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BRIEF REPORT

Trypanosomatids in European bison (*Bison bonasus*): insights from biting midges and tissue screening

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Abstract
While leishmaniases have long been established in southern Europe, increasing cases have also been reported in Central Europe. A recent serological study in Poland suggested the presence of *Leishmania* spp. in European bison (*Bison bonasus*). To verify this, we screened both *Culicoides* biting midges and European bison tissues for *Leishmania* DNA. Biting midges were collected in the Białowieska Forest, where we identified ten species, with *Culicoides obsoletus* and *C. pictipennis* being the most abundant. However, none of the 129 biting midges or 63 European bison tissue samples tested positive for *Leishmania* DNA. In contrast, *Culicoides* midges were found to carry *Trypanosoma theileri* and the monoxenous trypanosomatid *Herpetomonas ztiplika*. Moreover, *T. theileri* was detected in three European bison, marking the first molecular confirmation of this parasite in this host. Given the absence of *Leishmania* DNA in both European bison and biting midges, we propose that the previous serological findings may have resulted from cross-reactivity with *T. theileri* rather than a true *Leishmania* infection.

Keywords European bison · *Culicoides* · *Leishmania* · *Trypanosoma* · *Herpetomonas*

Introduction

Recently, four European bison were identified as seropositive for *Leishmania* antibodies in the Białowieska Forest and Gdańsk Zoo (Didkowska et al. 2024). However, Poland is not considered endemic for leishmaniasis, and phlebotomine sand flies (Diptera: Psychodidae), the typical vectors of *Leishmania* parasites (Kinetoplastida: Trypanosomatidae), are currently believed to be absent from the country. On the other hand, two trypanosome species are known to

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infect European bison: *T. theileri* Laveran, 1902 and *T. wrublewskyi* (*wrublewskii*) Wladimiroff & Yakimoff, 1909, now considered a synonym of *T. theileri* (Yakimoff 1915) (Karbowiak 2014). These findings highlight the need for further epidemiological investigation. Therefore, we investigated biting midges as potential alternative vectors of *Leishmania* parasites in the Białowieża Forest and screened tissue samples from Polish European bison for *Leishmania* DNA using PCR.

Leishmaniasis is a vector-borne disease transmitted by sand flies of the genera *Phlebotomus* and *Lutzomyia*, occurring predominantly in tropical and subtropical regions. It is caused by protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), with over 50 species described, approximately 20 of which are pathogenic to humans (Akhoundi et al. 2016). The primary causative agent of leishmaniasis in southern Europe is *Leishmania infantum*, with dogs serving as the main reservoir and sand flies (subgenus *Larroussius*) as vectors (Maia et al. 2023). Endemic to the Mediterranean region, *L. infantum* is expanding northwards due to climate change, with sporadic autochthonous cases reported in Central Europe (Kniha et al. 2023) and a few likely imported cases of canine leishmaniasis reported in Poland (Sapierzynski 2008; Adaszek et al. 2024).

In addition to *L. infantum*, autochthonous cases of cutaneous leishmaniasis in horses and cattle caused by *Leishmania* (*Mundinia*) *martiniquensis* have emerged in Central Europe over recent decades, with infections reported in Germany, Switzerland, Austria and Czechia (Müller et al. 2009; Lobsiger et al. 2010; Modrý et al. 2025). The vectors and reservoirs remain unknown, but evidence from Thailand and Australia suggests that biting midges (Diptera: Ceratopogonidae) may serve as vectors. Parasite DNA has been repeatedly detected in wild-caught females (Sunantaraporn et al. 2021; Songumpai et al. 2022; Kaewmee et al. 2023), and mature infections have been observed microscopically in *Forcipomyia* (*Lasiohelea*) from Australia and *Culicoides peregrinus* from Thailand (Dougall et al. 2011; Kaewmee et al. 2023). Laboratory experiments further support the role of biting midges in *L. martiniquensis* transmission (Becvar et al. 2021).

European bison (*Bison bonasus*), also called wisent, is classified as a "near-threatened" species by the International Union for Conservation of Nature (IUCN) (Plumb et al. 2020). Although the population is growing, the conservation of the wisent faces new challenges, such as excessive local density (Klich et al. 2021, 2023) and increasing risks of health threats, including local outbreaks of parasitic diseases. Recognizing these potential health threats is critically important for the conservation of this species.

Materials and methods

Study area. The Polish part of Białowieska Forest, spanning 625 km^2 at an altitude of 135–197 m, is a major biodiversity hotspot on the Polish–Belarusian border with a temperate continental climate influenced by the Atlantic (Boczon and Salachewitz 2022). Trapping was conducted at the following locations: No. (1) Mature climax high mixed forest in Budy (52°43 × 55"N, 23°45 × 10"E; 9 traps/nights) with diverse spatial structures, rich undergrowth, and coarse woody debris surrounded by meadows. No. (2) Mosaic habitats in Piaski hamlet (52°39 × 25"N, 23°31 × 41"E; 9 traps/nights), including mesic grasslands, young *Betula* forest, and semi-natural high forest. No. (3) Extensive wet meadows in the Narewka floodplain (52°43 × 10"N, 23°49 × 01"E; 3 traps/nights) are dominated by tall herbs, *Salix* scrub, and strips of *Alnus* forest. No. (4) Wet forest boundary in the Narewka River floodplain (52°42 × 18"N, 23°49 × 41"E; 3 traps/nights), with young *Alnus* trees, *Salix* shrubs, and tall herbs. No. (5) The swampy forest along the Łutownia floodplain (52°44 × 01"N, 23°47 × 13"E; 3 traps/nights) dominated by *Alnus* trees with dense herbaceous undergrowth.

Collection of biting midges and identification of morphological species. Insects were collected in May 2024 via nine CDC light traps (John W. Hock, USA) placed overnight ~ 1.5 m above ground. Insects were briefly anaesthetized at -4 °C, and biting midges were separated and preserved in 70% ethanol. Species identification was performed under a stereomicroscope using taxonomic keys (Chvála et al. 1980; Alwin and Szadziewski 2013).

European bison tissue samples. Organs and tissues from 63 European bison (34 females, 29 males) were collected between 2022 and 2024. In total, 112 spleen samples (from 56 animals), 6 liver samples, and 15 skin samples (the latter derived from an individual with skin lesions infected with *Sarcoptes scabiei* and *Demodex* sp.) were analysed. The animals originated from different locations in Poland: the Bieszczady Mountains ($n = 50$), Białowieska Forest (5), Knyszyńska Forest (5), Pszczyna (2), and Borecka Forest (1) (Fig. 1). No animal was sacrificed for this study. All the samples were transported immediately to the laboratory and stored at -20 °C until analysis.

Molecular species identification of biting midges. For damaged specimens or to verify morphological determination, mitochondrial cytochrome oxidase subunit I (*COI*) barcoding was performed (Folmer et al. 1994). The generated *COI* gene sequences were analysed against BINs in the Barcode of Life Data System (BOLD) (https://boldsystems.org/).

Molecular detection of trypanosomatids DNA in biting midges and European bison. The presence of *Leishmania* DNA was screened in blood-fed, gravid, and parous

Fig. 1 Map of Poland showing the five localities where the tested European bison originated, along with a graphical representation of the number of individuals analysed from each site

biting midges and European bison tissue samples. DNA was extracted via the High Pure PCR Template Preparation Kit according to the manufacturer´s instructions. The extracted DNA served as a template for PCR amplification with primers targeting the 434-bp region of the *Leishmania* HSP70 sequence (F: 5´-GAGCTGAACAAGAGCATCAAC-3´, R: 5´-GGTGATCTGGTTGCGCTTG-3´). Additionally, European bison tissue samples were tested via a more sensitive nested PCR assay targeting the kinetoplastid SSU (Seward et al. 2017; Lukeš and Votýpka 2020). The PCR products were purified via the Exo-CIP Rapid PCR Cleanup Kit, and Sanger sequencing was performed with the HSP70-R and 1000R (5´-ATGCCTTCGCTGTAGTTCGTCT-3´; for nested PCR) primers. The generated sequences were analysed via BLAST comparison with the GenBank database.

Table 1 Species composition of *Culicoides* biting midges collected via CDC light traps in Białowieska forest, Poland

Species	Locality No.	Nulliparous	Blood-fed	Gravid	Parous	Males	Total
C. (*Oecacta*) *achrayi*	**4**	2	0	0	0	0	2
C. (*Oecacta*) *furcillatus*	**3**	0	0	0	1	0	1
C. (*Culicoides*) *impunctatus*	**1**	0	0	0	2	0	2
C. (*Avaritia*) *obsoletus* complex	**1**, **2**, **3**, **4**	62	0	1	91	2	156
C. (*Oecacta*) *pictipennis*	**1**, **2**, **4**, **5**	61	6	2	23	4	96
C. (*Culicoides*) *pulicaris*	**1**	1	0	0	0	0	1
C. (*Culicoides*) *punctatus*	**1**, **2**, **5**	2	0	0	3	0	5

Results and discussion

Biting midge identification and trypanosomatid detection

A total of 263 biting midges (genus *Culicoides*) were captured across five sites. Seven species were identified (Table 1), with the *Culicoides obsoletus* complex (59.3%) and *C. pictipennis* (36.5%) being the most abundant and widespread, recorded at four out of the five sites.

The primary *Leishmania* vectors, phlebotomine sand flies, are absent in Poland. In temperate regions, biting midges—abundant in Central Europe—are considered potential vectors for species of the subgenus *Mundinia* (Becvar et al. 2021). In Poland, 220 biting midge species from 21 genera have been recorded (Szadziewski and Dominiak 2016).

The biting midges most frequently captured in this study belong to the *Culicoides obsoletus* complex, key vectors of the Bluetongue virus (BTV) and Schmallenberg virus (SBV) in Europe, including the Białowieska Forest (Kęsik-Maliszewska et al. 2018). The second most abundant species, *C. pictipennis*, is a generalist that feeds on birds and mammals, including humans and ungulates. Other recorded species (*C. punctatus*, *C. achrayi*, *C. impunctatus*, *C. pulicaris*, and *C. furcillatus*) are also known to feed on horses and ruminants, making their presence near European bison unsurprising. The relative abundances of *C. punctatus* and *C. achrayi* were lower than those reported in an SBV epidemiological survey conducted from 2014 to 2015 near bison resting sites (Kęsik-Maliszewska et al. 2018).

In addition to *Culicoides*, three other midge species were recorded: *Forcipomyia* (*Thyridomyia*) *monilicornis* ($n=2$) and two *Atrichopogon* species, *A.* (*Meloehelea*) *winnertzi* (6) and *A.* (*Meloehelea*) *oedemerarum* (3). *Meloehelea* species feed on the haemolymph of Meloidae and Oedemeridae beetles, whereas the feeding habits of *Thyridomyia* remain unknown but likely involve other insects (Grogan Jr et al. 2010).

None of the 129 tested blood-fed, gravid, or parous females, grouped into 35 pools (1–32 per pool), tested positive for *Leishmania* DNA via HSP70 PCR. As a control, all pools were also tested using a more sensitive nested PCR targeting trypanosomatid SSU rRNA. All tested pools remained negative for *Leishmania* DNA, but four *C. obsoletus* pools tested positive for *Herpetomonas ztiplika*, and *Trypanosoma* (*Megatrypanum*) *theileri* was detected in another four pools: 1× *C. furcillatus*, 1× *C. obsoletus* complex, and 2× *C. pictipennis*. Thus, although the number of screened insects was limited, the two trypanosomatid species mentioned above were detected quite frequently. Nevertheless, further vector screening in other localities of European bison occurrence in Poland would be beneficiary.

Trypanosomatid detection in European bison tissue samples

A total of 112 spleen, 6 liver, and 15 skin samples from 63 European bison were tested by *Leishmania* (HSP70)-specific PCR and trypanosomatid (SSU)-specific nested PCR; all tested negative for *Leishmania* DNA by both methods.

Highly sensitive nested PCR detected *Trypanosoma theileri* in three European bison, all originating from the Bieszczady Mountains. In two cases, only one of the duplicates tested positive, whereas in the third case both spleen duplicates tested positive. Despite its global distribution, *Trypanosoma theileri* remains largely overlooked due to its limited economic impact and lack of overt pathology. Infections are typically asymptomatic; however, fever, anorexia and anemia have been reported in bovids, particularly in cases of coinfection or physiological stress (Hoare 1972; Wells 1976). The *T. theileri* complex is common in European wild ruminants (Brotánková et al. 2022) and has also been reported in Poland (Filip-Hutsch et al. 2022; Orłowska et al. 2025). However, our findings (GenBank accession number PV759752), along with the sequences KF765799 and KJ397592 (unpublished but available in GenBank, identified as originating from *Bison bonasus* in Poland), represent the first molecular confirmation of *T. theileri* (subclade TthI) in the European bison (see Supplementary file 1). The detection of trypanosomes in three individuals corresponds to a prevalence rate of approximately 5%, although the actual rate may be higher, as parasitemia of *Megatrypanum* species is typically very low.

Confirmed vectors of *T. theileri* are tabanids; however, the parasite has been detected in a wide range of other potential vector groups, including keds, mosquitoes, sandflies, black flies and biting midges. The relevance of these groups in parasite transmission remains unclear, although metacyclic stages were reported in experimentally infected *Aedes aegypti* mosquitoes (Brotánková et al. 2022).

According to a recent seroprevalence study (Didkowska et al. 2024) suggesting the occurrence of *Leishmania* parasites in European bison in Poland, it is important to note that ELISA may not be conclusive, as it often yields false-positive or false-negative results (Zanette et al. 2014; Singh and Sundar 2015). Since the four previously reported positive cases presented borderline titres, they may represent false positives. Additionally, no control for cross-reactivity with trypanosomes infecting European bison was included. Therefore, we propose that the assumed seropositivity likely reflects cross-reactivity with *T. theileri*. This conclusion is further supported by the absence of *Leishmania* DNA in the tested *Culicoides* biting midges.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate All experimental procedures including the culling of European bison, were approved by the General Directorate for Environmental Protection in Poland, in accordance with the Act of 16 April 2004 on the Protection of Nature. Lethal control was conducted by local institutions responsible for European bison management, with each hunter holding the required permits. The collection and storage of samples from deceased individuals for research purpose were authorised by the Regional Director of Environmental Protection in Warsaw.

Competing interests The authors declare no competing interests.

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