Parasitology

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Molecular detection of *Trypanosoma* spp. in *Lipoptena cervi* and *Lipoptena fortisetosa* (Diptera: Hippoboscidae) and their potential role in the transmission of pathogens

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Review

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Abstract

The family Hippoboscidae is a less known group of blood-sucking flies. Deer ked are particularly important for animal health; they may act as potential vectors of disease to ungulates, and may transmit pathogens to animals and humans. The aim of this study was to investigate the presence of *Trypanosoma* (*Megatrypanum*) DNA in deer keds using molecular methods. Results prove the presence of *Megatrypanum* trypanosome DNA in the studied winged adult deer keds and this is the first detection of this pathogen in *Lipoptena fortisetosa*. In addition, this paper evidences the occurrence of *L. fortisetosa* in two new locations: one in the Białowieża Primeval Forest, and another in the Strzałowo Forest Inspectorate (Piska Forest), both in north-eastern Poland.

Introduction

Flies in the family Hippoboscidae (Diptera), known as 'louse flies' or 'keds' are a group of obligate parasites of mammals and birds (Rahola *et al*., 2011). A recent checklist of Hippoboscidae across the world retains three subfamilies (Ornithomyinae, Hippoboscinae and Lipopteninae) more than 213 species and 21 genera (Dick, 2006; Petersen, 2013). It has been shown that the two subfamilies Hippoboscinae and Lipopteninae are monophyletic groups (Petersen *et al*., 2007). From Europe, 30 species of Hippoboscidae are known (Petersen, 2013). In Poland, the hippoboscid fauna is relatively not well known. About 10 species are present in Poland including four which parasitize mammals: the forest fly *Hippobosca equina* L., 1758, the sheep ked *Melophagus ovinus* L., 1758 and two species of the *Lipoptena* – *Lipoptena cervi* L., 1758 and *Lipoptena fortisetosa* Maa, 1965 (Borowiec, 1984; Borowiec and Zatwarnicki, 1989; Kowal *et al*., 2016).

Worldwide, 30 *Lipoptena* species were recorded, five of them were described from Europe (Dick, 2006; Petersen, 2013). *Lipoptena cervi* and *L. fortisetosa* have a more northern range while the other three species have a restricted distribution in Southern Europe, including the Mediterranean islands (Petersen, 2013; Kurina *et al*., 2019). In Europe, the *L. fortisetosa* was first recorded from Czech Republic where it was initially described as a new species – *L. parvula* Theodore, 1967, but was later changed to *L. fortisetosa* by Grunin (1970) (Kurina *et al*., 2019).

Its appearance in Europe is probably associated with the introduction of sika and Siberian deer in 1839 (Bartoš, 2009). The species is also able to colonize the European roe deer population as a result of natural spread following contact with Siberian roe deer (Kowal *et al*., 2016). *Lipoptena fortisetosa* was reported for a few European countries, including Germany, Belarus, Moscow district in Russia, Austria, Lithuania, Moldova, Poland, the Czech Republic, Romania, Slovakia and Switzerland (Kurina *et al*., 2019; Oboňa *et al*., 2019). More recently the species has been also recorded in Italy and Estonia (Andreani *et al*., 2019; Kurina *et al*., 2019). The most important hosts of deer keds in Europe are mainly: red deer *Cervus elaphus* L., 1758, roe deer *Capreolus capreolus* (L., 1758), Eurasian elk *Alces alces* (L., 1758), sika deer *Cervus nippon* Temminck, 1838 and fallow deer *Dama dama* (L., 1758). In North America, the parasite is common on wapiti *Cervus canadensis* Erxleben, 1777 and white-tailed deer *Odocoileus virginianus* (Zimmermann, 1780). A wide range of animals can also be accidental hosts of *L. cervi*: horses, cattle, European bison, sheep, domestic dogs, red foxes, badgers and suids (Hermosilla *et al*., 2006; Karbowiak *et al*., 2014; Kowal *et al*., 2016). Deer keds occasionally bite also humans (Kortet *et al*., 2010). In Poland, while *L. cervi* has been observed throughout the country, *L. fortisetosa* has been found only in the Dolnośląskie, Małopolskie and Warmińsko-Mazurskie Voivodeships (Borowiec and Zatwarnicki, 1989; Kowal *et al*., 2009). Sokół and Gałęcki (2017) reported the presence of *L. fortisetosa* in dogs in cities in central Poland. Both

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L. fortisetosa and *L. cervi* have a specific development cycle. Upon settling on a suitable mammal host, deer keds shed their wings, remaining in a wingless form for the rest of their life. These flies are viviparous species: they generate fully-grown larvae that fall to the ground and pupate. Although the keds occur throughout the year, the winged adults only appear in high numbers from summer to early autumn (Haarløv, 1964; Borowiec, 1984).

Deer keds have an economic impact on the hunting economy. Their infestation can induce scratching and itching in free-ranging cervids, and this may impair the condition of the host following secondary bacterial infection (Dehio *et al.*, 2004). Besides, although deer keds do not reproduce on humans and accidental hosts, people who work in forestry, or visitors, are still particularly vulnerable to deer ked infestation. As a matter of fact, deer ked bites can result in the occurrence of severe dermatitis in humans (Härkönen *et al.*, 2009).

According to the life cycle of insect vectors, *Trypanosoma* species are divided into two sections: Stercoraria (subgenera: *Megatrypanum* Hoare, 1964; *Herpetosoma* Doflein, 1901; *Schizotrypanum* Hoare, 1972) and Salivaria (subgenera: *Duttonella* Chalmers, 1918; *Nannomonas* Hoare, 1964; *Trypanozoon* Luhe, 1906; *Pycnomonas* Hoare, 1964) (Hoare, 1972). Stercorarian trypanosomes develop only in the hindgut (rectum) of the host thus allowing the metacyclic forms to leave the vector organism with the feces. The infection takes place through damaged skin or mucous membrane of the host (Hoare, 1972). During their developmental cycle, Salivarian trypanosomes enter the salivary glands of the insect vector so that it can transmit the pathogen injecting saliva into vertebrate hosts. The subgenus *Megatrypanum* comprises a group of large trypanosomes that infect almost all mammalian orders (Hoare, 1972). Previous studies of these parasites have typically been restricted to morphological and morphometric examinations of samples from hosts' blood and cultured forms. More recently, complex phylogenetic analyses concerning trypanosomes of the subgenus *Megatrypanum* revealed two main lineages of *Trypanosoma theileri* (TthI and TthII) and 10 genotypes associated with the host species: four genotypes from cattle, one from water buffalo, one from deer, two from duikers and one from sitatunga (Rodrigues *et al.*, 2010*a*, 2010*b*; Garcia *et al.*, 2011*a*, 2011*b*). The species of *Megatrypanum* trypanosomes described in Poland are presented in Table 1. Although *Megatrypanum* infections in animals are typically subclinical, some clinical cases are reported; such disease or deaths tend to be associated with stressed cattle or with the presence of concomitant infections such as bovine leucaemia virus (Matsumoto *et al.*, 2011). *Trypanosoma theileri* can result in leucocytosis, neonatal death, anaemia, weight loss and a considerable drop in milking capacity (Matsumoto *et al.*, 2011). Several neurological manifestations associated with *T. wrublewskii* infection, including depressive neurological signs, apathy and oedema have been observed in European bison from the Białowieża Primeval Forest (Wrublewski, 1912; Kingston *et al.*, 1992). However, further investigation on infection with parasites of European bison did not confirm these clinical signs (Kingston *et al.*, 1992; Karbowiak *et al.*, 2014).

Megatrypanum trypanosomes are typically transferred to the vertebrate hosts by contamination of the oral mucosa with feces or the gut contents of the infected insects such as tabanid flies (Böse *et al.*, 1987). Hoare (1972) described that the entire life cycle of *Megatrypanum* trypanosomes takes place in the alimentary tract of the invertebrate host. Only a few reports have described the occurrence of *Megatrypanum* trypanosomes in deer keds; for example, Böse and Petersen (1991) documented the presence of trypanosomatids in the midgut and hindgut of *L. cervi*.

It is much more challenging to assess the effect of pathogenicity on arthropods as they tend to display higher tolerance to parasites, and thus less pronounced signs and symptoms (Lipa, 1968). Nonetheless, some studies highlighted the effect of *Trypanosoma* infections on the condition and survivability of the invertebrate host. Nelson (1956) reports high mortality of sheep keds, caused by obstruction of the intestine with a massive number of trypanosomes. However, Hoare (1972) did not notice any changes in the appearance and behaviour of sheep ticks in his research.

The Hippoboscidae, including deer keds, are potential vectors of a number of pathogens, including bacteria such as *Bartonella* spp., *Anaplasma* spp., *Coxiella* spp. and *Ehrlichia* spp., protozoa such as *Trypanosoma* (*Megatrypanum*) spp. and apicomplexan parasites such as *Theileria* spp. (Halos *et al.*, 2004; Lee *et al.*, 2016; Szewczyk *et al.*, 2017).

The occurrence of *Trypanosoma melophagium* in sheep ked was confirmed by Martinković *et al.* (2012). Billeter *et al.* (2008) identified *Bartonella melophagi* in sheep ked (*M. ovinus*) and *B. chomelii* in forest flies (*H. equina*) in Algeria. *Bartonella* spp. have also been identified in deer keds collected from deer in Poland (Szewczyk *et al.*, 2017), while the presence of *Anaplasma phagocytophilum* was confirmed in deer keds collected from deer in Slovakia (Víchová *et al.*, 2011). These findings underline the potential role of these blood-sucking hippobocids in the mechanical transmission of pathogenic bacteria within the population of wild animals. They also highlight the risk of transmission of pathogens to humans and animals *via* the bite of infected haematophagous ectoparasites.

Our previous study reported the presence of *Megatrypanum* trypanosomes in some species of blood-sucking flies belonging to the Tabanidae family (Werszko *et al.*, 2020). We hypothesize that the same trypanosomes could also be present in two species of *Lipoptena*: *L. cervi* and *L. fortisetosa*.

Materials and methods

Fly collection and taxonomical study

Hippoboscid specimens were collected manually from the fur of red deer during the autumn hunting seasons in the years 2018/2019, and from vegetation in autumn, using an entomological net. The flies were collected from the Strzałowo Forest Inspectorate (Piska Forest) (53°46′N, 21°27′E) and three localities in the vicinity of the Białowieża Primeval Forest (Białowieża 52°42′N, 23°52′E, Hajnówka 52°44′N, 23°35′E and Smolany Sadek 52°48′N, 23°36′E) (Fig. 1). All the locations where deer keds were collected before 2019 as well as those identified during the current study are marked on the map in Fig. 1, according to the UTM (the Universal Transverse Mercator) geographical grid. This method is commonly used for plotting the ranges of animals on a regional scale in faunistic research. Before the taxonomical identification, hippoboscid specimens were rinsed in ultrapure water (Direct-Pure® adept Ultrapure Lab Water Systems, RephiLe Bioscience, Ltd., China) and then air-dried and prepared for optical observations. Sex determination and species identification were carried out using taxonomic keys, according to Borowiec (1984) and Salvetti *et al.* (2020) under an OPTA-TECH microscope (Warsaw, Poland). In the current study, two dimensions were measured: the total length of the body and the largest width of the abdomen.

PCR and sequence analyses

DNA from each fly was extracted using a Genomic Mini AX Tissue kit (A&A Biotechnology, Gdynia, Poland), according to

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Table 1. *Trypanosoma* (*Megatrypanum*) species and their respective hosts, described in Poland, on basis of morphological and morphometric data

Trypanosoma species	Host	References
Trypanosoma wrublewskii	European bison (*Bison bonasus*)	Wrublewski (1912)
Trypanosoma theileri	Cattle (*Bos taurus*)	Demiaszkiewicz and Lachowicz (1991)
Trypanosoma stefanskii	Roe deer (*Capreolus capreolus*)	Kingston *et al.* (1992)
Trypanosoma cervi	Red deer (*Cervus elaphus*)	Wita and Kingston (1999)
Trypanosoma ornata	Water shrew (*Neomys fodiens*)	Karbowiak *et al.* (2005)

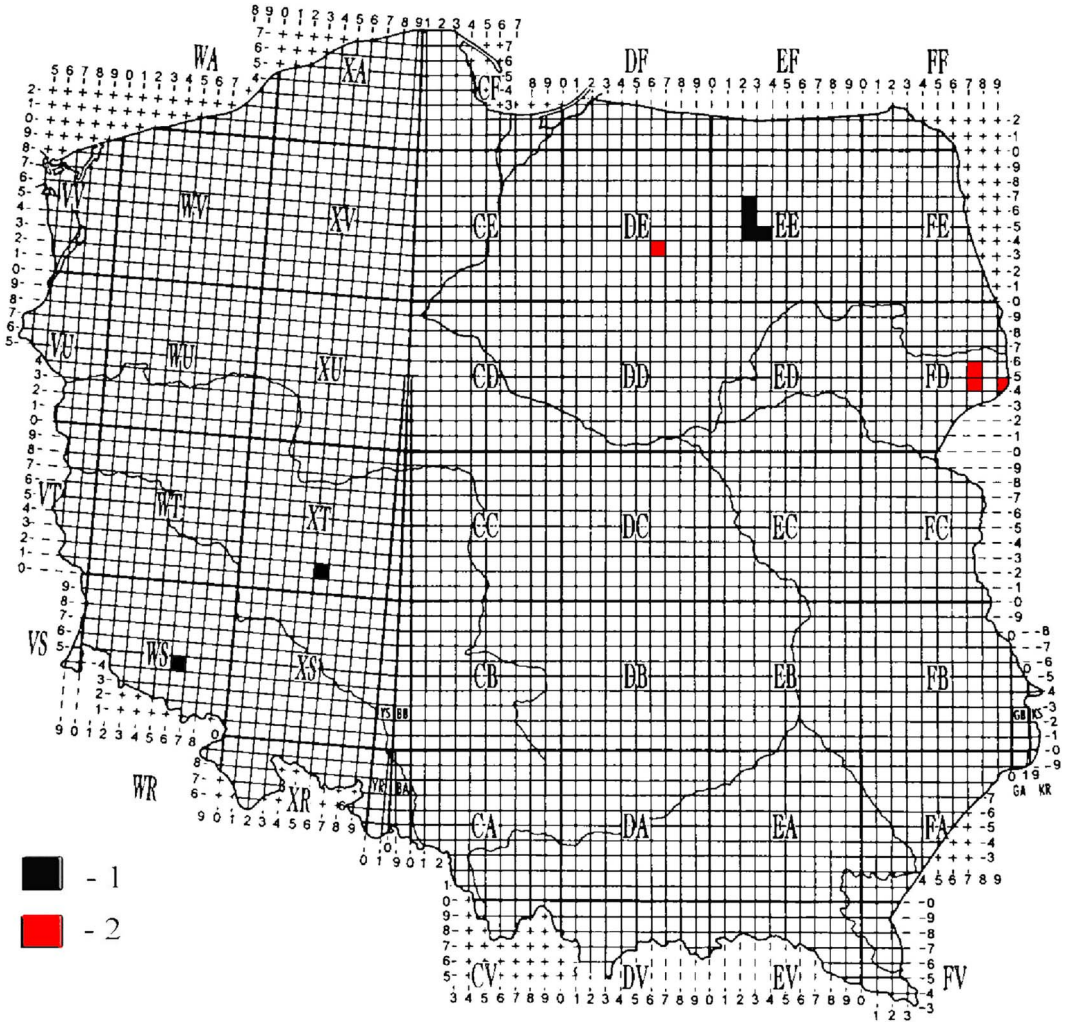

Fig. 1. The documented occurrence of *Lipoptena fortisetosa* in Poland. (1) Localities where *L. fortisetosa* was collected before 2019; (2) New record-sites described during the study.

the manufacturer's instructions and stored at − i °C until molecular analysis. Flies were individually screened for the presence of trypanosomes using polymerase chain reaction (PCR). The following 18S rDNA oligonucleotides TrypF 150 (5′-GAA ACA CGG GAG CGG TTC CTT-3′) and TrypR 800 (5′-ACC TCA AAG CTT TCG CGT GAA G-3′) were used as previously described (Werszko *et al.*, 2020). These primers amplified a 650 bp fragment of the 18S rRNA gene of *Trypanosoma* spp.

PCR reactions were conducted in a 50 μL reaction mixture containing 36 μL of deionized water, 3 μL of a 25 μM solution of $MgCl_2$, 0.5 μL of Allegro *Taq* DNA polymerase (5 U μL^{-1}) (Novazym, Poznań, Poland), 0.5 μL of dNTP-mix (10 mM), 5 μL of 10 × Taq DNA polymerase buffer (with 25 mM $MgCl_2$), 0.5 μL of each primer (20 pmol μL^{-1}) and 4 μL of template DNA. DNA from a *Trypanosoma* sp. (GenBank acc. no.: MK088728) isolated from *Haematopota pluvialis* (Tabanidae), was used as a positive control. As a negative control, nuclease-free water was added to the PCR mix instead of the DNA sample.

The amplification conditions include initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, primer extension at 72°C for 30 s and final extension at 72°C for 3 min. The final phase of PCR reaction included cooling the samples to 10°C.

PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Visualization was performed using ChemiDoc, MP Lab software (Imagine, BioRad, Hercules, USA). The resulting product was compared using the Nova 100 bp DNA Ladder Novazym (Poznań, Poland). PCR products were purified using

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Table 2. Specimens of *Lipoptena cervi* and *Lipoptena fortisetosa* collected from different localities in Poland, analysed for *Trypanosoma* spp. infection (number and percentage of infection)

Processed deer ked (species and sex)			Location				
			Nadleśnictwo Strzałowo (Puszcza Piska Forest)	Białowieża	Hajnówka	Smolany Sadek (winged deer ked)	Overall
L. cervi	♀	Analysed	27	6	9	10	52
		Infected	5	0	2	3	10
	♂	Analysed	38	3	8	17	66
		Infected	7	2	3	2	14
	Total	Analysed	65	9	17	27	118
		Infected	12	2	5	5	24 (20.33%)
L. fortisetosa	♀	Analysed	16	8	–	–	24
		Infected	10	3	–	–	13
	♂	Analysed	11	2	–	–	13
		Infected	4	1	–	–	5
	Total	Analysed	27	10	–	–	37
		Infected	14	4	–	–	18 (48.64%)

the QIAEX II Gel extraction kit (Qiagen, Hilden, Germany) and sequenced by Genomed (Warsaw, Poland). The purified PCR products were assembled into contigs using ContigExpress, Vector NTI Advance 11.0 (Invitrogen Life Technologies, New York, USA). The obtained sequences were compared by using BLAST (BasicLocal Alignment Search Tool) with sequences available in GenBank.

Results

In total, 155 flies (118 *L. cervi* and 37 *L. fortisetosa*) were collected, including 27 adult winged deer keds (*L. cervi*) from vegetation caught in one single location (Smolany Sadek) through sweeping net (Table 2). In the current study *L. fortisetosa* was recorded in two localities in Poland: one in the Białowieża Primeval Forest, and another in the Strzałowo Forest Inspectorate (Puszcza Piska Forest), both in north-eastern Poland (Fig. 1).

Morphological identification

The most significant difference between the two species is the body size: *L. fortisetosa* is smaller than *L. cervi*. In both species, females are larger than males. The mean length of *L. fortisetosa* is 4.43 ± 0.0963 mm for females and 3.75 ± 0.0172 mm for males. In *L. cervi*, the mean body length is 5.90 ± 0.070 mm for females and 5.55 ± 0.050 mm for males. The mean abdomen width of *L. fortisetosa* is 2.31 ± 0.099 mm for females and 2.079 ± 0.022 mm for males while in *L. cervi*, the abdomen width is 2.86 ± 0.052 mm for females and 2.96 ± 0.050 mm for males.

The sutural pattern and the distribution of bristles in the thoracic region reveal significant morphological features to distinguish the two species. *Lipoptena cervi* is hairier than *L. fortisetosa* and the dimensions of its bristles vary, whereas all bristles in *L. fortisetosa* are of equal dimensions (Fig. 2).

Female terminalia show other important taxonomical differences: the number of bristles on the genital opening, and the features of the pregenital sclerites and plates. *Lipoptena cervi* shows three pregenital aligned sclerites, with the central triangular sclerite bearing four or six bristles, and each oval external sclerites with three or four bristles. In contrast, *L. fortisetosa* has only one central pregenital sclerite with two long, strong bristles in the middle and one on both sides (Fig. 2).

PCR and sequence analyses

The overall positivity to *Trypanosoma* spp. DNA in deer keds was 27.09% (42/155). The presence of *Trypanosoma* spp. was detected in 24 out of 118 (20%) *L. cervi*. In the tested group of winged *L. cervi* 5 out of 27 (18.51%) were infected. The presence of trypanosomes was detected in 18 out of 37 (48.64%) *L. fortisetosa*. The males and females of both species of *Lipoptena* demonstrated similar prevalence of trypanosome infection. The positivity to trypanosome DNA among females and males *L. cervi* and *L. fortisetosa* from different locations is given in Table 2.

Four partial 18S rDNA nucleotide sequences were obtained from *L. fortisetosa* and three from *L. cervi*, including one sequence obtained from winged deer ked. Two isolates of *Trypanosoma* spp. obtained from *L. cervi* (isolates Lc99KG and Lc106KG) and one obtained from winged *L. cervi* (isolates Lc6SM) were identical each other, and all shared 100% similarity to *T. theileri* from a tse-tse fly *Glossina fuscipes fuscipes* Newstead, 1910 from Central Africa (KR024688) and *Trypanosoma* cf. *cervi* from white-tailed deer (*Odocoileus virginianus* Zimmermann, 1780) from the USA (JX178193).

Sequences of *Trypanosoma* spp. obtained from two *L. fortisetosa* (isolates Lf4KG and Lf15KG) were identical to each other and showed 100% similarity to *T. theileri* from European bison *Bison bonasus* from Poland (KF765799). These isolates also showed 100% identity with *T. theileri* from cattle from Poland (KF924257). In addition, they also shared a high identity (100%) with trypanosomes isolated in the USA and Brazil (JX853185, JX178162, JX178185, JX178188 and AY773679) and water buffalo *Bubalus bubalis* (L., 1758) from Brazil (AY773674). One nucleotide sequence from *L. fortisetosa* (isolate Lf10KG) infected with *Trypanosoma* spp. was 100% identical to *T. melophagium* from sheep ked (*M. ovinus*) from Croatia and the UK (HQ664912 and FN666409). One sequence derived from *L. fortisetosa* (isolate 1LfHka) infected with *Trypanosoma* spp. demonstrated high similarity (99.6%) to *T. theileri* from sitatunga *Tragelaphus spekii* (Sclater, 1863) from Cameroon (FM202489) and *Trypanosoma* sp. from horse fly *Hybomitra tarandina* (L., 1758) from Russia (MK156791). The derived sequences of *Trypanosoma* spp. were submitted to the GenBank database under the accession numbers: MT394044, MT393974, MT393977, MT393982, MT393983, MT393984 and MT393991.

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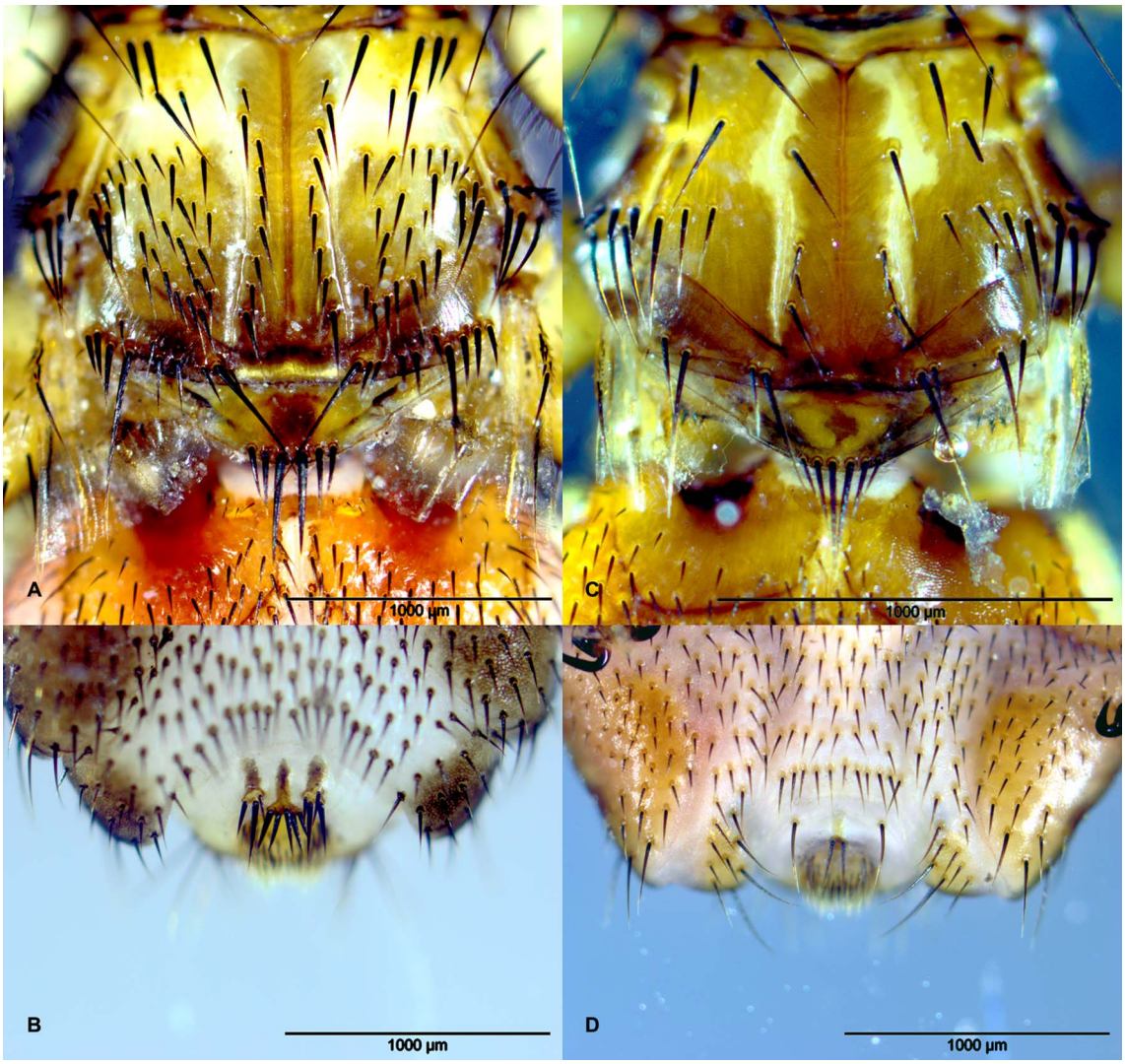

Fig. 2. Features on the thorax and female terminalia of *Lipoptena cervi* (A, B) and *L. fortisetosa* (C, D).

Discussion

First records of *L. fortisetosa* in Poland date back to the 1980s (Borowiec and Zatwarnicki, 1989). However, due to its dynamic spread observed in recent years, it can be considered an invasive species in the country; furthermore, owing to the possibility that it could be introduced to other European countries with its host, it could also be considered as an alien invasive species (Kowal *et al.*, 2016). In addition, the presence of these blood-sucking flies may play a significant role in the circulation and maintenance of vector-borne pathogens; they may also demonstrate significant vector competence for infectious agents, despite not being well recognized. Indeed, as the deer keds shed their wings when they find a suitable host and settle for the rest of their lives, the possibility of transmitting a pathogen from one host to another is believed relatively low. Nevertheless, deer keds are recognized as an important group of haematophagous insects for both veterinary parasitology and medical reasons since they have been proved able to transmit *Bartonella* spp., *Borrelia* spp. and *Trypanosoma* (*Megatrypanum*) spp. (Böse and Petersen, 1991; Dehio *et al.*, 2001; Vichová *et al.*, 2011; Szewczyk *et al.*, 2017), they are recognized as an important group of haematophagous insects for both veterinary parasitology and medical reasons. It is important to note that most part of the studies performed so far have concerned *L. cervi* (Halos *et al.*, 2004), while much less is known about the competence of *L. fortisetosa* as a pathogen vector, and less data have been acquired on trypanosomes in other *Lipoptena* species.

In the current study, the overall prevalence of infection with *Trypanosoma* spp. was 27.09% (42/155) among deer keds. The highest positivity was reported in the case of *L. fortisetosa* (48.64%, 18/37). The presence of *Trypanosoma* spp. was detected in 24 of the 118 (20%) *L. cervi*. Including the tested group of winged *L. cervi*, five out of 27 (18.51%) were infected.

Böse and Petersen (1991) report the identification of *Megatrypanum* trypanosomes in the midgut and hindgut of 9/37 *L. cervi* collected from deer. High levels of infection with other pathogens have also been observed in *L. cervi*; the presence of *Bartonella* spp. was observed in 75.12% of *L. cervi* collected from deer in Poland (Szewczyk *et al.*, 2017). Víchová *et al.* (2011) detected *A. phagocytophilum* infection in only two out of 19 tested deer keds collected from deer in Slovakia. Pathogens present in *L. fortisetosa* have not been well studied. Lee *et al.* (2016) report the presence of *Coxiella* spp., *Theileria luwenshuni* and *Theileria ovis* in *L. fortisetosa* in inland regions of South Korea.

The studies mentioned above were based on wingless flies collected from hosts. In this case it was not known whether the fly was infected or the pathogen was present only in the host blood withdrawn from the vertebrate. We provide the first molecular evidence for *Trypanosoma* spp. DNA in five out of 27 investigated winged deer keds (*L. cervi*) collected from vegetation. The winged

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flies had not fed since they left the pupae; therefore, we suppose the only explanation for the presence of trypanosome DNA is transovarial transmission. The present findings support the hypothesis of a transstadial transmission of *Trypanosoma* spp. in these species of *Lipoptena*, but the topic remains open at this stage of research. Korhonen *et al.* (2015) report the presence of *Bartonella* DNA in *L. cervi* pupae and winged adults, thus supporting the potential of deer ked for vector competence of *Bartonella* spp. and indicating their transstadial transmission; their findings also demonstrate that adult winged deer keds do harbour bartonellae. Víchová *et al.* (2011) found winged deer keds to be negative for the presence of *A. phagocytophilum*, indicating that they do not serve as competent vectors of this pathogen. Similarly, all *L. cervi* collected in a field study by de la Fuente *et al.* (2005) were found to be negative for the presence of *A. phagocytophilum* and *A. marginale*. In contrast, *Bartonella* spp. was found to be present in pools of winged unfed deer keds (Dehio *et al.*, 2004; Duodu *et al.*, 2013) in midgut bacterial aggregates of *Bartonella schoenbuchensis*. It is therefore possible that deer keds support the replication of the pathogen and serve as potential biological vectors (Dehio *et al.*, 2004).

Trypanosomes of the subgenus *Megatrypanum* do not show high specificity for the insect as a host, and the same species of trypanosomes may infect different flies (Böse *et al.*, 1987). The absence of *A. phagocytophilum* in winged deer keds collected from vegetation and the presence of *Bartonella* pathogens may indicate that these pathogens demonstrate host specificity for the vector. Until now, deer keds have not been shown to transmit any infectious agents to humans. The causes underlying the variation of prevalence and intensity of blood parasites are poorly known (Sol *et al.*, 2000). The prevalence of microparasite infection in an insect can arise both from factors intrinsic to the host, such as genotype resistance, biochemical immunity processes, behaviour or state of health and from extrinsic factors, such as differences in exposure to vectors (Sol *et al.*, 2000).

All isolates (except to isolate 1LfHka) obtained in this study were identical to the *Trypanosoma* spp. sequences in the GenBank database. Moreover, isolates Lf4KG and Lf15KG were identical to those of *T. theileri* isolates obtained previously from cattle and European bison inhabiting the same area.

It seems there is a growing range of arthropods that might serve as potential vectors for transmissible pathogens. The blood-sucking flies, including insects from the genus *Lipoptena*, are important potential vectors that can disseminate a variety of pathogens in natural foci of transmission disease.

In conclusion, the current study examines the presence of *Megatrypanum* trypanosomes using molecular methods in *L. cervi* and *L. fortisetosa* (Hippoboscidae). It describes the first detection of *Trypanosoma* spp. in *L. fortisetosa*, and first recorded presence of trypanosomes in an unfed winged *L. cervi* from vegetation. The study also identifies two new localities where *L. fortisetosa* was recorded in Poland: one in the Białowieża Primeval Forest, and another in the Strzałowo Forest Inspectorate (Puszcza Piska Forest), both in north-eastern Poland.

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Conflict of interest. The authors declare no conflicts of interest.

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