infected sandflies to canaries

All three canaries inoculated with metacyclic trypomastigotes of *T. avium*, isolated from experimentally infected sandflies, developed infection. In one case, trypanosomes were detected as early as 1 day after inoculation (Table 1). Trypanosomes were detected in the blood till 126–213 days after inoculation.

Infection of sandflies after feeding on canaries

Out of 27 sandflies that fed on the infected canaries and survived till dissection, 37% were positive for trypanosomes, with 19% heavy infections (see Fig. 1). Twelve dead flies were PCR negative. Two out of three infected canaries were infectious to the sandflies that took blood on them.

Localization and morphology of trypanosomes in sandflies

T. avium was found primarily in the hindgut and rectal ampulla of the infected sandflies with mature infections (Fig. 2A, B). Rarely, parasites have been found in abdominal midgut and malpighian tubes as well. The localization was the same after membrane-feeding of sandflies and after feeding on experimentally infected canaries. To test the stability of parasite dimensions in different vectors and modes of infection, body and flagellum lenghts were measured; the parasites in late stage infections were similar in size and shape (Table 2, Fig. 2C–F). These metacyclics tended to retain a strain specific size; the body length did not differ significantly between *T. avium* SIM3 metacyclics developed in *P. arabicus* or *L. longipalpis*, and was not influnced by the way of infection (membrane x canary) in *L. longipalpis*. However, there was a significant difference between *T. avium* SIM3 and BUT15 in *L. longipalpis* (see Tab. 2; ANOVA LSD post-hoc comparisons, $P < 0.001$, $F = 17.3$). Flagellum length was longer for BUT15 as well; moreover, flagellum was significantly shorter in *T. avium* SIM3 metacyclics developed after feeding on canaries in comparison with membrane feeding ($P < 0.001$, $F = 18.5$, Table 2).

DISCUSSION

Recent years have revealed several new kinetoplastid vectors. *Leishmania macropodum* is highly probably transmitted by *Forcipomyia* sp. biting midges in Australia, although phlebotomine sandflies are the main vectors of *Leishmania* spp. in the rest of the World (Dougall *et al.* 2011). The suspected role of biting midges as vectors of avian trypanosomes has been confirmed recently; interestingly, midges supported not only the development of *T. bennetti* but that of *T. avium* as well (Svobodová *et al.* 2017). Here, we confirm that phlebotomine sandflies have the potential to transmit avian trypanosomes, as has been suggested by findings

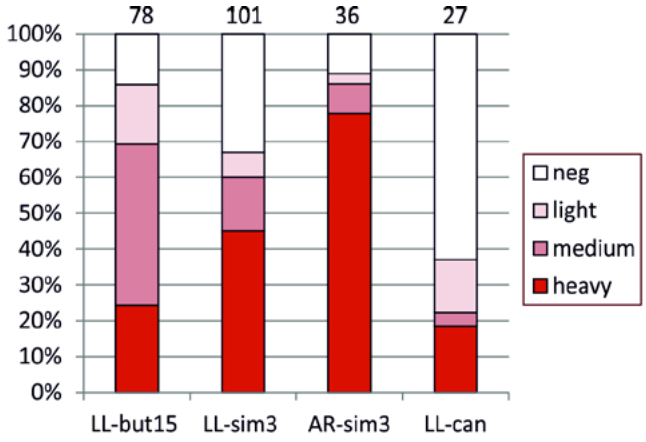

Fig 1. Infection rates and intensities in sandflies *L. longipalpis* and *P. arabicus* membrane-fed on *Trypanosoma avium* strains BUT15 and SIM3, and on canaries. Infection intensities: low – 1–100 parasites; medium – 100–1000 parasites; heavy – > 1000 parasites per gut. Numbers of dissected females shown above the columns. Data from repeated experimental feeding of *L. longipalpis* on SIM3 are pooled. Sandflies fed on membrane were dissected after 8 days, *L. longipalpis* fed on canaries were dissected 7–10 days after feeding.

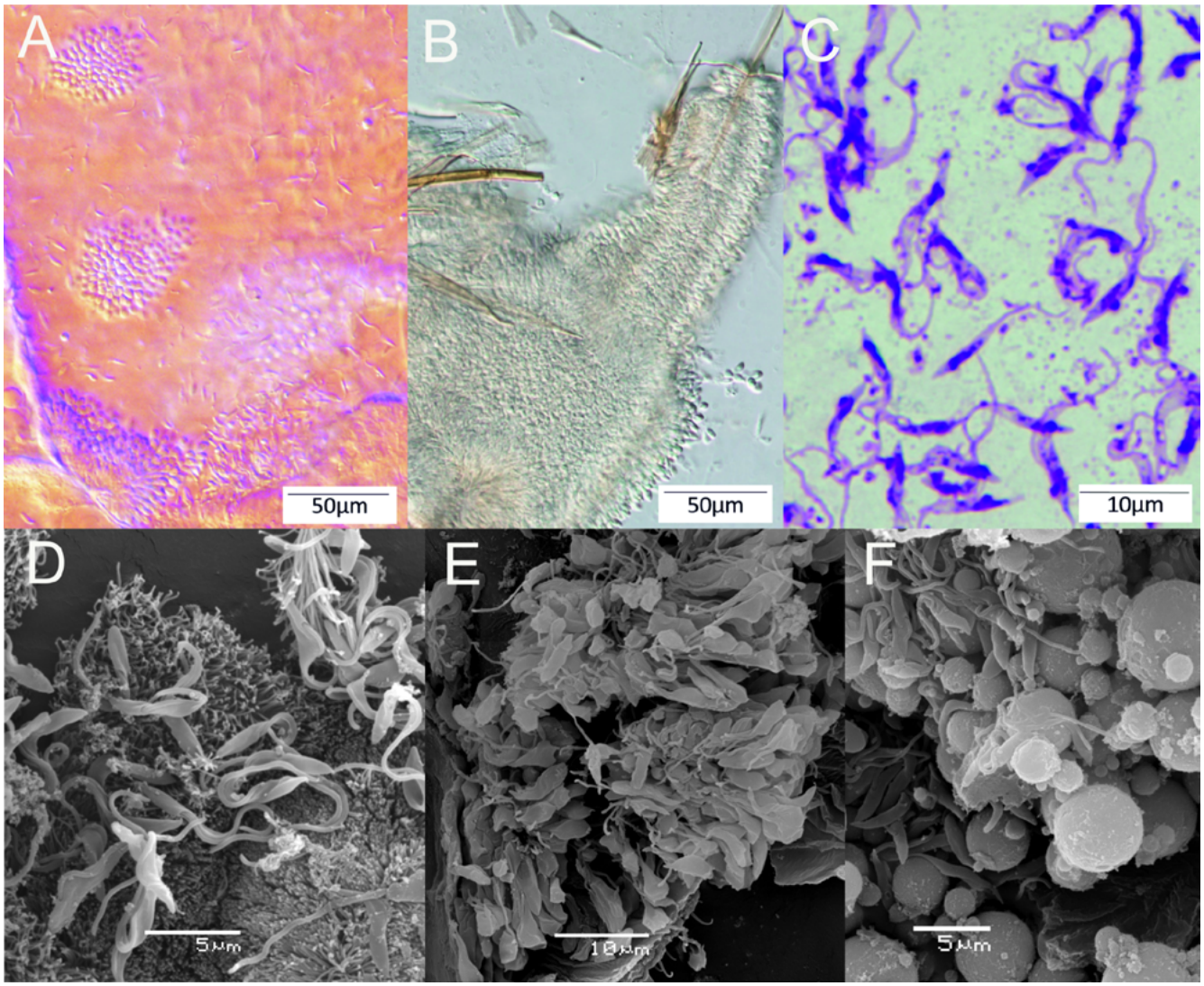

Fig. 2. Light microscopy of live *T. avium* SIM3 trypanosomes in *L. longipalpis* (A) and *P. arabicus* (B) gut; Giemsa stained metacyclic trypomastigotes of *T. avium* (s. s.) strain SIM3 from *L. longipalpis* hindgut (C). Scanning electron microscopy of *T. avium* (s. s.) SIM3 in the gut of sandfly *P. arabicus* (D, E) and *L. longipalpis* (F). A detailed view of trypomastigotes at the epithelium (D); note the massive infection of trypomastigots covering the gut (E); rounded objects are developmental stages of gregarines *Psychodiella chagasi* (F).

Table 1. Results of trypanosome cultivation from experimental canaries inoculated perorally (po) or transconjuctivally (tc) with *T. avium* s. s. strain SIM3 from guts of experimentally infected *L. longipalpis*. Pos – positive

Bird	Dose	Infection route	Result	Day first positive	Day last positive	Day last checked
1	14 guts	po	pos	21	213	213
2	6 guts	tc	pos	1	126	189
3	8 guts	tc + po	pos	7	189	189

Table 2. Morphometry of metacyclic trypomastigotes in sandfly gut

T. avium strain	Sandfly species	n	Body length	Flagellum length	Blood source
SIM3	*L. longipalpis*	27	9.7 ± 0.3 (9.1–10.3)	5.2 ± 0.2 (4.8–5.6)	canary
SIM3	*L. longipalpis*	15	10.5 ± 0.4 (9.6–11.4)	7.2 ± 0.3 (6.6–7.7)	feeder
SIM3	*P. arabicus*	20	10.5 ± 0.3 (9.8–11.2)	8.2 ± 0.3 (7.5–8.9)	feeder
BUT15	*L. longipalpis*	20	13.2 ± 0.5 (12.2–14.3)	11.1±1.2 (8.7–13.5)	feeder

Values in μm, average ± SE, range is given in parentheses.
Length: total length of the cell without free flagellum; Flagellum: free flagellum

of sandflies naturally infected with *T. avium* (Kato *et al.* 2011).

Blackflies, hippoboscids, mosquitoes, and biting midges can be accounted as confirmed vectors of avian trypanosomes (Baker 1956; Bennett 1961; Miltgen and Landau 1982; Votýpka *et al.* 2002; Votýpka and Svobodová 2004; Votýpka *et al.* 2012; Svobodová *et al.* 2015, 2017). We suggest that phlebotomine sandflies should be added to this list as potential vectors, based on 1. high susceptibility of both, *Lutzomyia* and *Phlebotomus* genera to artificial infection, 2. development of infectious parasite stages in sandflies, and 3. confirmed mature infection of sandflies after feeding on experimental or wild birds (this study; Kato *et al.* 2011).

T. avium group (group C sensu Zídková *et al.* 2012) consists of *T. avium* s. s. strains transmitted by blackflies as well as lineages transmitted probably by mosquitoes. The sequence of SSU rRNA of trypanosome isolated from *Lutzomyia* sandfly has 99% identity with that of the isolate SIM3. Therefore, we have chosen this *T. avium* isolate, originally obtained from a blackfly, to test the vector potential of *L. longipalpis*. Not only did this isolate developed mature infections with stages infective for passerine birds; the same was achieved with Old World sand fly genus *Phlebotomus*, namely, *P. arabicus*. This species has been selected because it belongs to the so called permissive sand fly vectors of *Leishmania* (Myskova *et al.* 2007), and has been confirmed as a vector of *L. tropica* possessing a modified lipophosphoglycan (Soares *et al.* 2004, Svobodová *et al.* 2006). To our knowledge, no avian trypanosomes have been isolated from *Phlebotomus* spp. to date; however, anuran trypanosomes have been found in *P. kazeruni* (Kato *et al.* 2010). Avian blood has been found in guts of several species of *Phlebotomus* (Maia *et al.* 2013, Velo *et al.* 2005, Svobodová *et al.* 2003, Bongiorno *et al.* 2003), confirming frequent feeding on birds that is a prerequisite of a transmission cycle between birds and sandflies belonging to the genus *Phlebotomus*. Feeding of *Lutzomyia* sandflies on avian host is well-known (e. g., Sant'Anna *et al.* 2008), and was best confirmed by natural infection of *L. caballeroi* with avian trypanosomes (Kato *et al.* 2011).

The localization of trypanosomes in the vector gut seems to be lineage-specific. In biting midges, *T. bennetti* trypanosomes were localized in the abdominal midgut while *T. avium* strains in the hindgut (Svobodová *et al.* 2017). In sandflies, *T. avium* mature stages were localized in the hindgut as well (this study; Kato *et al.* 2011), similarly to the usual vectors, blackflies (Bennett 1961, Votýpka and Svobodová 2004). The localization of trypanosome mature infectious stages in vector gut is tightly linked to the mode of transmission. Interestingly, although *T. culicavium* is localized on the stomodeal valve, it is fully dependent on transmission by vector ingestion, since no parasites are passed dur-

ing mosquito feeding (Votýpka *et al.* 2012); therefore, to date it has been found exclusively in passerines that eat mosquitoes (Zídková *et al.* 2012). *T. avium* has more transmission possibilities; besides ingestion of the vector, transconjunctival transmission has been experimentally confirmed (Votýpka and Svobodová 2004, Svobodová *et al.* 2017). This is not only a theoretical mode but it highly probably occurs in nature, since infective kinetoplastid parasite stages are passed during vector feeding with faeces in the process called prediuresis (Sádlová and Volf 1999).

The morphology and size of infectious forms is also similar in different vectors: trypomastigote forms found in naturally infected blackflies had body length 9.6 ± 1.9 μm and ranged between 4.6–14.4 (Votýpka and Svobodová 2004); in sandflies they were 10.1 ± 0.4 μm long, ranging between 9.1–14.3. There was no difference between cell length of *T. avium* SIM3 strain infecting two sandfly genera; however, BUT15 strain was significantly longer that SIM3 strain (10.1 vs 13.2 μm). Interestingly, similar body length was found in *C. nubeculosus* infected with the same *T. avium* strain BUT 15 (13.2 ± 0.5 μm; Svobodová *et al.* 2017). This might suggest that morphology is stable not only in the same vector subfamily, but even across vector families.

The potential of sandflies to transmit avian trypanosomes seems to be restricted to *T. avium* s. s. Attempts to infect sandflies with trypanosomes from *T. bennetti* clade were unsuccessful. These trypanosomes are transmitted by biting midges; besides being susceptible to *T. bennetti* infection, midges supported the development of *T. avium* strains as well (Svobodová *et al.* 2017). However, this extended permissiveness obviously does not apply to sandflies. In light of these results, we did not try to infect sandflies with trypanosomes from the third group, namely, *T. culicavium* clade specific for *Culex* spp.; these trypanosomes not only did not develop in *C. nubeculosus* midges but infection attempts were unsuccessful even for *Aedes aegypti* mosquitoes (Svobodová *et al.* 2017, Votýpka *et al.* 2012).

Sandflies may be used as model hosts producing metacyclic avian trypanosome stages infective for avian hosts. We have shown that *T. avium* forms derived from cultures were not infective, while trypomastigotes from naturally infected blackflies were (Votýpka and Svobodová 2004). Sandflies experimentally infected with *T. avium* may serve as a source of stages infective for birds.

In conclusion, our study further extends the spectrum of avian trypanosomes vectors; moreover, it has a broader implication for the future studies of potential vectors of parasites and pathogens. We can expect the emergence of new, unexpected host-parasite combinations.

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