Biting mosquito data from the Lake Balaton region for the surveillance of West Nile virus infection

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Abstract: During the 2013–2014 period, mosquitoes of the Lake Balaton region were collected and examined for the presence of West Nile virus (WNV). Our test area contained biting mosquito habitats of considerable expanse and variety, as well as large numbers of presently residing or migrating bird fauna. Specimens of five potential vector species of WNV (*Anopheles maculipennis* s.l., *Aedes vexans*, *Coquillettidia richiardii*, *Culex p. pipiens*, *Culex modestus*) were involved in the virological studies. Our investigation consisted of 4,197 mosquito specimens. The RNA of WNV was not detected by qRT-PCR in any of the mosquito pools. We concluded that during the above mentioned time period there was not WNV activity around Lake Balaton. Although, during the same time period there were West Nile encephalitis cases found in the Eastern and Central part of the country, precisely 31 and 11 cases.
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Introduction

Lake Balaton is the largest lake in Central Europe. Due to its unique natural values, it is one of the oldest and most popular tourist destinations of Hungary. The lake and its surroundings have also long been given a priority in zoological research. Therefore, the local mosquito fauna is one of the best-known Hungarian subareas in terms of exploration (Tóth, 2004b, Tóth & Kenyeres, 2012). The first known data on the area of mosquitoes occurring in the Lake Balaton region came from Badacsony (*Aedes vexans*) (Kertész, 1904), but the first specimens were regularly collected in the region by Lajos Gammel between 1926 and 1931 (Tóth & Sáringer, 2002). Research activity gained major momentum by the establishment of the Mosquito Research Institute of Balaton in 1938. As a researcher of the institute, Ferenc Mihályi carried out intensive studies, which resulted in detection of 26 mosquito species (Mihályi, 1941). The post-World War II period of the Lake Balaton region biting mosquito research was mainly based upon Mihályi's work, whereby the number of species recorded increased to 32 (Mihályi et al., 1956). The thematic study of the region revived again in the 1970s, primarily due to the work of Sándor Tóth and Gyula Sáringer (Kecskeméti & Tóth, 1981; Tóth, 1991, 1996; Tóth & Sáringer, 1997, 2002). Decades of research resulted in the record of 40 taxa of biting mosquitos (some 90% of the Hungarian fauna) in the region.

Based on the above mentioned examinations, *Coquillettidia richiardii*, *Ae. annulipes*, *Culex p. pipiens* and *Ae. vexans* occurred in the largest numbers at the lake in recent decades. Biting mosquito fauna of an adjacent swamp of Lake Balaton, called Kis-Balaton, having a fundamentally different habitat structure than that of Lake Balaton was also examined by Tóth (1996, 2004a-b). The local species number (26) in this subarea proved to be lower than at Lake Balaton. Based on the research of Sáringer-Kenyeres & Kenyeres (2014) the biting mosquito-fauna of Kis-Balaton shows no significant difference as compared to Lake Balaton. Mosquito-fauna of Kis-Balaton is dominated by *Cq. richiardii*, *Ae. annulipes* and *Cx. p. pipiens*, but a significant presence of *Culiseta annulata*, *Cx. modestus*, *Ae. vexans* and *Ae. cinereus* was also recorded.

Larval stages and adults of biting mosquitoes, as nutrition sources, play an important role in the ecosystem of Lake Balaton. However, the bites of females often significantly disturb the people living or relaxing in this region. In addition, the biting mosquitoes might bear public and animal-health significance, as they can be vectors of viruses (e.g. alpha-, flavi- and bunyaviruses) as well as unicellular parasites (e.g.: *Plasmodium* spp.) and multicellular parasites (e.g.: *Dirofilaria* spp.) (Sáringer-Kenyeres, 2018).

Among the viruses transmitted by mosquitoes, a flavivirus causing West Nile fever (West Nile virus, WNV) has been the most important in Europe in the past decade (Bakonyi et al., 2013). This virus was first detected in 1937, in the West Nile district of Uganda in a patient symptomatic with high fever (Smithburn et al., 1940). The virus occurs in Africa, Europe, Asia and Australia. The pathogen emerged in the United States in 1999 and spread over both the North and the South American continents in a few years (Gratz, 2004). In Europe, its occurrence has been identified in most southern and eastern countries. Birds are the natural hosts of the virus. In the biting mosquito–bird–biting mosquito circle, the primary vector in Europe is *Cx. p. pipiens*, however, its presence has been detected in many other biting mosquito species as well (eg. *Cx. modestus*, *Ae. cantans*, *Cq. richiardii*, *Ae. caspius*, *Ae. cinereus*, *Ae. vexans*, *Anopheles maculipennis* s.l.) (Hubalek & Halouzka, 1999).

Based on phylogenetic analysis, at least nine genetic lineages of the virus have been identified (Kemenesi et al., 2014), of which viruses belonging to genetic lineage 1 are the most common. Earlier, the viruses classified in genetic lineage 2 had occurred in sub-Saharan Africa and Madagascar, but some

strains have recently emerged and spread in Central, Southern and Eastern Europe (Kovács, 2014).

The presence of the virus in Hungary has been recorded since the 1960s; serological tests gave evidence of the presence of antibodies in human sera (Koller et al., 1969). For the first time in 2003, clinical occurrence of the disease was diagnosed in Hungary. Following animal studies, neurological disease occurred in a stock of geese (*Anser anser f. domestica*) in the southern part of the Hungarian Great Plain. A virus strain belonging to genetic lineage 1 of WNV was detected in the brain samples of the geese. In 2004, in the south-eastern region of Hungary, another West Nile virus, belonging to genetic lineage 2, was detected in a northern goshawk (*Accipiter gentilis*) specimen which died from encephalitis. The latter case was the first emergence of the lineage 2 WNV strain described in Europe (Bakonyi et al., 2006). In 2007 the virus was detected in the south-eastern region of Hungary as one causing disease in birds and horses (Kovács, 2014). In 2008, a large-scale spread of the virus was recorded in Hungary. In 2009, further birds, horses and people were infected by this neuroinvasive WNV strain (Bakonyi et al., 2013).

In order to better understand the vector-ecological role of Hungarian biting mosquitoes in WNV transmission cycle, a targeted survey was carried out during the years 2011–2012 (Szentpáli-Gavallér et al., 2014). In the southern and eastern regions of Hungary, a total of 23,193 biting mosquito specimens were collected, of which (based on species, sex, place and time of collection) 645 pools were created. WNV nucleic acid was detected in 3 pools: in a pool of *Ae. annulipes* (Fényeslitke, collected in June, 2011), a pool of *Cq. richiardii* (Debrecen, collected in July, 2011) and a pool of *Cx. p. pipiens* (Kardoskút, collected in September, 2011) proved to be WNV-infected (Szentpáli-Gavallér et al., 2014) (Fig. 1).

In the southwest regions of Hungary, a total of 23,029 biting mosquito specimens were collected by Hungarian researchers during the years 2011–2013, of which 586 pools were created. WNV nucleic acid was detected in 1 pool of *Uranotaenia unguiculata* (Pécs, collected in August, 2011) proved to be WNV-infected (Kemenesi et al., 2014) (Fig. 1).

In the subsequent years, the surveys have been extended to Lake Balaton and its surroundings. This is justified by the fact that in the above territory (a) no studies for the presence of West Nile virus have been carried out (although West Nile encephalitis cases occurred in horses kept near the lake in 2009 (Bakonyi et al., 2013)), (b) biting mosquito-habitats of significant expanse and variety are found, (c) a migrating and resident bird fauna of a large number of individuals can also be found.

Materials & Methods

We carried out regular collections of adult mosquitoes in seven sampling areas between March and November (2013), June and November (2014). Samplings were taken twice a month in the summer, and once a month during the rest of the sampling periods. The sampling areas included barns (Balatonmagyaród, Szigliget), buildings (Badacsonytördemic, Keszthely, Szigliget) and natural habitats, such as swamp forests and reed beds (Szigliget, Zalavár) (Fig. 1). All sampling areas are surrounded with temporary and permanent waters. These habitats are ideal conditions for all mosquito species having different habitat-requirements.

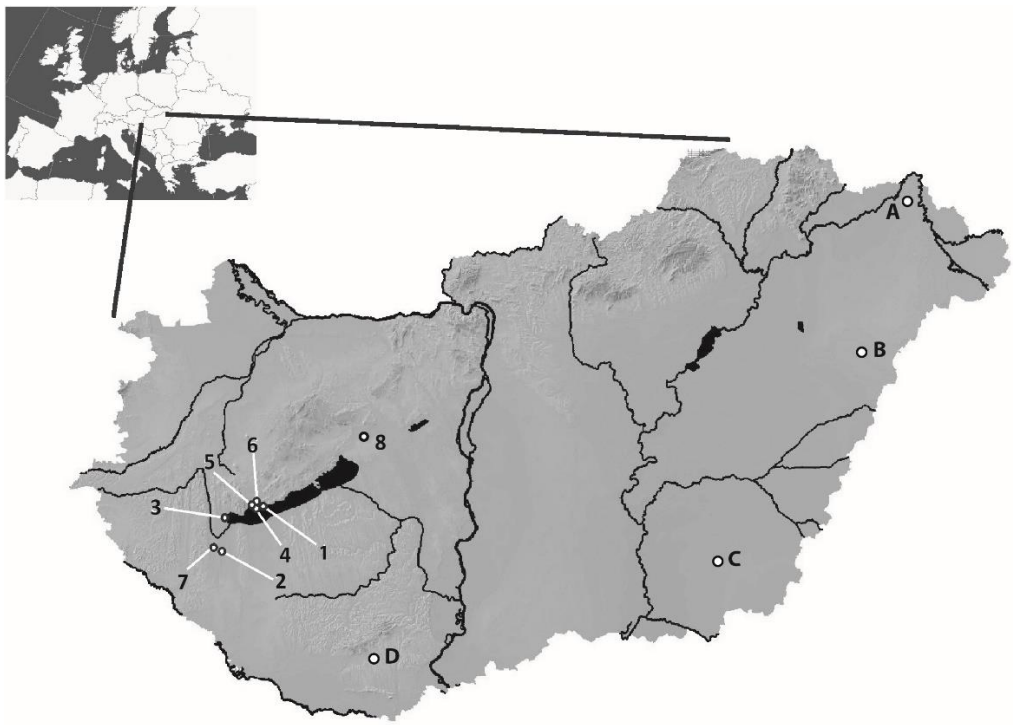

Figure 1: Map of the studied sties (1–8) and the points of WNV detections from mosquito species.
1: Badacsonytördemic; 2: Balatonmagyaród (Fenyvespuszta); 3: Keszthely; 4: Szigliget (building); 5: Szigliget (reedbeds); 6: Szigliget (barn); 7: Zalavár (Kápolnapuszta); 8: Ősi; A: Fényeslitke; B: Debrecen; C: Kardoskút; D: Pécs

Table 1: Individual numbers of the collected species in the overall material

Species	Abundance
Anopheles claviger (Meigen, 1804)	23
Anopheles hyrcanus (Pallas, 1771)	2
Anopheles maculipennis Meigen, 1818 s.l.	2269
Aedes cinereus Meigen, 1818	24
Aedes rossicus Dolbeshkin, Goritzkaja & Mitrofanova, 1930	2
Aedes vexans Meigen, 1830	148
Aedes annulipes (Meigen, 1830)	314
Aedes cantans (Meigen, 1818)	189
Aedes flavescens (Müller, 1764)	1
Aedes sticticus (Meigen, 1838)	316
Coquillettidia richiardii (Ficalbi, 1889)	365
Culex modestus Forskal, 1775	17
Culex pipiens pipiens Linnaeus, 1758	1393
Culiseta annulata (Schrank, 1776)	197
Uranotaenia unguiculata Edwards, 1913	4
Total number of individuals:	**5264**

The samplings were carried out by aspirator, carbon dioxide and light traps (Becker *et al*., 2003). The primary method of collecting was catching by aspirator from the walls of the studied barns and buildings, as well as catching the mosquitoes during human encounters. Carbon dioxide traps (BG-Sentinel) with gas and MO-EL KYOTO light traps were occasionally used on one sampling site (Keszthely) during the summer of 2013 and 2014. Mosquitoes were collected by aspirator during daytime, by carbon dioxide trap between 7 p.m. and 10 p.m., and by light trap between 10 p.m. and 2 a.m.

The collected specimens were treated separately by sample areas and samplings and were identified to species level (Becker et al., 2003; Kenyeres & Tóth, 2008). Until virological tests were performed, the mosquitoes were stored in plastic tubes in dry ice cooler boxes and were transported to the virology laboratory.

Analyses were carried out on samples divided into pools of up to 25 individuals. Each pool contained mosquito specimens of the same category (species, sex, place of collection, collection time). The pools were homogenized and RNA was extracted. As described in previous research (Bakonyi *et al*., 2013), real-time reverse transcription polymerase chain reaction (real-time RT-PCR) with WNV-specific oligonucleotide primers and probes were used to detect viral genomic RNA. Briefly, the SuperScript III Platinum One-Step Quantitative RT-PCR System kit (Invitrogen, USA) was used for the amplifications. Each 25 µl reaction volume contained 12.5 µl of 2×Reaction Mix buffer, 5 pmol of forward and reverse primers (WNV5009f, 5'-GAACGTCAGGTTCCCCCATT-3', and WNV 5103r, 5'-GGCGCTTATGTATGAACCATTAGG-3', respectively) and the TaqMan probe (WNV 5050p, FAM-ATTGGATTGTATGGGAACGGCGTCATC-TAMRA), 0.5 µl ROX reference dye, 0.5 µl SuperScript III RT/Platinum Taq Mix, 0.25 µl RNasin (Promega, USA) RNase inhibitor, and 2.5 µl template RNA. Amplifications were performed in an Applied Biosystems 7300 Real-Time PCR System using 96-well plates. Reverse transcription (48 ºC, 15 min) was followed by an RT denaturation and Taq activation step (95 ºC, 2 min) and cyclic amplification (45× [95 ºC, 15 s; 60 ºC for 30 s]). Fluorescence was detected at the annealing/elongation step. Results were recorded and processed in Absolute Quantification mode; 10-fold dilutions of RNA extracts from the cultivated and titrated. Hungarian lineage 2 WNV strain was used as standard.

Scientific nomenclature follows Sáringer-Kenyeres *et al.* (2018).

Results

Altogether we collected 5,264 individuals of 15 species (Table 1). For the virological processing we prepared 4,192 specimens from the collected samples. Our observation was that three species showed extremely high frequency: *An. maculipennis* s.l. (43.1 % of the collected materials), *Cx. p. pipiens* (26.4 %), *Cq. richiardii* (6.9 %). Only five species, considered to be potential vectors of the West Nile virus (*An. maculipennis* s.l., *Ae. vexans*, *Cq. richiardii*, *Cx. p. pipiens*, and *Cx. modestus*). Pooled data of the examined species are shown in Table 2 and 3.

The number of specimens of *An. maculipennis* s.l. showed steady growth until August in 2013 and July in 2014. The flight peak fell on the above throughout the rest of the months. In the case of *Cq. richiardii*, the number of individuals collected in 2013 remained low throughout, and no serious swarming was detected. By contrast, a powerful flight peak was noted in July of 2014. Two small peaks were observed in the case of *Cx. p. pipiens* in August and October of 2013. We noted a marked peak in July of 2014. According to the more detailed figures, lower and higher abundances alternated, respectively.

We did not detect any RNA of West Nile virus by qRT-PCR method in any of the mosquito pools.

Discussion

Summarizing our research, we can conclude that two of the most frequent species (*Cq. richiardii*, *Cx. p. pipiens*) were the same as previous data indicated several decades ago (Tóth & Sáringer, 2002). The fact that the species of the largest number

of individuals caught was *An. maculipennis* s.l., should be related to the high proportion of collections by aspirator from the walls of the stables. The high abundance of *An. maculipennis* s.l.

Table 2: Individual numbers of collected potential vectors of West Nile virus

2013

	March–May								June–August								September–November								
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	Σ
Anopheles maculipennis Meigen, 1818 s.l.		81					41			930				249		19		267					27		**1614**
Aedes vexans (Meigen, 1830)		1				1	1	2		7		3	12	14	4										**45**
Coquillettidia richiardii (Ficalbi, 1889)										82		1	2	3	9	2		6							**105**
Culex modestus Ficalbi, 1890												2	15												**17**
Culex pipiens pipiens Linnaeus, 1758													4		131	3		89		7			2		**236**

2014

	June–August								September–November								
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	Σ
Anopheles maculipennis Meigen, 1818 s.l.		517	4							134							**655**
Aedes vexans (Meigen, 1830)		2	17							84							**103**
Coquillettidia richiardii (Ficalbi, 1889)		17	243														**260**
Culex modestus Ficalbi, 1890																	**0**
Culex pipiens pipiens Linnaeus, 1758			786						75	296							**1157**

detected in each site is also probably due to the proximity of lakes (Balaton and Kis-Balaton), and high proportion of marshlands. *Cx. p. pipiens* and *Ano. maculipennis* s.l. lay their eggs on the water surface, and have several generations within a year. Therefore, the lakes and marshlands with permanent waters are particularly favourable to these species (Becker *et. al*, 2003; Kenyeres *et al*., 2011). On the other hand, *Cq. richiardii* laying their eggs on water surfaces prefers marshy areas with permanent waters rich in plants. Their larvae develop for nine months fixed to underwater regions of rushes. *Coquillettidia richiardii* usually has one generation per year and is primarily characterised by the end of summer gradation (Tóth, 1991). Between 2013 and 2014, an inversion was seen in the number of collected specimens of *An. maculipennis* s.l. and *Cx. p. pipiens*. In 2013 *An. maculipennis*, and in 2014 *Culex p. pipiens*, was the dominant species of the collected material. Probably it was caused by differences in annual weather. Precipitation in 2014 was much higher than in 2013. It has resulted many more breeding sites of different sized artificial containers and water-pits being suitable for the habitat-requirements of *Culex p. pipiens.*

Our virology analyses results showed a remarkable correlation with the previously found fact that all human West Nile encephalitis cases were only diagnosed in the eastern and central part of the country during the years of 2013 and 2014 (31 and 11 respectively). Our research area as the western part of the country, including the Lake Balaton region, where WNV was not active. However, it should be noted that in later years in 2015 (Fejér County) and in 2016 (Fejér, Veszprém and Győr-Moson-Sopron counties) human illnesses had been reported. It might indicate the geographical spread of the pathogen. However, it could be due to the fact that the weather conditions of 2015–2016 changed and were more favourable to the proliferation of mosquito vectors and the spread of viral infections. Current research following formers (Tóth, 1996; 2004a; Sáringer-Kenyeres & Kenyeres, 2014), confirmed that most of the potential vector species of WNV occur at western part of Lake Balaton and region of Kis-Balaton with robust populations. Based on this, continuation of WNV vector monitoring and surveillance activities in the Lake Balaton region is justified.

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Table 3: Individual numbers of the species in the analysed pools

Collection date	Species	Collection site	Number of pools
26.04.2013	*Anopheles maculipennis* s.l.	Balatonmagyaród (Fenyvespuszta), barn	1
	Anopheles maculipennis s.l.	Szigliget, barn	1
03.05.2013	*Anopheles maculipennis* s.l.	Szigliget, barn	1
	Aedes vexans	Zalavár (Kápolnapuszta), swamp forest	1
26.05.2013	*Anopheles maculipennis* s.l.	Szigliget, barn	2
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	4
	Aedes vexans	Szigliget, barn	1
	Aedes vexans	Balatonmagyaród (Fenyvespuszta), barn	1
	Aedes vexans	Zalavár (Kápolnapuszta), swamp forest	1
	Aedes vexans	Szigliget, reedbeds	1
09.06.2013	*Anopheles maculipennis* s.l.	Ősi, cattle farm	1
	Anopheles maculipennis s.l.	Szigliget, barn	2
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	5
	Aedes vexans	Szigliget, reedbeds	1
	Aedes vexans	Szigliget, barn	1
	Aedes vexans	Balatonmagyaród (Fenyvespuszta), barn	1
	Coquillettidia richiardii	Balatonmagyaród (Fenyvespuszta), barn	1
23.06.2013	*Anopheles maculipennis* s.l.	Ősi, cattle farm	1
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	5
	Aedes vexans	Balatonmagyaród (Fenyvespuszta), barn	1
	Aedes vexans	Szigliget, reedbeds	1
	Coquillettidia richiardii	Ősi, cattle farm	1
	Coquillettidia richiardii	Balatonmagyaród (Fenyvespuszta), barn	2
	Coquillettidia richiardii	Zalavár (Kápolnapuszta), swamp forest	1
	Culex p. pipiens	Ősi, cattle farm	1
07.07.2013	*Anopheles maculipennis* s.l.	Balatonmagyaród (Fenyvespuszta), barn	1
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	1
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	5
	Anopheles maculipennis s.l.	Szigliget, barn	2
	Aedes vexans	Szigliget, reedbeds	1
	Aedes vexans	Szigliget, barn	1
	Coquillettidia richiardii	Szigliget, reedbeds	1
	Coquillettidia richiardii	Szigliget, barn	1
	Culex p. pipiens	Szigliget, reedbeds	1
21.07.2013	*Anopheles maculipennis* s.l.	Szigliget, barn	2
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	9
	Aedes vexans	Szigliget, barn	1
	Aedes vexans	Szigliget, building	1
	Aedes vexans	Szigliget, barn	1
	Aedes vexans	Balatonmagyaród (Fenyvespuszta), barn	1
	Coquillettidia richiardii	Szigliget, barn	1
	Coquillettidia richiardii	Szigliget, building	1
	Coquillettidia richiardii	Szigliget, reedbeds	1
	Coquillettidia richiardii	Balatonmagyaród (Fenyvespuszta), barn	2

	Culex modestus	Szigliget, building	1
	Culex modestus	Szigliget, reedbeds	1
08.08.2013	*Anopheles maculipennis* s.l.	Balatonmagyaród (Fenyvespuszta), barn	5
	Anopheles maculipennis s.l.	Szigliget, reedbeds	3
	Aedes vexans	Szigliget, reedbeds	1
	Aedes vexans	Zalavár (Kápolnapuszta), swamp forest	1
	Coquillettidia richiardii	Zalavár (Kápolnapuszta), swamp fores	1
	Culex p. pipiens	Zalavár (Kápolnapuszta), swamp fores	6
28.08.2013	*Anopheles maculipennis* s.l.	Szigliget, barn	2
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	11
	Coquillettidia richiardii	Balatonmagyaród (Fenyvespuszta), barn	1
10.09.2013	*Anopheles maculipennis* s.l.	Szigliget, barn	2
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	11
	Coquillettidia richiardii	Balatonmagyaród (Fenyvespuszta), barn	1
	Culex p. pipiens	Szigliget, barn	1
	Culex p. pipiens	Ősi, cattle farm	1
	Culex p. pipiens	Balatonmagyaród (Fenyvespuszta), barn	1
16.10.2013	*Anopheles maculipennis* s.l.	Szigliget, barn	1
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	1
	Culex p. pipiens	Szigliget, barn	1
	Culex p. pipiens	Balatonmagyaród (Fenyvespuszta), barn	4
05.06.2014	*Anopheles maculipennis* s.l.	Balatonmagyaród (Fenyvespuszta), barn	3
14.07.2014	*Anopheles maculipennis* s.l.	Balatonmagyaród (Fenyvespuszta), barn	7
	Aedes vexans	Balatonmagyaród (Fenyvespuszta), barn	1
	Coquillettidia richiardii	Balatonmagyaród (Fenyvespuszta), barn	1
23.07.2014	*Culex p. pipiens*	Keszthely, building	7
	Coquillettidia richiardii	Keszthely, building	3
27.07.2014	*Coquillettidia richiardii*	Keszthely, building	4
	Culex p. pipiens	Keszthely, building	7
28.07.2014	*Coquillettidia richiardii*	Keszthely, building	2
	Culex p. pipiens	Keszthely, building	4
30.07.2014	*Coquillettidia richiardii*	Keszthely, building	1
	Culex p. pipiens	Keszthely, building	1
	Anopheles maculipennis s.l.	Balatonmagyaród (Fenyvespuszta), barn	2
26.08.2014	*Anopheles maculipennis* s.l.	Balatonmagyaród (Fenyvespuszta), barn	9
	Anopheles maculipennis s.l.	Keszthely, building	1
	Aedes vexans	Keszthely, building	1
	Coquillettidia richiardii	Balatonmagyaród (Fenyvespuszta), barn	1
	Coquillettidia richiardii	Keszthely, building	1
	Culex p. pipiens	Keszthely, building	17
19.09.2014	*Anopheles maculipennis* s.l.	Balatonmagyaród (Fenyvespuszta), barn	3
	Aedes vexans	Balatonmagyaród (Fenyvespuszta), barn	4
19.10.2014	*Anopheles maculipennis* s.l.	Balatonmagyaród (Fenyvespuszta), barn	1
	Culex p. pipiens	Balatonmagyaród (Fenyvespuszta), barn	5
29.10.2014	*Culex p. pipiens*	Badacsonytördemic, building	3
07.11.2014	*Anopheles maculipennis* s.l.	Balatonmagyaród (Fenyvespuszta), barn	1
	Culex p. pipiens	Balatonmagyaród (Fenyvespuszta), barn	8

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