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SHORT COMMUNICATION

Trypanosomatids in ornithophilic bloodsucking Diptera

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Abstract. Trypanosomes are known as widespread blood parasites of birds; however, knowledge of their prevalences in vectors and their overall biodiversity is rather limited. To assess the prevalences in potential vectors, we have microscopically examined ornithophilic bloodsucking Diptera (Culicidae, Simuliidae and Hippoboscidae) for the presence of trypanosomatids in their guts. In total, 3270 specimens were dissected, namely *Culex pipiens* Linnaeus, 1758 ($n = 898$), *C. modestus* Ficalbi, 1890 (136), *Simulium vernum* (Macquart, 1838) (1455), *S. angustipes* Edwards, 1915 (221) and *Ornithomyia avicularia* (Linnaeus, 1758) (560). All insect species were found to be infected with trypanosomatids, and the prevalence ranged from 4 to 8% but reached 60% in *S. vernum*. Blackflies and hippoboscids exclusively harboured trypanosomes (both *T.* cf. *avium* s.s. Danilewsky, 1885; *T. corvi/culicavium* group in hippoboscids). Mosquitoes were infected with *T. culicavium* Votypka, 2012 and *T. avium s. l.* but also with monoxenous parasites, namely *Crithidia brevicula* Frolov and Malysheva, 1989, and *Paratrypanosoma confusum* Votypka and Lukes, 2013. Only 4% of the isolated parasite strains were monoxenous whereas the majority were avian trypanosomes, confirming the vectorial status of the studied insects.

Key words. *Crithidia*, *Culex*, *Ornithomyia*, *Paratrypanosoma*, *Simulium*, *Trypanosoma*, kinetoplastida, prevalence.

Screening of avian vector-borne blood parasites is usually biased towards vertebrate hosts. In birds, two main groups of dixenous blood protists studied are haemosporidia (Haemosporida: *Plasmodium*, *Leucocytozoon*, *Haemoproteus*) and trypanosomes (Kinetoplastea, Trypanosomatida: *Trypanosoma)*, with a higher emphasis on the former. Although many authors begin to acknowledge the importance of vector studies, these still remain scarce as concerns trypanosomatids, and are mostly based on PCR diagnosis, which estimates prevalences in pooled insects (Van Dyken *et al.*, 2006; Reeves *et al.*, 2007).

Vectors of trypanosomes include various bloodsucking insects, as well as other haematophagous invertebrates. Avian trypanosomes have been shown to represent three major clades, each consisting of several lineages; vectors of some remain unknown (Zídková *et al.*, 2012). Previously, we have described or confirmed life cycles of several avian trypanosome species which differ in vectors and transmission modes. Two of them, *Trypanosoma* cf. *avium* and *T. corvi*, have mature infections localized in the hindgut of the insect. While *Trypanosoma* cf. *avium* is transmitted by blackflies (*Simulium* spp.) by ingestion or via conjunctiva (Bennett, 1961; Votýpka & Svobodová, 2004) *Trypanosoma corvi* is transmitted by ingestion of hippoboscid flies (*Ornithomyia avicularia*) (Baker, 1956; Votýpka *et al.*, 2004). Mature infections of the third species, *T. culicavium*, were found on the stomodeal valve of *Culex* mosquitoes (Volf *et al.*, 2004) but transmission to birds surprisingly occurs by ingestion of the vector as well (Votýpka *et al.*, 2012).

Nevertheless, bloodsucking insects can also host monoxenous trypanosomatids which complete their life cycle in a single host (e.g. Podlipaev *et al.*, 2004; Svobodova *et al.*, 2007). Their findings in insects can be confused with dixenous species, and must, therefore, be analysed carefully. Moreover, there are several documented cases of mammals and birds being infected with monoxenous species of the genera *Herpetomonas*, *Crithidia* and *Leptomonas* (for review see Lukeš *et al.*, 2014), most probably transmitted to accidental vertebrate hosts by bloodsucking insects. In this paper, we present data on kinetoplastid prevalences in the guts of bloodsucking insects (mosquitoes, blackflies and hippoboscids) as revealed by dissection and direct

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microscopical analysis. Identifying the maturity and location of infection and various developmental forms of trypanosomes is necessary for demonstrating vectorial competence of vectors. It also allows the isolation of parasites and the establishment of strains for further use in experimental infections.

Bloodsucking insects attacking raptor nestlings were collected in the Czech Republic between 1998 and 2002. Insects were caught overnight by air-sucking miniature CDC (Centre for Disease Control) traps (John W. Hock, Gainesville, FL, U.S.A.) without a light bulb, placed at the level of the nests as described (Votypka *et al.*, 2002; Votýpka & Svobodová, 2004). Hippoboscid flies were caught directly on hosts. Insects were determined using several taxonomic keys.

European Sparrowhawk [*Accipiter nisus* (Linnaeus, 1758)] and Common Buzzard [*Buteo buteo* (Linnaeus, 1758)] study sites were described elsewhere (Svobodová *et al.*, 2015). Briefly, sparrowhawk was studied in Prague (1010 dissected insects), and the buzzard in Southern Moravia and Prague suburbs (1515 and 473 insects dissected, resp.). Marsh Harrier [*Circus aeruginosus* (Linnaeus, 1758)] was studied in Southern Bohemia, Třeboň basin (48.54°–49.10°N, 14.39°–14.56°E, area 700 km^2), a flatland with 465 fishponds. The region is a mosaic of woods (45%) and farmland habitats (30%) interspersed with wetlands (15%). Harriers breed in reed on the ground (272 dissected insects).

Dissection of insects and light microscopical examination of their guts for the presence of parasites was performed within 3 days after capture. Insects were immobilized on ice, washed in 70% ethanol followed by sterile saline, and dissected in a drop of sterile saline. The gut was placed in a new saline drop and checked at 100–400 magnification. Dissecting tweezers were sterilized in a flame between each individual insect to prevent crosscontamination by both the gut microbial fauna and trypanosomatid parasites. Localization of the parasites was recorded in positive specimens.

For *in vitro* cultivation, trypanosomatid-positive guts were inoculated into 2-mL volume glass cultivation vials containing rabbit blood agar (SNB-9) overlayed with RPMI1640/Schneider Drosophila Medium 1 : 1 supplemented with 10% (v/v) foetal calf serum, 2% sterile human urine, gentamicin (100 µg/mL), penicillin (10 000 IU/mL) and fluorocytosine (1500 µg/mL). Cultivation was performed at room temperature; cultures were checked weekly for 1 month, and growing cultures were subcultured once and cryopreserved.

DNA was isolated, RAPD analysis and SSU rRNA sequencing were performed as described previously (Zídková *et al.*, 2012). Briefly, insect isolates have been preliminarily sorted to groups using four primers: OPA3 (AGTCAGCCAC), OPA9 (GGGTAACGCC), OPD5 (TGAGCGGACA), OPD13 (GGGGTGACGA). Eighteen isolates (6 from *Culex* spp., 8 from *Simulium* spp., and 4 from *Ornithomyia avicularia*) were chosen for thorough RAPD analysis using 30 primers. Isolates representing new clades of trypanosomes or monoxenous species were then sequenced (Zídková *et al.*, 2012; Flegontov *et al.*, 2013; E. Suková, unpublished data, 2009) Three cryostabilates contaminated by bacteria or fungi were sequenced directly from cryostabilates using kinetoplastid-specific primers (J. Rádrová, unpublished data, 2014).

In total, 3270 bloodsucking insects belonging to five different species and three families of Diptera were dissected (Table 1). In addition to five abundant species summarized in Table 1, eight other species were trapped very rarely, reaching a maximum of three caught specimens during the whole study. These rare species comprise *Anopheles plumbeus* Stephens, 1828, *An. maculipennis* Meigen, 1818, *Aedes cantans* (Meigen, 1818), *Ae. cinereus* Meigen, 1818, *Culiseta annulata* (Schrank, 1776), *Simulium lundstromi* (Enderlein, 1921) and *S. aureum* (Fries, 1824).

We assessed the infection status by microscopical examination; strains thriving in cultures were then characterized by molecular biology methods. Lineage prevalences might, therefore, be biased owing to unequal *in vitro* growth of different parasite lineages. By contrast, PCR diagnosis prevents to assess the localization and maturity of infection within the insect digestive tract, and can give false-positive results. First, parasites can thrive for some time in the gut of unspecific vectors before they are defaecated with the bloodmeal remnants; moreover, vectors can stay PCR-positive days after defaecation of unspecific parasites, possibly owing to the presence of a small amount of dead or live parasites that are, however, not able to infect a vertebrate host (Šeblová *et al.*, 2012). After defaecation, there is no difference in infection detectability between microscopy and PCR (Myskova *et al.*, 2008).

Prevalence of trypanosomatids ranged from 4 to 8% with a notable exception of *Simulium vernum* where it reached 60%. However, prevalences have to be considered with caution, as the insects were trapped at different localities, and at different dates. The extreme difference in trypanosome prevalences between *S. angustipes* and *S. vernum*, however, occurs even in specimens caught simultaneously in the same trap. In three traps where abundances of both species exceed 10 specimens, prevalences in *S. angustipes* ranged from 4 to 8% whereas in *S. vernum* from 58 and 65% ($n = 102$ and 193, respectively). This discrepancy might be done by different seasonal dynamics of these species. *Simulium vernum* is known to occur earlier in the season and is usually restricted to a single spring generation. By contrast, *S. angustipes* can produce up to three generations per year but, importantly, its first generation emerges later than that of *S. vernum* (Chvála, 1980; Bass, 1998). Therefore, sampled specimens of *S. vernum* were probably older than those of *S. angustipes*. As black fly females feed on hosts repeatedly and the infection is lifelong, higher infection rates are expected in older females.

The efficacy of strain establishment ranged from 40 to 52% across species. Most of the established strains belonged to genus *Trypanosoma*, whereas only 4.3% of them represented other kinetoplastid genera (Table 2). By contrast, biting midges captured on raptors nests have been found infected exclusively with monoxenous trypanosomatids. In 2508 specimens of different species belonging to the genus *Culicoides*, the average infection rate in the gut was 1.4%, ranging from 0 to 8.6% in individual species. Eight strains were established out of 39 cultured; three different species belonging to two monoxenous genera (*Herpetomonas* and *Sergeia*) have been described (Podlipaev *et al.*, 2004; Svobodova *et al.*, 2007; Zídková *et al.*, 2010). As the number of examined midges well exceeds that of other bloodsucking Diptera occuring at the same study sites and nevertheless the midges were negative for trypanosomes, it seems

Table 1. Prevalence of trypanosomatids in the gut of ornithophilic bloodsucking Diptera

Species	Examined	Infected	Prevalence (%)	Localization
Culex pipiens	898	74	8.2	60 sv 3 sv and am 1 sv and hg 7 hg 1 am 1 mt 1 cysts in am
Culex modestus	136	7	5.1	2 sv 4 hg 1 am - bloodfed
Simulium angustipes	1455	61	4.2	59 hg 2 am and mt
Simulium vernum	221	132	59.7	132 hg
Ornithomyia avicularia	560	28	5.0	28 ra

Sv, stomodeal valve; am, abdominal midgut; hg, hindgut; mt, malpighian tubes; ra, rectal ampulla.

Table 2. Trypanosomatid lineages isolated from ornithophilic bloodsucking insects.

Species	*n* cultured	*n* strains established	*n*, **trypanosome group**, species/lineage
Cx. pipiens	69	28	23 ***T. corvi/culicavium/****T.culicavium* (B V) 1 ***T. avium*** C II 2 ***T. avium*** C III 2 *Paratrypanosoma confusum*
Cx. modestus	7	2	1 ***T. corvi/culicavium/****T.culicavium* (B V) 1 *Crithidia brevicula* cul26
S. angustipes	55	22	22 ***T. avium*** C X + XI
S. vernum	14	6	6 ***T. avium*** C X
O. avicularia	23	12	2 *T.* ***corvi/culicavium*** (B I) 9 ***T. corvi/culicavium*** B IV *T. corvi* 1 ***T. avium*** C XI

Species/lineages according to (Zídková *et al.*, 2012): group B - *T. corvi/culicavium*, lineages I, IV, V, XII, group C - *T. avium* s. l., lineages II, III, X, XI.

that biting midges do not act as vectors of avian trypanosomes in the present area.

Most of the trypanosomatids that we have found in bloodsucking Diptera belong to different lineages of avian trypanosomes, whereas only 4% (3/69) represent monoxenous trypanosomatids, and those were found only in culicine mosquitoes (7% of established strains). This may be linked to different adult and/or larval biology; hippoboscids are viviparous and both sexes feed exclusively on blood, whereas blackflies larvae live in running water where transmission of kinetoplastids seems unprobable.

The habitat has a great influence on parasite prevalences in bloodsucking insects, probably owing to (infected) avian host availability. In mosquitoes caught in reed, the prevalence of *T. culicavium* was only 0.3 and 0.05% in *Cx. pipiens* and *Cx. modestus*, respectively (Votýpka *et al.*, 2012) whereas in our sample (mostly obtained in forests or wooded areas) the estimated prevalence is 5.4 in *Cx. pipiens*, and 1.4% in *Cx. modestus*, as based on strain localization and relative lineage occurence. Prevalences of parasites seem to be influenced by mosquito host preferences in connection to host availability; whereas *Cx. modestus* fed equally on Anseriformes and Passeriformes, *Cx. pipiens* preferred passerines (Radrova *et al.*, 2013). Other factors potencially influencing prevalences detected in insects are time and year of sampling and location of traps in the habitat (e.g. Černý *et al.*, 2011).

Studies on kinetoplastid prevalences in vectors are scarse. In a study conducted in Colorado, 1 out of 456 *Cx. pipiens* and 1 out of 1155 *Cx. tarsalis* Coquillett, 1896 were positive for kinetoplastids belonging to *T. avium* s. l. clade, 1 *Cx. tarsalis* for *T. corvi* and 1 *Cx. tarsalis* harboured an unclassified trypanosome strain (Van Dyken *et al.*, 2006). Most of the sequences (24) formed a new kinetoplastid clade that was later shown to be *Paratrypanosoma confusum* (Flegontov *et al.*, 2013). As concerns blackflies, two *Simulium lyra* (Lundström, 1911) and one *S. vernum* from Finland harboured *T*. cf *avium* parasites (Reeves *et al.*, 2007); unfortunately, parasites were enclosed within the bloodmeal, so the vectorial status of these black fly species is uncertain.

In our study, the localization of mature infection between vectors is variable but it seems quite conservative in some of the trypanosome lineages. Out of 26 blackfly strains that have been cultured and molecularly characterized, all were localized in the hindgut with one exception owing to incomplete boodmeal digestion. Mature infections in hippoboscids were also localized in the posterior part of the gut. In mosquitoes, all except one isolates belonging to *T. culicavium* (22) were localized on the

stomodeal valve. By contrast, isolates belonging to group C (*T. avium* s. l.) were localized in the hindgut, abdominal midgut or stomodeal valve, with different localization even among isolates belonging to a single lineage (CIII). Experimental infections of *C. quinquefasciatus* with a strain originally localized in the hindgut resulted in infections on the stomodeal valve (Szabová, 2008, master thesis). The stomodeal valve and hindgut are both lined by a chitin cuticle, whereas the midgut epithelium is lined by microvilli (Volf *et al.*, 2004). Trypanosomes attach to the chitin layer by structures called hemidesmosoms. A similar mechanism of attachment may explain alternative localization in these two rather distant parts of the digestive tract.

In conclusion, we show that vectors of avian trypanosomes are diverse within one study area, prevalences may be extremely high and individual vector species potencially transmit several avian trypanosome lineages.

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