

Blood-meal analysis and avian malaria screening of mosquitoes collected from human-inhabited areas in Hungary and Serbia

Kornélia Kurucz¹, Anett Kepner^{1,2}, Bosiljka Krtinic³, Dávid Hederics^{1,2}, Fanni Földes^{1,2}, Brigitta Zana^{1,2}, Ferenc Jakab^{1,2}, Gábor Kemenesi^{1,2}

¹Virological Research Group, Szentágothai Research Centre, University of Pécs, Pécs, Hungary

²Institute of Biology, Faculty of Sciences, University of Pécs, Pécs, Hungary

³Ciklonizacija Ltd, Novi Sad, Serbia

Corresponding author: kornelia.kurucz@gmail.com

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Abstract: Mosquito-borne pathogens have a growing medical importance in several European countries. The emergence and invasion of exotic vector species increases the likelihood that additional vector-borne diseases may appear in regions that have not encountered them previously. In the last few years, different emerging pathogens affecting humans, livestock and wildlife were detected in mosquitoes in the Eastern European region; moreover, several invasive mosquito species were recorded in the area. The importance of particular mosquito species for the transmission of vector-borne pathogens is strongly determined by several factors such as their feeding behaviour. However, there is a huge knowledge gap regarding the ecological background of host use by mosquitoes and their capacity to act as vectors. Derived from a transboundary mosquito surveillance programme, a total of 84 engorged mosquitoes were subjected to PCR-based blood meal identification analysis and screened for *Plasmodium* species. The majority of tested mosquitoes were *Aedes vexans* and *Culex pipiens*, in addition to an exotic species *Aedes koreicus*, which was detected last summer for the first time in the vicinity of Balkans region. Hereby, we provide the first explicit data regarding the feeding behaviour of this exotic species in Europe. The human origin of blood-meal may highlight the possible medical importance of the species and its important role in mosquito nuisance. *Aedes vexans* mosquitoes exhibited mostly zoophilic behaviour compared to *Cx. pipiens*, which was found to be primarily anthropophilic and secondarily ornithophilic, along with amphibian host preference. We detected *Plasmodium relictum* in five *Cx. pipiens*, which had fed on humans. This study provides additional insight into the ectothermic host preference of *Cx. pipiens* in nature and highlights the human and veterinary health importance of these species. In addition, these results provide novel insight for the feeding patterns of the investigated mosquitoes. *Journal of the European Mosquito Control Association* 36: 3-13, 2018

Keywords: host-feeding, invasive species, digestion status, vector competence, *Plasmodium*, amphibian host

Introduction

Mosquito-borne pathogens have a growing medical importance in several European countries (Zeller *et al.*, 2013). The invasion of exotic vector species, probably introduced passively by global trading activities, was already published for several countries in Europe (ECDC 2012a, b). The emergence of further exotic vector species increases the likelihood that additional vector-borne diseases may appear in regions that have not encountered them previously (Fischer *et al.*, 2010). Through intensified surveillance over the last few years, different pathogens were detected in mosquito specimens from the eastern part of the Central European region. Multiple lineages of West Nile virus (WNV) (Kemenesi *et al.*, 2014) and Usutu virus (USUTV) (Kemenesi *et al.* unpublished data), filarial parasites like *Dirofilaria* spp. and *Setaria tundra* (Kemenesi *et al.*, 2015; Zittra *et al.*, 2015; Kurucz *et al.*, 2016a), and protozoan parasites like *Plasmodium* spp. (Zittra *et al.*, 2015; Ionică *et al.*, 2017) were identified in wild-caught mosquitoes. Besides *Aedes albopictus* (Skuse, 1894) and *Aedes japonicus* (Theobald, 1901), which were already detected in South Hungary and the Austrian-Hungarian border retrospectively (Scheidel *et al.*, 2015; ECDC, 2016), another invasive mosquito species *Aedes koreicus* (Edwards, 1917) was detected for the first time in Hungary and in the vicinity of the Balkans region (Kurucz *et al.*, 2016b).

Several aspects of mosquito-borne diseases are less well known. For instance, there is a huge knowledge gap regarding the vector competence of many mosquito species along with several behavioural factors, such as host preference, which together contribute to the epidemiology and ecology of vector-borne diseases (Chaves *et al.*, 2010; Roiz *et al.*, 2010). In the past decade, several studies identified the host species of mosquitoes (e.g. Muñoz *et al.*, 2012; Osório *et al.*, 2012; Radrova *et al.*, 2013; Martínez-de la Puente *et al.*, 2015; Brugman *et al.*, 2017). Most of them focused on already known vector species or invasive mosquitoes in Europe, but only a few studies collected data on a wide range of European mosquito species (Börstler *et al.*, 2016; Schönenberger *et al.*, 2016). Furthermore, there is a lack of knowledge regarding the blood-feeding habits of common mosquito species in the eastern part of Europe.

Therefore, a molecular approach was utilised to characterise the general host-feeding patterns of mosquitoes that occur in Hungary and Serbia. To better understand the human and veterinary importance of these mosquitoes, it is important to classify host-feeding groups, particularly in human-inhabited areas. Regarding those mosquitoes as possible vectors of avian *Plasmodium* species, the most prevalent and widespread vertebrate malarial agents, with an almost worldwide distribution (Pigeault *et al.*, 2015), we aimed to identify avian *Plasmodium* lineages in blood-fed mosquitoes as

well. Avian *Plasmodium* screening was included in our study in order to investigate the connection between ornithophilic *Culex pipiens* (Linnaeus, 1758) mosquitoes and *Plasmodium* positivity to reveal local enzootic cycles of the pathogen. The current study contributes to the general knowledge of the ecological background of endemic or emerging mosquito-borne diseases in the region.

Materials and Methods

Study area, mosquito collection, identification, and storage

As part of the seasonal mosquito control activities in Baranya county (Hungary) and Vojvodina province (Serbia), a total of 34,295 adult female mosquitoes were collected from urbanized, human-inhabited areas and typical mosquito breeding sites within cities and small villages. Center for Disease Control and Prevention (CDC) light traps baited with dry ice were placed at 71 sampling sites (9 from Hungary and 62 from Serbia) from May to October 2014-2016, and operated overnight from 19:00 to 07:00. Altogether, 54 overnight sampling events were conducted during the study period. All collected mosquitoes were transported to the laboratory on dry ice and kept frozen at -80 °C until further processing. Each specimen was determined by species according to their taxonomic keys (Becker *et al.*, 2010; ECDC, 2012a) using a stereomicroscope. As it is difficult to morphologically distinguish between *Cx. pipiens* and the closely related species *Culex torrentium* (Martini, 1925), or identify rarely observable species, all samples belonging to *Culex* genus along with specimens of *Aedes koreicus* were further confirmed at molecular level also. We performed DNA barcoding targeting mitochondrial *Cytochrome oxidase I gene* (COI) segment, using the Culicidae specific primer sets (F: 5'-GGATTGGAATTGATTAGTTCCTT-3' and R: 5'-AAAAATTTTAATTCCAGTTGGAACAGC-3') (-700 bp) and following the methods described previously (Kumar *et al.*, 2007; Kurucz *et al.*, 2016b). Briefly, PCR reactions were performed with the GoTaq® G2 Flexi DNA Polymerase Kit (Promega Ltd., USA) with the following cycling conditions: incubation at 95 °C for 5 min, 40 cycles at 94 °C for 40 s, 51 °C for 1 min, and 72 °C for 1 min, final elongation at 72 °C for 10 min. PCR product was purified with Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan) and was bi-directionally sequenced (BigDye Terminator v1.1 Cycle Sequencing Kit, ABI Prism 310 DNA Sequencer-Applied Biosystems). For this study, we selected blood-fed females only, identified visually by abdominal blood contents. The digestion status of mosquito blood meals was scored according to the Sella score from zero (unfed mosquito) to seven (female without visible blood and eggs fully developed in their abdomen), following Detinova (1962) and Martínez-de la Puente *et al.* (2013).

Molecular identification of blood meal origin

The abdomens of engorged mosquitoes were separated from the head-thorax using sterile tips and were homogenized

manually in 200 µL sterile PBS. Genomic DNA of the abdomen of each mosquito was extracted from 200 µL of supernatants with QIAamp DNA Micro Kit (Qiagen Ltd., Germany) according to the manufacturer's protocol. Polymerase chain reaction (PCR) amplification of mitochondrial cytochrome b gene was conducted with the vertebrate-specific primers L14841: 5'-CCATCCAACATCTCAGCATGATGAAA-3' and H15149: 5'-CCCTCAGAAATGATATTTGTCCTCA-3' (-358 bp) published by Kocher *et al.* (1989). PCR reactions were performed with the GoTaq® G2 Flexi DNA Polymerase Kit with the following cycling conditions: incubation at 95 °C for 5 min, 40 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, final elongation at 72 °C for 5 min (Börstler *et al.*, 2016). Amplicons were visualized by agarose-gel electrophoresis in a 2 % agarose gel stained with ethidium bromide (EtBr). Blood samples from humans (*Homo sapiens*), domestic dog (*Canis lupus familiaris*), red deer (*Cervus elaphus*) and striped field mouse (*Apodemus agrarius*) were used as positive controls and nuclease-free water (Promega Ltd.) as a negative control. PCR products were purified with Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan) and were bi-directionally sequenced with BigDye Terminator v1.1 Cycle Sequencing Kit according to the manufacturers' protocol on the ABI Prism 310 DNA Sequencer platform (Applied Biosystems, USA). Blood-meal sources were finally determined via nucleotide BLAST database search of the sequences (Madden, 2002).

Molecular identification of avian malaria

For the screening of all blood-fed mosquito samples for *Plasmodium* DNA, a nested-PCR targeting a portion (-480 bp) of the mitochondrial cytochrome b gene of the parasite was performed using the primer sets: HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3'), HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') as initial primers, and HaemF (5'-ATGGTGCTTTCGATATATGCATG-3'), HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') for the second PCR, as previously described by Bensch *et al.* (2000) and Hellgren *et al.* (2004). The first and nested PCRs were also performed with the GoTaq® G2 Flexi DNA Polymerase Kit (Promega Ltd.) using the following conditions: incubation at 94 °C for 3 min, 20/35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s, final elongation on 72 °C for 10 min. All PCR-positive products were purified and sequenced as described above. Mitochondrial DNA lineages were identified by comparison with published sequences available on the GenBank database using BLAST analysis, and sequences were submitted to GenBank (Accession Nos. MF988744 - MF988748).

Data analysis

Analysis of frequencies for the blood meal source of engorged females was conducted using Pearson's Chi-squared Test performed with R 3.3.3 software (R Development Core Team, 2017).

Table 1: List of analysed blood-fed mosquito specimens and data related to the localities and date of collection, Sella stages and identified host species.

ID	Mosquito species	Sella score	Country	Settlement (Municipality)	Sampling site	Location	Year	Month	Host species
1	<i>Aedes vexans</i>	2	Hungary	Pécs	2	city	2016	August	<i>Canis lupus familiaris</i>
2	<i>Aedes vexans</i>	3	Hungary	Pécs	6	city	2016	June	<i>Homo sapiens</i>
3	<i>Aedes vexans</i>	2	Hungary	Pécs	6	city	2016	June	<i>Sus scrofa</i>
4	<i>Aedes vexans</i>	2	Hungary	Pécs	6	city	2016	June	<i>Homo sapiens</i>
5	<i>Aedes vexans</i>	2	Hungary	Pécs	6	city	2016	June	<i>Homo sapiens</i>
6	<i>Aedes vexans</i>	3	Hungary	Pécs	6	city	2016	June	<i>Sus scrofa</i>
7	<i>Aedes vexans</i>	2	Hungary	Pécs	6	city	2016	June	<i>Sus scrofa</i>
8	<i>Aedes vexans</i>	2	Hungary	Pécs	6	city	2016	June	<i>Sus scrofa</i>
9	<i>Aedes vexans</i>	4	Hungary	Pécs	6	city	2016	June	<i>Lepus europaeus</i>
10	<i>Aedes vexans</i>	4	Hungary	Pécs	6	city	2016	June	<i>Homo sapiens</i>
11	<i>Aedes vexans</i>	4	Hungary	Pécs	6	city	2016	June	<i>Lepus europaeus</i>
12	<i>Aedes vexans</i>	4	Hungary	Pécs	6	city	2016	July	<i>Homo sapiens</i>
13	<i>Aedes vexans</i>	2	Hungary	Pécs	6	city	2016	July	<i>Equus caballus</i>
14	<i>Aedes vexans</i>	4	Hungary	Pécs	6	city	2016	July	<i>Capreolus capreolus</i>
15	<i>Aedes vexans</i>	2	Hungary	Pécs	6	city	2016	July	<i>Bos taurus</i>
16	<i>Aedes vexans</i>	3	Hungary	Pécs	6	city	2016	July	<i>Homo sapiens</i>
17	<i>Aedes vexans</i>	5	Hungary	Pécs	6	city	2016	August	other <i>Cervidae</i>
18	<i>Aedes vexans</i>	2	Hungary	Pécs	6	city	2016	August	<i>Homo sapiens</i>
19	<i>Aedes vexans</i>	3	Hungary	Pécs	6	city	2016	August	<i>Capreolus capreolus</i>
20	<i>Aedes vexans</i>	3	Hungary	Pécs	6	city	2016	August	<i>Lepus europaeus</i>
21	<i>Aedes vexans</i>	3	Hungary	Pécs	6	city	2016	August	<i>Lepus europaeus</i>
22	<i>Aedes vexans</i>	6	Serbia	Begeč (Novi Sad)	7	village	2014	October	<i>Sus scrofa</i>
23	<i>Aedes vexans</i>	2	Serbia	Čurug (Žabalj)	8	village	2014	July	<i>Bos taurus</i>
24	<i>Aedes vexans</i>	2	Serbia	Farkaždin (Zrenjanin)	9	village	2014	August	<i>Bos taurus</i>
25	<i>Aedes vexans</i>	3	Serbia	Farkaždin (Zrenjanin)	9	village	2014	August	<i>Bos taurus</i>
26	<i>Aedes vexans</i>	2	Serbia	Futog (Novi Sad)	10	city	2014	August	<i>Canis lupus familiaris</i>
27	<i>Aedes vexans</i>	4	Serbia	Kisač (Novi Sad)	11	village	2014	August	<i>Homo sapiens</i>
28	<i>Aedes vexans</i>	3	Serbia	Klek (Zrenjanin)	12	village	2014	August	other <i>Cervidae</i>
29	<i>Aedes vexans</i>	2	Serbia	Knićanin (Zrenjanin)	13	village	2014	August	<i>Capra hircus</i>
30	<i>Aedes vexans</i>	6	Serbia	Novi Sad	14	city	2015	August	<i>Homo sapiens</i>
31	<i>Aedes vexans</i>	6	Serbia	Novi Sad	14	city	2015	September	<i>Canis lupus familiaris</i>
32	<i>Aedes vexans</i>	2	Serbia	Orlovat (Zrenjanin)	16	village	2014	August	<i>Bos taurus</i>
33	<i>Aedes vexans</i>	2	Serbia	Subotica	17	city	2015	September	<i>Bos taurus</i>
34	<i>Aedes vexans</i>	5	Serbia	Titel	18	city	2014	August	<i>Sus scrofa</i>
35	<i>Aedes vexans</i>	6	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Homo sapiens</i>
36	<i>Aedes vexans</i>	2	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Bos taurus</i>
37	<i>Aedes vexans</i>	4	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Capra hircus</i>
38	<i>Aedes vexans</i>	6	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Ovis aries</i>
39	<i>Aedes vexans</i>	3	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Homo sapiens</i>
40	<i>Aedes vexans</i>	2	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Bos taurus</i>
41	<i>Aedes vexans</i>	3	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Bos taurus</i>
42	<i>Aedes vexans</i>	2	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Homo sapiens</i>
43	<i>Aedes vexans</i>	3	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Anser erythropus</i>
44	<i>Aedes vexans</i>	2	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Capra hircus</i>
45	<i>Aedes vexans</i>	2	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Sus scrofa</i>

46	<i>Aedes vexans</i>	2	Serbia	Zrenjanin	21	city	2014	August	<i>Canis lupus familiaris</i>
47	<i>Culex pipiens</i>	5	Hungary	Pécs	1	city	2016	August	<i>Homo sapiens</i>
48	<i>Culex pipiens</i>	4	Hungary	Pécs	3	city	2016	August	<i>Meleagris gallopavo domesticus</i>
49	<i>Culex pipiens</i>	3	Hungary	Pécs	4	city	2016	August	<i>Homo sapiens</i>
50	<i>Culex pipiens</i>	4	Hungary	Pécs	4	city	2016	August	<i>Homo sapiens</i>
51	<i>Culex pipiens</i>	6	Hungary	Pécs	6	city	2016	June	<i>Homo sapiens</i>
52	<i>Culex pipiens</i>	6	Serbia	Begeč (Novi Sad)	7	village	2014	August	<i>Pelophylax</i> spp.
53	<i>Culex pipiens</i>	5	Serbia	Čurug (Žabalj)	8	village	2014	July	<i>Bos taurus</i>
54	<i>Culex pipiens</i>	2	Serbia	Farkaždin (Zrenjanin)	9	village	2014	August	<i>Bos taurus</i>
55	<i>Culex pipiens</i>	5	Serbia	Farkaždin (Zrenjanin)	9	village	2014	August	<i>Felis catus</i>
56	<i>Culex pipiens</i>	2	Serbia	Klek (Zrenjanin)	12	village	2014	August	<i>Sus scrofa domesticus</i>
57	<i>Culex pipiens</i>	3	Serbia	Novi Sad / shoreline	15	city	2015	June	<i>Felis catus</i>
58	<i>Culex pipiens</i>	4	Serbia	Novi Sad	14	city	2015	August	<i>Gallus gallus</i>
59	<i>Culex pipiens</i>	2	Serbia	Novi Sad	14	city	2015	August	<i>Passer domesticus</i>
60	<i>Culex pipiens</i>	6	Serbia	Novi Sad	14	city	2015	August	<i>Columba livia</i>
61	<i>Culex pipiens</i>	5	Serbia	Novi Sad	14	city	2015	September	<i>Gallus gallus</i>
62	<i>Culex pipiens</i>	5	Serbia	Novi Sad	14	city	2015	September	<i>Homo sapiens</i>
63	<i>Culex pipiens</i>	4	Serbia	Novi Sad	14	city	2015	September	<i>Homo sapiens</i>
64	<i>Culex pipiens</i>	4	Serbia	Orlovat (Zrenjanin)	16	village	2014	August	<i>Homo sapiens</i>
65	<i>Culex pipiens</i>	2	Serbia	Subotica	17	city	2015	September	<i>Homo sapiens</i>
66	<i>Culex pipiens</i>	4	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Pelophylax</i> spp.
67	<i>Culex pipiens</i>	2	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Homo sapiens</i>
68	<i>Aedes koreicus</i>	2	Hungary	Pécs	4	city	2016	July	<i>Homo sapiens</i>
69	<i>Anopheles hyrcanus</i>	5	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Gallus gallus</i>
70	<i>Anopheles hyrcanus</i>	3	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Homo sapiens</i>
71	<i>Anopheles maculipennis</i>	6	Hungary	Pécs	5	city	2016	June	<i>Canis lupus familiaris</i>
72	<i>Anopheles maculipennis</i>	2	Serbia	Novi Sad	14	city	2015	August	<i>Homo sapiens</i>
73	<i>Anopheles maculipennis</i>	6	Serbia	Subotica	17	city	2015	July	<i>Bos taurus</i>
74	<i>Coquillettidia richiardii</i>	3	Hungary	Pécs	2	city	2016	July	<i>Vulpes vulpes</i>
75	<i>Coquillettidia richiardii</i>	2	Hungary	Pécs	6	city	2016	July	<i>Homo sapiens</i>
76	<i>Coquillettidia richiardii</i>	3	Serbia	Novi Sad	14	city	2015	August	<i>Lepus europaeus</i>
77	<i>Coquillettidia richiardii</i>	3	Serbia	Novi Sad	14	city	2015	August	<i>Homo sapiens</i>
78	<i>Ochlerotatus annulipes</i>	2	Hungary	Pécs	6	city	2016	June	<i>Capra hircus</i>
79	<i>Ochlerotatus caspius</i>	4	Serbia	Tomaševac (Zrenjanin)	19	village	2014	August	<i>Homo sapiens</i>
80	<i>Ochlerotatus riparius</i>	2	Hungary	Pécs	6	city	2016	June	<i>Canis lupus familiaris</i>
81	<i>Ochlerotatus rusticus</i>	2	Hungary	Pécs	6	city	2016	May	other Cervidae
82	<i>Ochlerotatus sticticus</i>	2	Hungary	Pécs	6	city	2016	July	<i>Homo sapiens</i>
83	<i>Ochlerotatus sticticus</i>	2	Hungary	Pécs	6	city	2016	August	<i>Capreolus capreolus</i>
84	<i>Ochlerotatus sticticus</i>	2	Serbia	Žabalj	20	city	2015	July	<i>Ovis aries</i>

Results

In total, 84 blood-fed mosquitoes belonging to 11 different mosquito species, representing five genera were investigated in this study. The specimens came from 21 different collection sites (within 8 villages and 7 cities) belonging to 6 municipalities (Table 1). The most abundant species sampled was *Aedes vexans* (Meigen, 1830) (n=46, 54.8% of all mosquito specimens), followed by *Culex pipiens* (Linnaeus, 1758) (n=21, 25% of all mosquito specimens). All other species were included with a considerably lower number (1-4 specimens, <5% respectively) (Table 2). Notably, one blood-fed female of

the recently detected, invasive species *Aedes koreicus* was also included in this study (Kurucz *et al.*, 2016). To our knowledge, this represents the first analysed specimen from European *Ac. koreicus* populations, regarding its blood source. The Sella stage classified digestion status of the specimens was determined as follows: Sella stage II (n=31, 40.3%); stage III (n=13, 16.9%); stage IV (n=14, 18.2%); stage V (n=9, 11.7%) and stage VI (n=10, 13%). The blood meal source was successfully identified in 100% of the female mosquitoes involved in the study, regardless of the digestion status of mosquito blood meals.

Table 2: Frequency and percentage of each mosquito species with information on the frequency of detected host-feeding groups (amphibians, birds, non-human mammals and humans) and number of detected host species.

Mosquito species	No. mosquito specimens	Percentage of all collected specimens	Amphibians	Birds	Non-human mammals	Humans	Host species
<i>Aedes koreicus</i>	1	1.2	0	0	0	1	1
<i>Aedes vexans</i>	46	54.8	0	1	33	12	11
<i>Anopheles hyrcanus</i>	2	2.4	0	1	0	1	2
<i>Anopheles maculipennis</i>	3	3.6	0	0	2	1	3
<i>Coquillettidia richiardii</i>	4	4.8	0	0	2	2	3
<i>Culex pipiens</i>	21	25.0	2	5	5	9	9
<i>Ochlerotatus annulipes</i>	1	1.2	0	0	1	0	1
<i>Ochlerotatus caspius</i>	1	1.2	0	0	0	1	1
<i>Ochlerotatus riparius</i>	1	1.2	0	0	1	0	1
<i>Ochlerotatus rusticus</i>	1	1.2	0	0	1	0	1
<i>Ochlerotatus sticticus</i>	3	3.6	0	0	2	1	3
Total (percentage)	84 (100%)		2 (2.4%)	7 (8.3%)	47 (56%)	28 (33.3%)	

Altogether, 18 host species were determined including 12 mammalian (including humans), five bird and one unspecified amphibian species (*Pelophylax* spp.) (Table 3). Non-human mammals represented the majority of hosts with 47 specimens,

56% of all mosquito samples, followed by humans (n=28, 33.3%). Avian and amphibian hosts were detected at a lower proportion (n=7, 8.3% and n=2, 2.4% respectively) (Table 2, Table 3).

Table 3: Frequency and percentage for each host species classified by four host-feeding groups: amphibians, birds, non-human mammals and humans.

Host group	Host species	No. mosquito specimens	Percentage of all collected specimens
Amphibians	<i>Pelophylax</i> spp.	2	2.4
	Total	2	2.4
Birds	<i>Anser erythropus</i>	1	1.2
	<i>Columba livia</i>	1	1.2
	<i>Gallus gallus</i>	3	3.6
	<i>Meleagris gallopavo domesticus</i>	1	1.2
	<i>Passer domesticus</i>	1	1.2
	Total	7	8.3
	Non-human mammals	<i>Bos taurus</i>	12
<i>Capra hircus</i>		4	4.8
<i>Capreolus capreolus</i>		3	3.6
<i>Ovis aries</i>		2	2.4
<i>Sus scrofa</i>		7	8.3
<i>Sus scrofa domesticus</i>		1	1.2
other <i>Cervidae</i>		3	3.6
<i>Equus caballus</i>		1	1.2
<i>Lepus europaeus</i>		5	6.0
<i>Canis lupus familiaris</i>		6	7.1
<i>Felis catus</i>		2	2.4
<i>Vulpes vulpes</i>		1	1.2
Total		47	56.0
Humans		<i>Homo sapiens</i>	28
	Total	28	33.3

Beyond the most frequent host species (*Homo sapiens*), common hosts were domestic cattle (*Bos taurus*) (12, 14.3% of total), wild boar (*Sus scrofa*) (7, 8.3%) and dogs (*Canis lupus familiaris*) (6, 7.1%) (Table 3). Moreover, less than 6% of mosquitoes fed on eight further mammalian species individually: domestic goat (*Capra hircus*), European roe deer (*Capreolus capreolus*), sheep (*Ovis aries*), domestic pig (*Sus scrofa domesticus*), horse (*Equus caballus*), European hare (*Lepus europaeus*), domestic cat (*Felis catus*) and red fox (*Vulpes vulpes*) (Table 3). The major avian blood host was domestic chicken (*Gallus gallus domesticus*) (n=3, 3.6%). The other four bird species, the lesser white-fronted goose (*Anser erythropus*), rock dove (*Columba livia*), domestic turkey (*Meleagris gallopavo*

domesticus) and house sparrow (*Passer domesticus*) were identified in single blood meals respectively (Table 3). Mixed blood meals were not identified.

Aedes vexans samples showed a mainly zoophilic and secondary anthropophilic feeding preference ($\chi^2=9.8$, df=1, p=0.002). Avian host origin (lesser white-fronted goose) was detected only in one sample of the species (Table 2, Fig. 1). In contrast, *Cx. pipiens* mosquitoes were firstly anthropophilic, and then ornithophilic feeding preference was secondary along with zoophilic feeding behaviour ($\chi^2=1.68$, df=2, p=0.431). Interestingly, ectothermic host (*Pelophylax* spp.) for *Cx. pipiens* was detected in two samples (Table 2, Fig. 2).

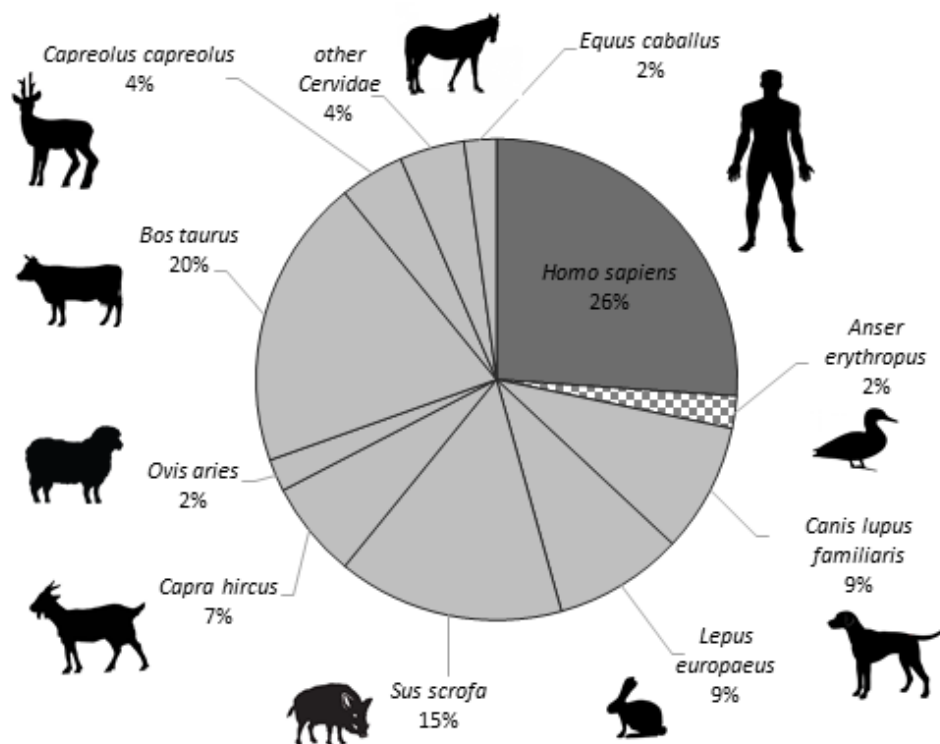


Figure 1: Blood meal sources of engorged *Aedes vexans* mosquitoes collected in Vojvodina province (Serbia) and Baranya county (Hungary). Spotted – birds; light grey – non-human mammals; dark grey – humans.

Out of the 84 blood-fed mosquitoes, five (5.9%) *Cx. pipiens* were found positive for avian *Plasmodium* infection; interestingly all of them were fed on humans. One of them was collected in Vojvodina, while the remaining four specimens originated from Hungary. Based on nucleotide BLAST homology search, our sequences most likely represent the *Plasmodium relictum* species with a nucleotide sequence homology of 100% to Turkish samples (MF101827-MF101832) and unspecified *Plasmodium* species from northwest Africa, northwestern Iberia, western Greater Caucasus and Transcaucasia with a homology of 96-100% (Drovetski *et al.*, 2014).

Discussion

The importance of mosquitoes for the transmission of vector-borne pathogens is strongly determined by, among other factors, their feeding behaviour (Simpson *et al.*, 2012). This study presents an important dataset from the Southern Central European region regarding the feeding preferences of local mosquitoes. Despite a wide range of mosquito samples

counted in different Sella stages (II-VI), the success of host identification was independent of the digestion status of the blood meal, whereas Martínez-de la Puente *et al.* (2013) found that the success of host identification decreased as the digestion status of the blood meal increased.

Although 11 species were investigated in this study, the two most represented species were *Ae. vexans* and *Cx. pipiens*, with 46 and 21 specimens tested respectively, which are the most common species composing the mosquito fauna of Vojvodina province in Serbia (Vujić *et al.*, 2010) and Hungary (Tóth & Kenyeres, 2012). Both species (*Ae. vexans* and *Cx. pipiens*) were found to be mostly generalist with a major human host preference. Similar results were reported from Germany with the same mosquito species, where both species were principally reported with anthropophilic behaviour (Börstler *et al.*, 2016). In the present study, *Ae. vexans* was mainly zoophilic, feeding on a variety of non-human mammals. These results are consistent with studies that considered the feeding behaviour of *Ae. vexans* in other geographic areas, where this species shows

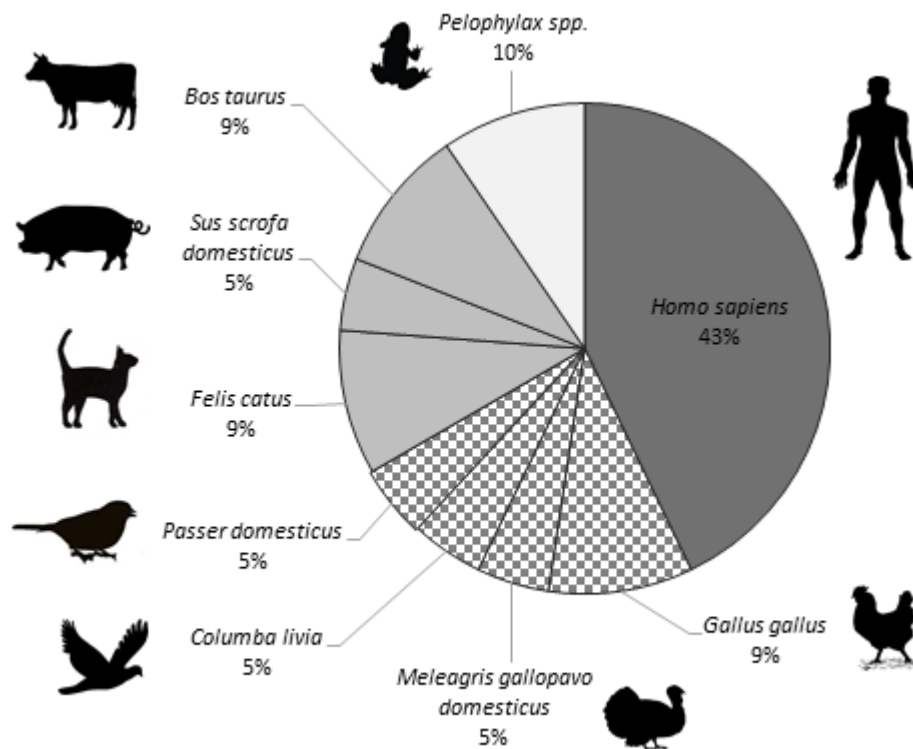


Figure 2: Blood meal sources of engorged *Culex pipiens* mosquitoes collected in Vojvodina province (Serbia) and Baranya county (Hungary). White – amphibians; spotted – birds; light grey – non-human mammals; dark grey – humans.

strong tendency to feed on mammals (Greenberg *et al.*, 2013; Lujan *et al.*, 2015; Börstler *et al.*, 2016). The feeding behaviour reflects the veterinary and human health significance of the species. Namely, mosquito species that exclusively feed on mammalian hosts including humans, making them potential vectors for pathogens transmitted between mammals, e.g. Batai virus (BATV) or filarial parasites such as *Dirofilaria* spp. (Börstler *et al.*, 2016). Moreover, vector competence studies highlighted the transmission capability of further significant viruses, such as WNV (Tiawsirisup *et al.*, 2008) or Tahyna virus (TAHV) (Lundström, 1999; Lebl *et al.*, 2015).

In the Mediterranean region, the most important hosts for *Cx. pipiens* are the common blackbird (*Turdus merula*) and house sparrow (*Passer domesticus*), but *Cx. pipiens* fed on other Passeriformes, e.g. house martin (*Delichon urbica*), European robin (*Erithacus rubecula*), European serin (*Serinus serinus*) or Columbiformes, e.g. collared dove (*Streptopelia decaocto*), as well (Roiz *et al.*, 2010; Osório *et al.*, 2012; Rizzoli *et al.*, 2015; Börstler *et al.*, 2016; Martínez-de la Puente *et al.*, 2016). Interestingly, *Cx. pipiens*, which is generally classified as an ornithophilic species (Molaei *et al.*, 2012; Radrova *et al.*, 2013; Brugman *et al.*, 2017), was found to be primarily anthropophilic and secondarily ornithophilic. However, *Cx. pipiens* can be subdivided into two distinct biotypes (*Cx. p. pipiens* and *Cx. p. molestus*), which are morphologically indistinguishable but show remarkable physiological and behavioural differences. The *Culex p.* biotype *pipiens* rarely bites humans and seems to be strictly ornithophilic (i.e. bird biting host preference), whereas females of the biotype *molestus* are mainly anthropophilic (Becker *et al.*, 2012). At the same time, humans were identified as hosts of *pipiens* and *molestus* forms and their hybrids as well (Martínez-de la Puente *et al.*, 2016). The adaptation of *Cx. pipiens* mosquitoes to human-altered environments led to their global distribution through dispersal via humans and, combined with

their mixed feeding patterns on birds and mammals (including humans), predestine them as bridge vectors for pathogens transmitted between mammals and birds, e.g. WNV (Fonseca *et al.*, 2004; Farajollahi *et al.*, 2011; Fortuna *et al.*, 2015; Brustolin *et al.*, 2016), USUTV (Ashraf *et al.*, 2015; Fros *et al.*, 2015; Nikolay, 2015; Cadar *et al.*, 2017) and Japanese Encephalitis virus (Wispelaere *et al.*, 2017). Although we did not identify *Cx. pipiens* to ecoform (*pipiens* or *molestus*) level, mosquitoes of the *molestus* form are more frequently found in urban than in natural areas (Martínez-de la Puente *et al.*, 2016) and biotype *molestus* was identified already in Novi Sad, Serbia (Becker *et al.*, 2012). Besides, it is important to consider the potential impacts of differences between biotypes of the *Cx. pipiens* complex, as vector competence of the *Cx. pipiens* biotypes and their hybrids for WNV is differentially affected by environmental factors, e.g. temperature. Namely, transmission rates of biotype *pipiens* and hybrids increased with higher temperatures, but not so for biotype *molestus* (Vogels *et al.*, 2016). Notably, our results confirm *Cx. pipiens* feeding on amphibian hosts in nature, which was detected by Radrova *et al.* (2013) in wetland areas of the Czech Republic already, and reported with other genus members also, such as *Culex peccator* (Dyar & Knab, 1909) and *Culex territans* (Walker, 1856) from the USA (Burkett-Cadena *et al.*, 2008). Furthermore, members of the *Culex* genus (*Cx. pipiens*, *Cx. hortensis* (Ficalbi, 1890) and *Cx. modestus* (Ficalbi, 1980)) were found to feed on reptiles, such as common wall lizard (*Podarcis muralis*) (Roiz *et al.*, 2010; Muñoz *et al.*, 2012), but rarely on amphibians. Nevertheless, amphibian feeding behaviour may facilitate the enzootic circulation of WNV, which was previously described in laboratory experiments (Kostiukov *et al.*, 1986) and reported under natural conditions in North American reptiles and amphibians (Klenk & Komar, 2003).

Multiple mosquito genera transmit avian malaria parasites, including different species of the genus *Culex*, which may play a central role in the transmission dynamic of *Plasmodium* parasites under natural conditions (Santiago-Alarcon *et al.*, 2012). Similar to previous reports from Europe (Glaizot *et al.*, 2012; Lalubin *et al.*, 2013; Zélé *et al.*, 2014; Zित्रा *et al.*, 2015; Ionică *et al.*, 2017), we identified the protozoa in *Cx. pipiens* mosquitoes. Interestingly none of the avian blood-fed mosquitoes was positive for avian malaria species, only the human blood-fed specimens. Due to the sampling methodology (as we investigated blood-fed mosquitoes only), our results are not suitable for estimating prevalence or infection rate, although provide evidence for local transmission of *Plasmodium* spp. in the sampling area. To investigate further the epizootic characteristics of *Plasmodium* species additional examinations involving several bird and mosquito species are needed in the region.

These data reflect the complex and mainly undiscovered role of *Cx. pipiens* complex in the ecology of several diseases (Turell, 2012), by acting as a possible bridge vector between varieties of vertebrate species, including several ectothermic hosts. Our results provided here further highlight the human and veterinary health significance of the species complex.

Little information on the biology and the behaviour of *Aedes koreicus* in an area outside of its native range is available (Versteirt *et al.*, 2012; Marcantonio *et al.*, 2016). Based on previous observations, the cold season is not a limiting factor to the establishment and spread of this vector-competent invasive mosquito species, thus extending the risk of invasion all over central Europe (Monstari *et al.*, 2013). Although there is evidence of cattle and human feeding by *Ae. koreicus* in their native areas, in Japan (Tanaka *et al.*, 1979) and Korea (Kim *et al.*, 2003), except for humans (human biting behaviour in Italy confirmed by Montarsi *et al.*, 2013) the host preference spectrum in Europe remains unknown. To our knowledge, our study provides the first data regarding the blood-meal source of *Ae. koreicus* in this area. Although only one specimen was tested, we showed important evidence of human feeding, which may highlight the possible medical importance of the species. In Europe, *Aedes koreicus* is a confirmed vector of the parasitic nematode *D. immitis* (Montarsi *et al.*, 2015), which is endemic in Hungary and Serbia as well, and presumed to be a potential vector of several arboviruses also (Cameron *et al.*, 2010). Furthermore, as a diurnal biting species, it has an important role in mosquito nuisance as well. These findings stress the importance of implementing entomological surveillance for early detection of invasive species, which is necessary for eradication or limitation of its further spread.

In summary, the present study provides the first insights to the feeding preferences of Southern Central European mosquitoes of urban environments, including the evidence of human host preference of the invasive mosquito *Aedes koreicus* for the first time in Europe. Based on our results about the host preferences of the investigated mosquitoes, these species may play a significant role in the enzootic cycle of several pathogens. However, further blood-fed collections (using gravid traps instead of CDC light traps) from the region are required, supplemented with vector competence studies to provide a complete picture of the risk of pathogen transmissions.

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