Detection of Flavivirus (West Nile Fever Virus and Usutu Virus) in the samples of mosquitoes collected in Hungary

By

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List of abbreviations

WNV: West Nile Virus

WNF: West Nile fever

WNND: West Nile neuroinvasive disease

USUV: Usutu virus

RVFV: Rift Valley fever virus

JEV: Japanese encephalitis virus

SINV: Sindbis virus

TAHV: Tahyna virus

CHIKV: Chikungunya virus

DENV: Dengue virus

YFV: Yellow fever virus

ZIKV: Zika virus

CB: catch bag

PBS: phosphate buffered saline

PCR: polymerase chain reactions

qRT-PCR: real-time reverse transcription polymerase chain reaction

RT: reverse transcription

ECDC: European Centre for Disease Prevention and Control

MIR: Minimum Infection Rate

Introduction

As vectors of various pathogens, mosquitoes hold a significant role in human and veterinary medicine. Their capacity to transmit a wide spectrum of potentially deadly diseases, including viruses belonging to the Flaviviridae family, highlights their importance concerning public health. These arboviruses can spread when vector mosquito species feed humans, birds, and mammals. The behaviour, feeding habits, preferred habitats, and geographical distribution of these vector mosquito species play a critical role in understanding the epidemiology of mosquito-borne diseases. Researchers rely on this knowledge to gain insights into the potential spread of diseases. Moreover, it is essential to consider the influence of various environmental factors on mosquito populations. [1] Variables such as humidity, temperature, flooding patterns, and the availability of shaded areas all contribute to the conditions that influence mosquito abundance and distribution.

The global distribution of mosquito-borne arboviruses has been expanding in recent years, and this is thought to result from the increased urbanisation, the global mobility of populations, and environmental alterations brought by rising temperatures and shifting precipitation patterns. Notably, their vector mosquitoes are highly sensitive to climatic conditions, relying on temperature and water resources within their habitats. With global surface temperatures exceeding pre-industrial levels by 0.8–1.2°C and evidence of extreme variations in precipitation patterns, the implications for mosquito-borne arbovirus distribution are significant. [36]

Consequently, the diligent study and continuous monitoring of the vector mosquito species responsible for transmitting arboviruses hold great importance. Such efforts facilitate the prompt detection of disease outbreaks

and provide insights into the trends of the global spread of mosquito-borne arboviruses, thereby contributing to response strategies in the field of public health and veterinary medicine.

Literature review

Mosquito species found in traps distributed in Hungary

Culex pipiens is called "common house mosquito", indicating its wide distribution shown in Figure 1, and is characterised by two distinct forms: the *Culex pipiens pipiens* and *Culex pipiens molestus* biotypes. These two variants possess the ability to interbreed, yielding offspring with altered habitat and host preferences. These hybrid offspring are recognised as bridge vectors, as they feed on both birds and mammals, consequently serving as key players in transmitting West Nile Virus (WNV) to humans and horses during epizootic events [1]. The *pipiens* biotype primarily displays ornithophilic tendencies, though it occasionally bites humans. It tends to seek outdoor spaces for feeding and resting and requires a blood meal for egg laying, entering a mandatory winter diapause as adults.

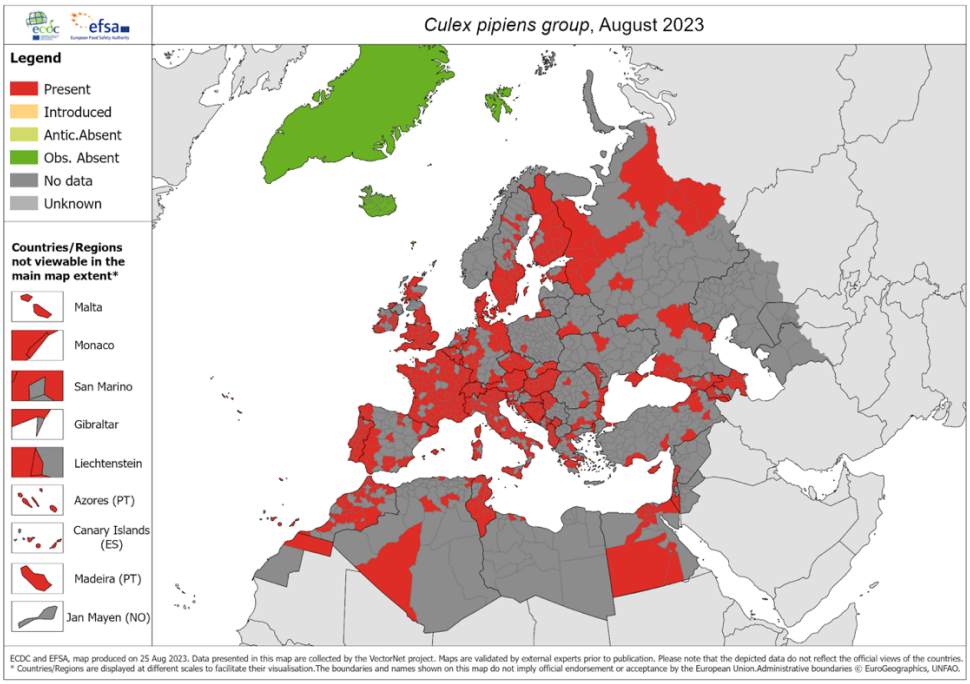

Figure 1.: Current known distribution of the Culex pipiens group in Europe. European Centre for Disease Prevention and Control and European Food Safety Authority. Mosquito maps [internet]. Stockholm: ECDC; 2023. Available from: https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps

In contrast, the *molestus* form exhibits a stronger preference for mammals, including humans, while occasionally targeting birds. This biotype often rests indoors, engages in indoor and outdoor feeding, and can lay its initial batch of eggs without a blood meal. Notably, it lacks a compulsory winter diapause [2]. *Culex pipiens* larvae can be found in any habitat with water ranging from clear and fresh to organic-rich subterranean water [3]. Importantly, *Culex pipiens* is a well-recognised vector for various pathogens, including West Nile virus (WNV), Usutu virus (USUV), Rift Valley fever virus (RVFV), Japanese encephalitis virus (JEV), Sindbis virus (SINV), Tahyna virus (TAHV), and avian malaria parasites [4].

Culex hortensis exhibits a strong affinity for birds and amphibians, being ornithophilic and herpetophilic in its feeding habits. [5] While it is recognised as a vector for West Nile virus and Usutu virus, it is important to note that it does not play a role in transmitting these flaviviral diseases to humans [6]. This mosquito species thrives in thermophilic conditions, making it a common sight in the Mediterranean region and widely distributed across Europe. Regarding reproduction, *Culex hortensis* relies on freshwater sources, which can be either stagnant or found in various locations like cement drinking troughs, pond edges, small ponds, and puddles [7].

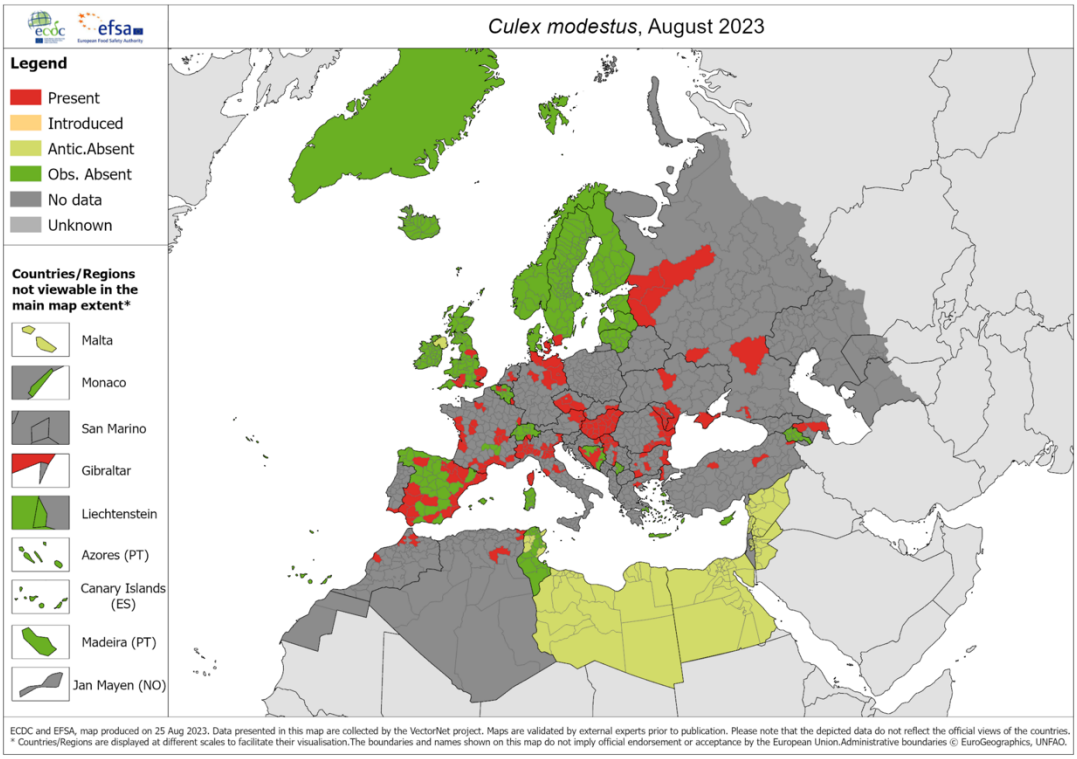

Figure 2.: Current known distribution of Culex modestus in Europe. Derived from European Centre for Disease Prevention and Control (ECDC): Mosquito maps [2023].

Culex modestus feeds on birds and mammals, and they are considered a WNV vector in Europe since WNV was first isolated from this species in France in 1964 [8]. It is widely spread in the Palaearctic region and is considered a bridge vector of WNV between birds and humans in southern France. Its distribution in Europe is shown in Figure 2. Its larvae are found in fresh to organic-rich water in rice fields and marshes. These mosquitoes peak in early August [9].

Aedes cinereus is widely distributed in North America and Europe. It is found in open floodwater, wetland, wet woodland, and flooded grassland [13]. *Ae. cinereus* larvae need temperature of 12°C to 13°C and 14°C to 15°C to develop, while the optimal temperature lay between 24°C and 25°C. It is a bridge vector, transmitting the virus from birds to humans, and is a potential vector for WNV and SINV. It is important to note that differentiating *Ae. cinereus* from *Ae.*

geminus is a challenge, therefore, researchers combine data to *Ae. cinereus/geminus* [14].

Aedes vexans, commonly called the "floodwater mosquito", holds a leading status among mosquito species in the Mediterranean and Central European regions. Its distribution in Europe is shown in Figure 3. Its population surge following flood events has earned it this moniker. Notably, this species is a carrier for bunyaviruses like the RVFV in Africa and the TAHV in Europe. Remarkably, *Ae. vexans* have even been identified as a vector for the WNV despite its preference for feeding on humans and mammals over avian hosts. When it comes to reproduction, *Ae. vexans* exhibits a unique behaviour of laying its eggs above the waterline. The critical factor for successful hatching is water temperature, with eggs failing to hatch in excessively cold or hot conditions. This mosquito species thrives in temperatures around 25°C, with its peak abundance observed at approximately 26.4°C. [10,11,12]

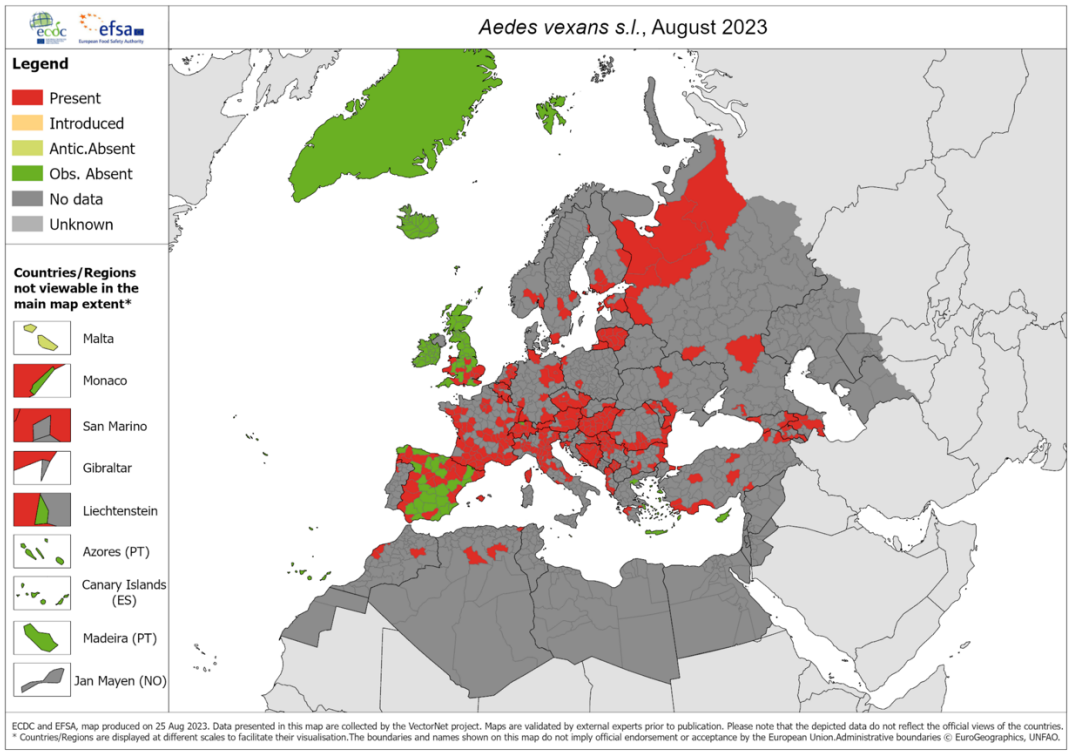

Figure 3.: Current known distribution of Aedes vexans s.l. in Europe. Derived from European Centre for Diseas e Prevention and Control (ECDC): Mosquito maps [2023].

Aedes albopictus, aka the „Asian tiger mosquito", originates from the tropical forests of Southeast Asia and has made its way to Europe through passive transportation methods like used car tires and items like "lucky bamboo," as well as through public or private vehicles and tourism [7]. In Hungary, the first specimens were detected in 2014. Its distribution in Europe is shown in Figure 4 [15]. Unlike other *Aedes* species, *Ae. albopictus* has a broad range of hosts, including birds, mammals, and reptiles [16]. Female mosquitoes of this species can lay eggs indoors and outdoors, and these eggs only require a small amount of water to hatch. They attach their eggs just above the waterline, making them highly resistant to drying out. Adult mosquitoes of this species are most active in temperatures ranging from 22°C to 28°C [17]. *Ae. albopictus* is considered a competent or potential carrier of various arboviruses, such as Chikungunya virus (CHIKV), Dengue virus (DENV), Japanese encephalitis virus, Yellow fever virus (YFV), Rift Valley fever virus, West Nile virus, Zika virus (ZIKV) and Sindbis virus. [18]

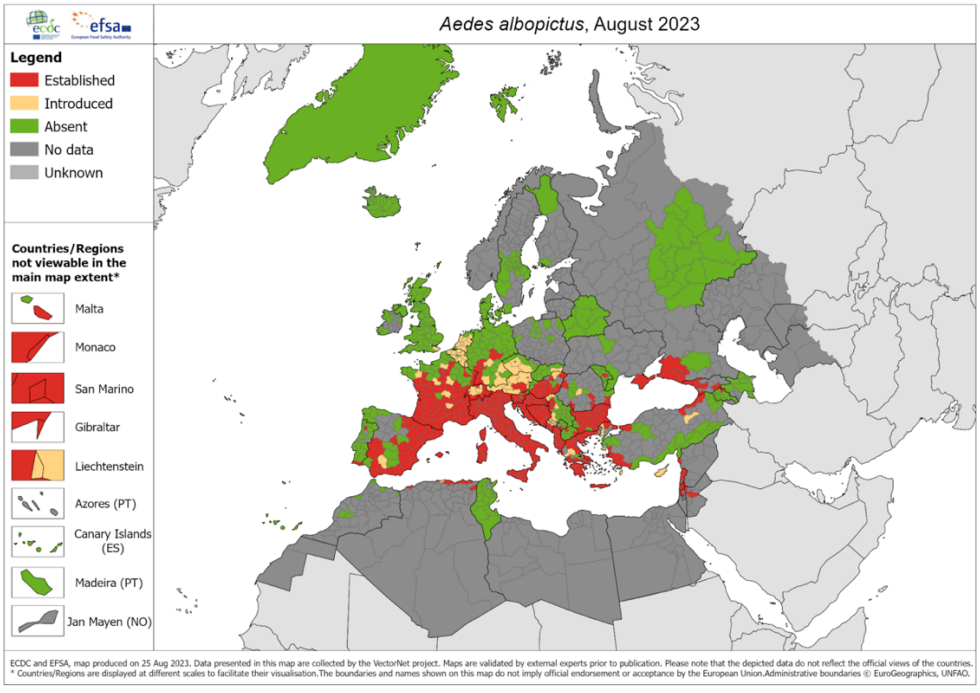

Figure 4.: Current known distribution of Aedes albopictus in Europe. Derived from European Centre for Disease Prevention and Control (ECDC): Mosquito maps [2023].

Aedes koreicus, commonly known as the "Korean bush mosquito", originated in Korea and was first detected in Europe (in Belgium) in 2008 [19]. Then, its first collection in Hungary was from the urban area of Pecs in 2016. However, the route of its introduction into Hungary has not been identified [15]. Its distribution in Europe is shown in Figure 5. The survival rate of its pupae and adults is optimum at temperatures between 23°C and 28°C. *Ae. koreicus*' anthropophilic behaviour, combined with its adaptability to urban settings, highlights the potential for transmission of diseases via human–mosquito contact. The introduction of this invasive mosquito species raises concern in public health, considering that it is potentially involved in the Chikungunya virus, *Dirofilaria immitis*, and heartworm transmission [19,20].

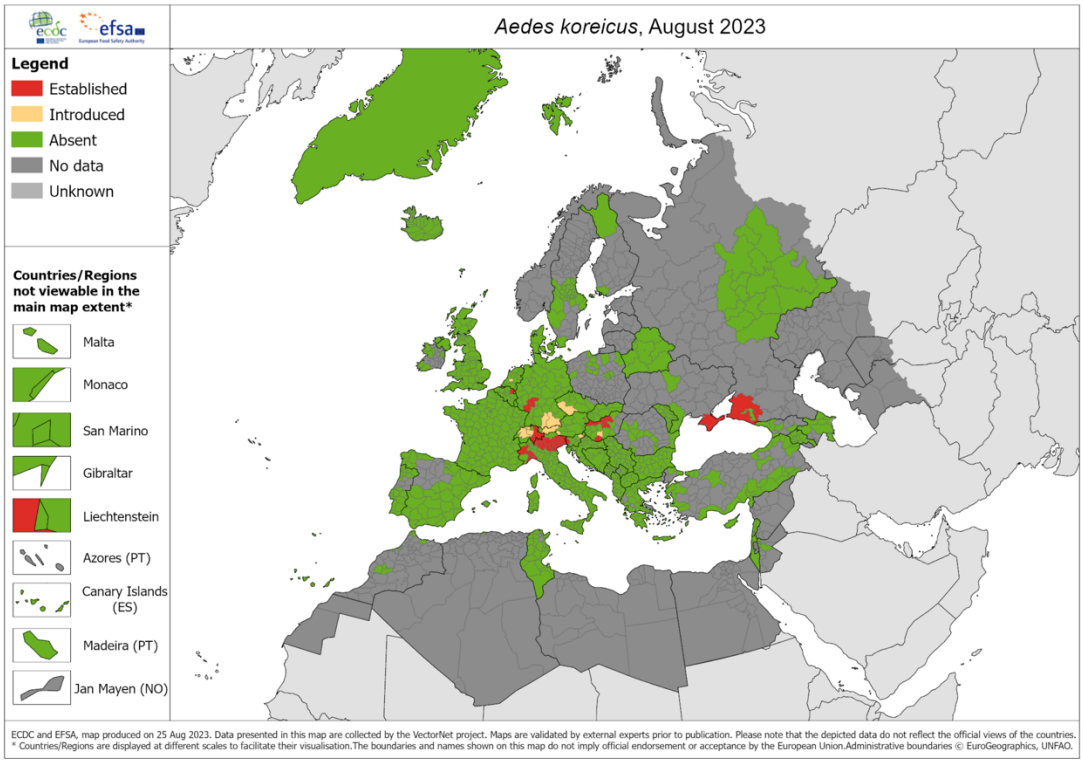

Figure 5.: Current known distribution of Aedes koreicus in Europe. Derived from European Centre for Disease Prevention and Control (ECDC): Mosquito maps [2023].

Aedes japonicus, called the "Asian bush mosquito" or the „Asian rock pool mosquito" in English, is originally native to Japan, Korea, Taiwan, and China,

appeared in Europe with initial reports in France (2000) and Belgium (2002) and later in Hungary in 2012 [15]. This introduction was likely facilitated through the transportation of used car tires [21]. Its distribution is shown in Figure 6. Notably, *Ae. japonicus* primarily feeds on mammals, showing a preference for animals over humans, earning it the moniker of a "bird biter." Similar to its *Aedes* counterparts, this mosquito requires just a small volume of water for egg-laying and hatching, and it can survive through the winter season in both larvae and egg stages. Thriving in temperatures of up to 30°C, *Ae. japonicus* has the capacity for multiple reproductive cycles throughout the year [22]. Its role as a vector extends to the transmission of CHIKV and several flaviviruses, including DENV, WNV, and JEV [23].

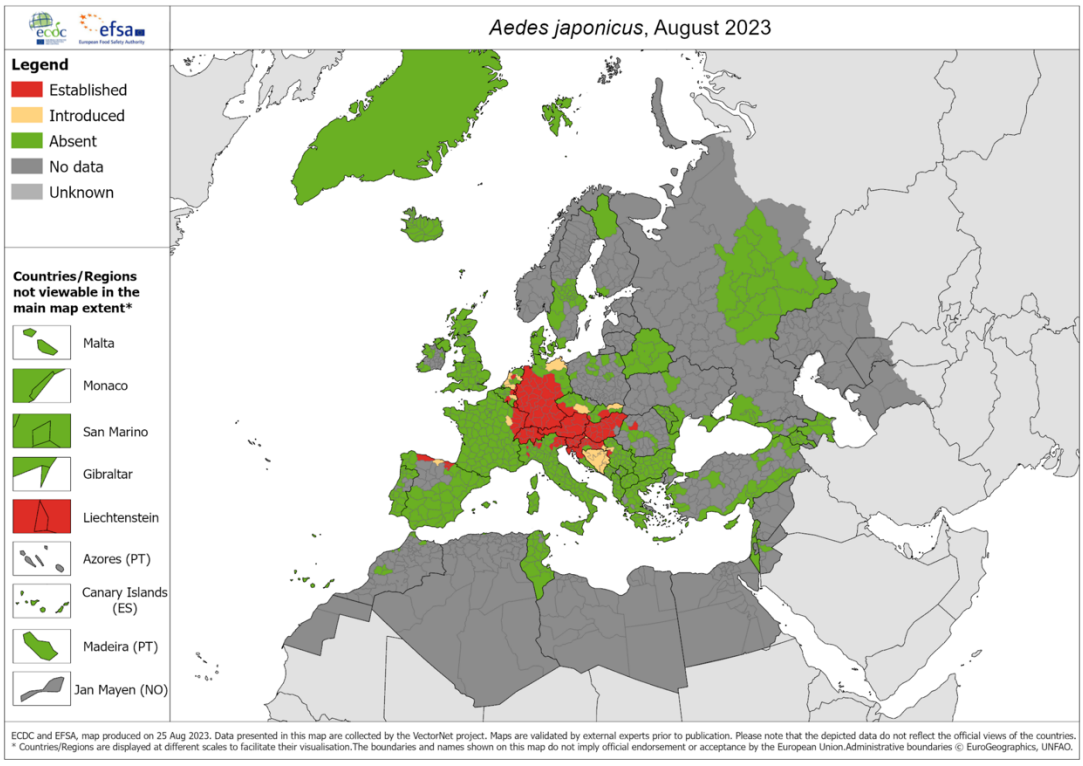

Figure 6.: Current known distribution of Aedes japonicus in Europe. Derived from European Centre for Diseas e Prevention and Control (ECDC): Mosquito maps [2023].

Ochlerotatus dorsalis is a widespread mosquito species that breeds freely in freshwater. Temperature is the principal environmental factor in stimulating and inhibiting diapause development in *Oc. dorsalis*. When the temperature

drops below 15.5°C, it stimulates diapause. It prefers salty ground that gets flooded periodically, and it overwinters in egg form and hatches larvae in early spring. Eggs hatch after two to five soaking, and eggs without flooding can survive for more than one year. Adults appear in March and feed on humans throughout summer until November, showing zoophilic feeding habits. A wide range of hosts was identified from *Oc. dorsalis* bloodmeal, including cattle, rabbit, horse, pig, and human. *Oc. dorsalis* being an infrequent bird feeder, it has a lower risk of transmitting WNV [24,25].

Ochlerotatus annulipes is a Palaearctic species commonly found in the forest. It overwinters in the egg stage, and the larvae develop in small water bodies like ponds and ditches. This anthropophilic mosquito is very aggressive and attacks humans in open spaces [26]. *Oc. annulipes* is a potential vector of *Dirofilaria repens* and WNV, but considering its infrequent bird-feeding habit, it does not pose importance in transmitting WNV [25,27].

Ochlerotatus geniculatus, a tree-hole breeding mosquito, is distributed in Europe, North Africa, and Southeast Asia. This species has shown vector competence to the YFV, CHIKV, and Eastern equine encephalitis virus in the lab. It feeds on mammals and stays outdoors. *Oc. geniculatus* breeds in semi-shaded water with vegetation in slow-flowing clay water, and the common habitat of the larvae is tree-holes. Its most preferred temperature is 11°C to 14°C [28,29,30].

Anopheles plumbeus is known for its aggressive biting behaviour, feeding on various mammals, encompassing humans, birds, and reptiles. This dietary versatility causes it to be an efficient bridge vector, facilitating the transmission of viruses from avian hosts to humans. This mosquito species holds significance as a vector for diseases such as malaria and the West Nile virus [32]. Regarding reproduction, *Anopheles plumbeus* lays its eggs just above the

waterline. These breeding sites can encompass natural habitats and artificial containers, including tree rot holes, water-filled pits, liquid manure repositories, cemetery vases, discarded car tires, and septic tanks. Adult mosquitoes are prevalent in European regions from late spring through the end of September, while their eggs and larvae can endure very cold weather for a long time, which helps them survive through the winter successfully. [33] Its distribution in Europe is shown in Figure 7.

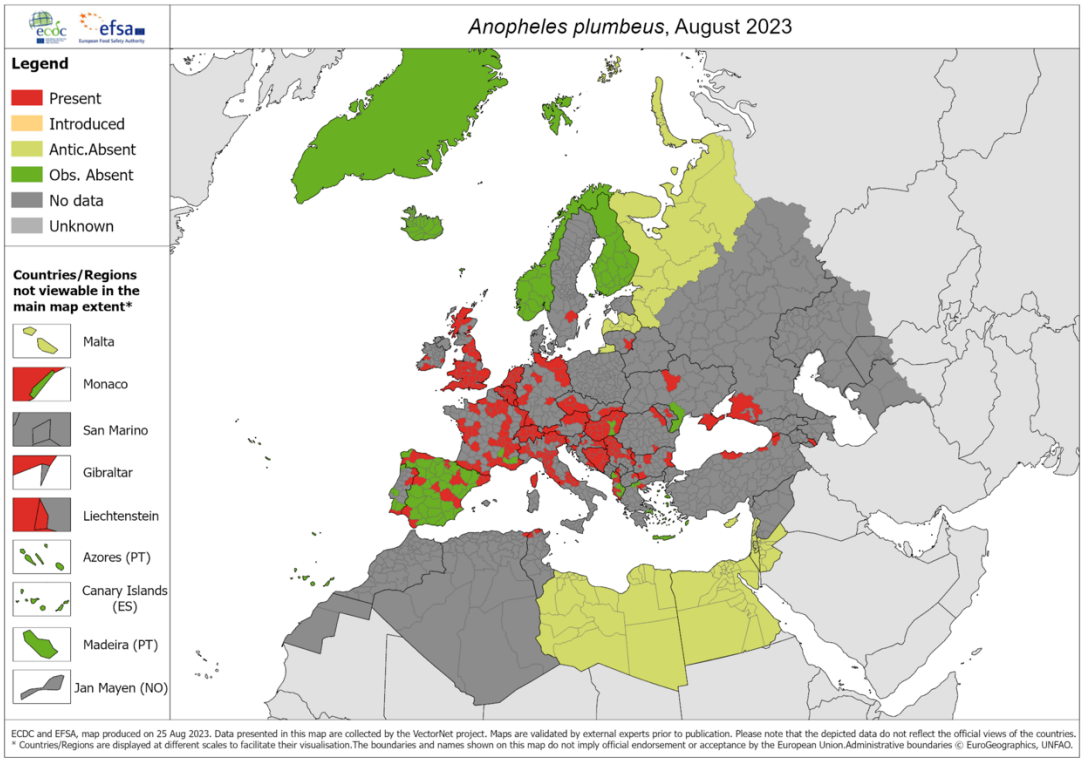

Figure 7.: Current known distribution of Anopheles plumbeus in Europe. Derived from European Centre for Disease Prevention and Control (ECDC): Mosquito maps [2023].

Anopheles hyrcanus, a species native to the Palearctic region, is widely distributed across Europe, the Mediterranean, and central Asia. Its population is notably abundant in irrigated rice-growing areas, reflecting a consistent increase since 2000, paralleling rice production growth. This mosquito species is a key malaria vector and also poses a potential risk for local filarial infections. Its impact highlights the need for targeted disease management and vector control in affected regions. [27, 35]

Anopheles maculipennis shows zoo-anthropophilic behaviour, and its tendency to blood suck on humans rises in case of inadequate amount of animal source. Its preferred habitat is freshwater. Vegetable and fruit production fields such as rice fields and watermelon fields are suitable habitats for the larvae development. Adult density accumulates in animal barns and chicken farms due to its zoophilic tendency. These animal barns allow resting and blood feeding for the adult mosquitoes. *An. maculipennis* is the main and potential vector of malaria; therefore, it poses high importance in monitoring this species regarding public health concerns. [34] Its distribution in Europe is shown in Figure 8.

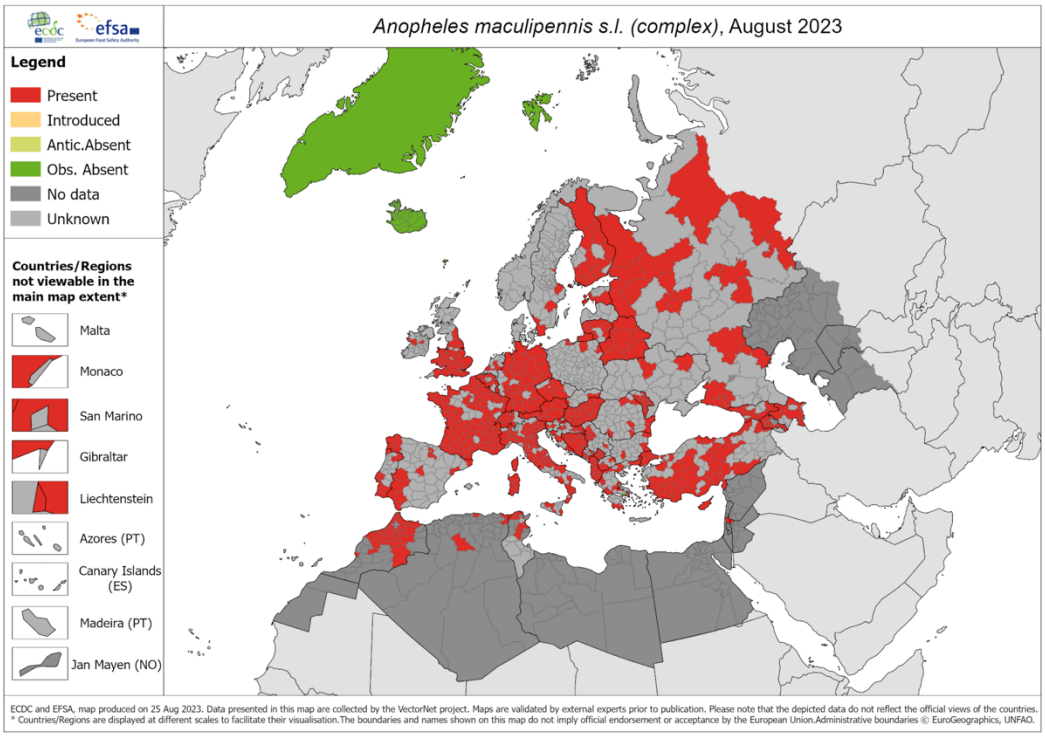

Figure 8.: Current known distribution of Anopheles maculipennis s.l. in Europe. Derived from European Centre for Disease Prevention and Control (ECDC): Mosquito maps [2023].

Coquillettidia richiardii prefers habitats characterised by abundant permanent water sources, such as marshlands and even urban areas. During their immature stages, these mosquitoes are closely associated with host plants

within nutrient-rich and oxygen-depleted aquatic environments, where they attach themselves to the stems and roots of aquatic vegetation. This strategic positioning below the water surface protects them against predators and human larvicidal control efforts. Importantly, *Coquillettidia* mosquitoes are believed to act as vectors for various diseases, including lymphatic filariasis, *Brugia malayi*, Eastern equine encephalitis virus, RVFV, Omsk hemorrhagic fever virus, and WNV, emphasising their significance in public health and epidemiological contexts [17, 25, 27]. Its distribution in Europe is shown in Figure 9.

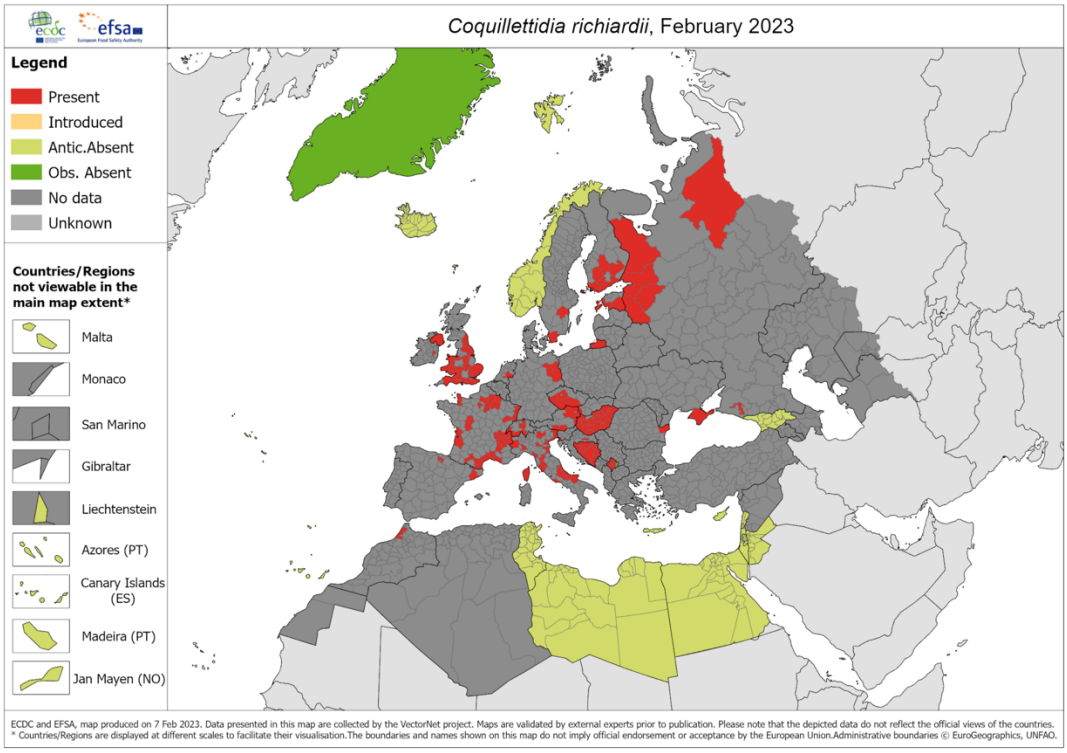

Figure 9.: Current known distribution of Coquillettidia richiardii in Europe. Derived from European Centre for Disease Prevention and Control (ECDC): Mosquito maps [2023].

Ochlerotatus pulcritarsis is a species of western Palaearctic, distributed mainly in the Mediterranean region, and it can be found in many countries in Central Europe. Regarding reproduction, it uses small water-filled cavities in plants, especially tree-holes, for its breeding sites. Unlike many other mosquito species that like to breed in small water hollows, there were no records of *Oc. pulcritarsis* breeding in tyres or road drains. The temperature of water at the

breeding sites should not exceed 21°C. It is anthropophilic and also zoophilic, but having its breeding sites in the woodlands limits its contact with humans [31].

Ochlerotatus caspius, as a Palaearctic species, is distributed in the Mediterranean region, shores of Great Britain, freshwater and salty marshes in the continental part of Europe, Russia, Mongolia, Northern China, Northern Africa, and Asia. It lays eggs in mud along the edges of pools and rivers, 2 cm under the surface and can overwinter in the egg stage. Higher temperature stimulates embryogenesis, while flooding and anoxia stimulate hatching. Adults appear in early April, increasing in number in the summer and decreasing in October–November. It survives in temperatures ranging between 11.5°C and 36°C. *Oc. caspius* has a zoophilic feeding habit, and in the natural population, WNV, TAHV and *Francisella tularensis* were detected [24].

Viruses investigated in the study

West Nile Virus

West Nile Virus (WNV; *Orthoflavivirus nilense*; *Flaviviridae* family, *Orthoflavivirus* genus) is a member of the Japanese encephalitis group of flaviviruses and was initially discovered in 1937 when it was isolated from human encephalitis cases in Uganda, subsequently identified as the causative agent behind the zoonotic illness known as West Nile fever. [37,52] It is considered one of the most widespread flaviviruses distributed in Africa, Asia, Europe, and Australia. In Europe, the first isolation was from ticks in Russia in 1963. In 2021, researchers have classified WNV into nine distinct genetic lineages, with lineages 1 and 2 being pathogenic. Lineage-1 is primarily found in regions such as Africa, Europe, the Middle East, and Australia, with its

emergence in the United States dating back to 1999. [38] In contrast, Lineage-2 viruses have been detected in southern Africa and Madagascar and were introduced in central Europe around 2004–2005. Since then, Lineage 2 strains have spread to countries like Austria and Southern Europe, resulting in the observation that WNV strains affecting humans, horses, birds, and mosquitoes in Europe are largely affiliated with WNV lineage-2. [39] Notably, Hungary has only identified Lineage 2 viruses in samples since 2004.

Birds are the natural hosts of WNV because the virus persists in their bodies for a long time, and they have a lot of it in their bloodstream. Some birds of prey are very sensitive to the infection, with encephalitis as the outcome, having a high chance of dying [40]. Other birds can carry the virus without getting sick, and birds that migrate over long distances seem to help the virus spread far and wide [41]. Among all the potential mosquito species that can carry the virus, *Culex pipiens* (especially its *pipiens* and *pipiens-modestus* hybrid types) and *Culex modestus* are thought to be the primary carriers of WNV in Europe because these mosquitoes feed on both birds and mammals. While *Aedes albopictus* is an invasive mosquito species in Europe and can transmit WNV in the lab, it is considered less important in real-life transmission because it mainly bites mammals and is unlikely to get an infection from birds [42]. WNV spreads when mosquitoes are active (usually between spring and autumn), and the number of human and horse cases tends to be highest between July and September.

Most WNV infections in humans and horses often go unnoticed without any apparent symptoms. Among humans, approximately 20% of cases manifest as West Nile fever (WNF), while less than 1% progress to a more severe condition known as West Nile neuroinvasive disease (WNND). On occasion, the presence of the virus may coincide with other neurological disorders, such as Guillain-Barré syndrome. In parallel, clinical disease in the form of WNND is observed

in roughly 10% of WNV infections in horses [43]. The fatality rate among humans afflicted with WNND can be as high as 17%, whereas a case fatality ratio ranging from 30% to 44% has been documented in horses. In Europe, vaccines for WNV are only available for horses [44,45,46].

Usutu virus

The Usutu virus (USUV; *Orthoflavivirus usutuense*, *Flaviviridae* family, *Orthoflavivirus* genus), originally isolated in South Africa back in 1959, found its way to Europe in 1996, triggering a rapid expansion in its geographic distribution [52]. To date, researchers have identified eight distinct genetic lineages of the virus, with three categorised as “African” and five as “European” . Like WNV, the enzootic transmission cycle of the Usutu virus involves *Culex* mosquitoes as vectors and birds as amplifying reservoir hosts, while humans and other mammals are most likely dead-end hosts [47,48].

In Europe, the Usutu virus has notably impacted bird populations, particularly affecting species like blackbirds (*Turdus merula*) and great grey owls (*Strix nebulosa*). Susceptibility to the infection has been confirmed through serological testing or virus detection in various animals, including rodents, squirrels, wild boar, deer, dogs, horses, and bats. Although the number of human cases remains relatively low, with most infections displaying no symptoms, a few cases of neurological complications, such as encephalitis or meningoencephalitis, have been reported in healthy individuals and those with lowered immune systems [49,50,51].

Materials and methods

In this study, the mosquito collection took place from May to November 2022. The samples were collected in 33 settlements located in 14 out of the 19 counties of Hungary, as summarised in table 1. The locations of the hot-spot sampling are indicated in red.

County	Settlement	WNV case
Bács-Kiskun	Dunavecse	
	Harta	
Békés	Battonya	
	Kardoskút	
Borsod-Abaúj-Zemplén	Szerencs	human
Csongrád-Csanád	Mórahalom	human
	Szeged	human
	Szentes	human
Fejér	Dunaújváros	
	Kisapostag	
	Székesfehérvár	
	Tatabánya	horse, blackbird
Győr-Moson-Sopron	Győr	penguin, human
	Jánossomorja	human
Hajdú-Bihar	Debrecen	
Heves	Eger	human
Komárom-Esztergom	Lábatlan	
Pest	Budaörs	
	Budapest	
	Domonyvölgy	horse
	Dunakeszi	human
	Göd	
	Szentendre	
	Vác	human
	Vácrátót	
	Veresegyház	
Somogy	Marcali	
Szabolcs-Szatmár-Bereg	Nyíregyháza	human
	Tiszabercel	human
Tolna	Dunaföldvár	
	Paks	
	Szekszárd	
Veszprém	Balatonalmádi	

I. Table Locations in Hungary where the samples were collected. The settlements wher hot-spot sampling was conducted are indicated in red.

Mosquito samples were collected in zoos (Győr, Szeged, Nyíregyháza, Debrecen), national parks (Lajta-Hanság National Park and Körös-Maros National Park) and urban environments. Among the locations, permanent traps were operated continuously or intermittently in Budapest, Vác, Vácrátót and Debrecen, Kardoskút, and the Körös-Maros National Park, while in the other premises, one sample was taken either on the basis of a public report on the detection of the presence of invasive mosquitoes or because in cooperation with the animal health and human epidemiology authorities, we conducted a targeted, so-called "hot spot" survey related to the reported cases of horse or human West Nile fever. The sampling in Kardoskút was conducted at red-footed falcon (*Falco vespertinus*) and common kestrel (*Falco tinnunculus*) nesting sites.

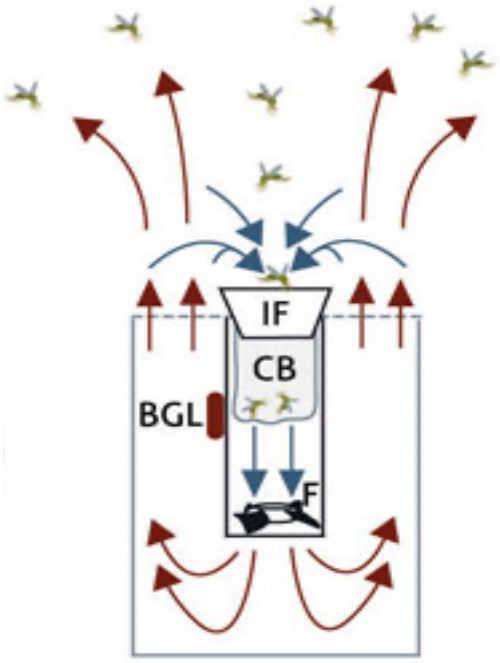

Figure 10.: The functioning of the BG sentinel trap used in the study. Source: www.biogents.com; date of acc ession: 01. 11. 2023.

Mosquitoes were trapped using BG-Sentinel traps that emit both CO_2 gas and human scents, the working principle of which is illustrated in Error! Reference source not found.: the airflow maintained by the fan placed in the trap inhales

the mosquitoes attracted by the CO_2 and scents into the IF (intake funnel) through an opening, through which they enter the collection net marked CB (catch bag). Due to the airflow and the design of the IF inlet, mosquitoes cannot fly out of the trap (https://eu.biogents.com/capture-method/). The net was emptied every 24-48 hours by the handling staff. The mosquitoes were euthanised by placing them at -20 ℃, and then, marking the place and time of collection, the samples were stored frozen until taxonomic identification.

The identification was carried out by dipterologist Dr. Zoltán Soltész (ELKH Ecological Research Center, Lendület Ecosystem Service Research Group) using a microscopic examination based on morphological features characteristic of mosquitoes. During the taxonomic identification, the collected mosquitoes were sorted into samples (pools) according to the place and time of trapping, as well as species, sex, and the blood content of the abdomen. A maximum of 20 mosquitoes were placed in a pool, and female mosquitoes with blood-filled abdomens were processed as individual samples.

After the identification, the numbered samples in the microcentrifuge tubes were stored at -80 ℃ until the beginning of the tests. Prior to the nucleic acid purification, sterile PBS (phosphate buffered saline) was measured into the microcentrifuge tubes containing the mosquitoes. In the case of female mosquitoes with a belly containing blood forming a unique sample, and if the tube contained for other reasons (e.g. there was only one specimen of the given species among the mosquitoes caught in the trap at the given collection location and time) there was only one mosquito, 300 µl, if there were more (maximum 20) individuals in the tube, 500 µl of PBS was added into the tube. The mosquitoes were homogenised with a Qiagen TissueLyser LT device: a sterile metal ball was placed in the tube containing the sample, and during shaking at a frequency of 50 Hz for 3 minutes, the mosquito bodies were destroyed to the

extent necessary for nucleic acid purification. Afterwards, the samples were centrifuged at 3060 × g for 10 minutes, and viral RNA was purified from the supernatant using the QIAmp Viral RNA Mini Kit (QIAgen, cat. no.: 52906) according to the manufacturer's instructions.

Viruses were detected by multiplex real-time reverse transcription polymerase chain reaction (qRT-PCR). The composition of the reaction mixture using the Takyon™ One-Step No Rox Probe 5X MasterMix dTTP (Eurogentec, cat. no.: UF-NP5X-RT0501) is listed in Table 2.

Takyon 1-step 5×MM	15 µl/tube
Components	µl
RNase & Dnase-free water	6,675
5×MasterMix	3,000
Enzyme mix	0,150
Additive	0,150
Primer F+R (WNV) 10 pM	1,200
Primer F+R (USUV) 10 pM	1,350
Probe (WNW L1 m3) 10 pM	0,300
Probe (WNW L2 m3) 10 pM	0,300
Probe (USUV) 10 pM	0,375
RNA	1,5

II. Table qRT-PCR reaction mixture for the multiplex detection of WNV Lineage 1, WNV Lineage 2 and USUV in the investigated samples.

The primers used in the reactions are based on methods published by Del Amo et al., 2013 and Cavrini et al., 2011 [53,54]. The primers detecting WNV lineage 1 and lineage 2 strains were slightly modified to make them more specific for the virus strains present in Hungary. In our tests, WNV lineage 1, WNV lineage 2, and USUV virus strains were detected in the same reaction in the same sample, thus, we were able to filter the collected samples in a shorter

time and with the use of fewer reagents. The sequences of the primers and probes are shown in Table 3.

WNV	Primer F	5'-GTG ATC CAT GTA AGC CCT CAG AA-3'
	Primer R	5'-TTT GCC TTT GTT AAC CCA GTC C-3'
	Lineage 1 probe	5'-/6FAM/AGG A+ CC + CCA + CAT + GTT/3IABkFQ/-3'
	Lineage 2 probe	5'-/5HEX/AGG+ ACC C + CA CGT + GCT/3IABkFQ/-3'
USUV	Primer F	5'—AAA AAT GTA CGC GGA TGA CAC A-3'
	Primer 2	5'-TTT GGC CTC GTT GTC AAG ATC—3'
	Probe	5'-/CY5/CGG CTG GGA CAC CCG GAT AAC C/BHQ-2/-3'

II. Table: Primers and probes used in the qRT-PCR reactions.

The temperature protocol of the reactions was the following: a reverse transcription (RT) step at 48 ℃ for 30 minutes, an activation step in which the RT enzyme was deactivated and the polymerase enzyme was activated at 95 ℃ for 3 minutes, that was followed 50 cycles of dehydration (separation of DNA strands) at 95 ℃ for 15 seconds and a combined annealing-extension step at 60 ℃ for 1 minute. The detection of the fluorescent emission was conducted during the annealing-extension step in each cycle. Based on the sensitivity tests conducted by the workgroup, samples with Ct lower than 36 were considered positive. For positive controls, RNA extracted from WNV Lineage 1, WNV Lineage 2 and USUV isolates were used. In the no-template negative control, instead of RNA, RNase & DNase-free water was added to the tube.

Results

Places

In the sampling sites all around Hungary (with the exception of the Kardoskút sampling site), altogether 1198 pools of mosquitoes were collected, of which 1056 contained female mosquitoes.

Species	Pools with no blood in the belly	Pools with blood in the belly	WNV lineage 2 positive samples	USUV positive samples
Aedes albopictus	112	12	0	0
Aedes koreicus	36	2	0	0
Aedes japonicus	2	0	0	0
Aedes vexans	58	3	0	0
Aedes cinereus/geminus	1	0	0	0
Anopheles maculipennis	5	1	0	0
Anopheles plumbeus	6	0	0	0
Coquillettidia richiardii	14	2	0	0
Culex hortensis	1	0	0	0
Culex modestus	4	1	0	0
Culex pipiens	608	143	6	9
Culiseta annulata	8	0	0	0
Ochlerotatus annulipes	1	0	0	0
Ochlerotatus caspius	5	0	0	0
Ochlerotatus dorsalis	13	0	0	0
Ochlerotatus geniculatus	15	0	0	0
Ochlerotatus pulchritarsis	2	0	0	0
Urotaenia unguiculata	1	0	0	0

IIIV. Table Results of the mosquito sampling collected in Hungary (except Kardoskút)

The following results are summarised in Table 4. The pools contained individuals of 18 species, i.e. *Aedes albopictus, Aedes koreicus, Aedes japonicus, Aedes vexans, Aedes cinereus/geminus, Anopheles maculipennis,*

Anopheles plumbeus, Coquillettidia richiardii, Culex hortensis, Culex modestus, Culex pipiens, Culiseta annulata, Ochlerotatus annulipes, Ochlerotatus caspius, Ochlerotatus dorsalis, Ochlerotatus geniculatus, Ochlerotatus pulchritarsis and *Urotaenia unguiculata*. WNV Lineage 2 (6 pools) and USUV (9 pools) positive samples were found only among the empty-bellied *Culex pipiens* pools.

Among the positive samples, some were collected in 2 permanent traps in Vác (2 samples collected in July containing USUV) and Debrecen (1 sample collected in July and 4 samples collected in August containing USUV, 2 samples containing WNV Lineage 2 viruses, 1 collected in July, 1 collected in August). The other positive samples were collected in the „hot spot" samplings, i.e. at places where human or horse WNV infections were reported: USUV was detected in Győr Zoo (1 sample), WNV Lineage 2 virus was detected in Szeged Zoo (1 sample), in Jánossomorja 1 sample was positive for USUV and another one for WNV Lineage 2, and in Mórahalom, 2 WNV Lineage positive samples were collected.

Among the 1018 pools of mosquitoes collected in Kardoskút, 961 contained female animals. The samples contained individuals of 10 species, i.e. *Aedes vexans, Anopheles hyrcanus, Anopheles maculipennis, Coquillettidia richiardii, Culex modestus, Culex pipiens, Culiseta annulata, Culiseta longioreolata, Ochlerotatus dorsalis* and *Phlebotomus*. Viruses were detected only in *Culex pipiens*: 11 samples contained WNV Lineage 2, and 9 samples contained USUV strains. The results are summarised in Table 5, where only the female mosquitoes are listed.

Species	Pools with no blood in **the** belly	Pools with blood in **the** belly	WNV lineage 2 positive samples	USUV positive samples
Aedes vexans	4	1	0	0
Anopheles hyrcanus	1	0	0	0
Anopheles maculipennis	10	6	0	0
Coquillettidia richiardii	27	5	0	0
Culex modestus	3	0	0	0
Culex pipiens	584	241	11	9
Culiseta annulata	1	3	0	0
Culiseta longioreolata	1	0	0	0
Ochlerotatus dorsalis	49	8	0	0
Phlebotomus	7	0	0	0

IV. Table Results of the mosquitoes collected in Kardoskút.

Discussion

Human WNV infections in Hungary were reported between W34 (22 August) – W42 (18 October) in 2022. Because of financial and HR reasons, for the Mosonmagyaróvár case, the mosquito collection was conducted in Győr, where in the Zoo animal cases were detected and in Jánossomorja, where we had a cooperation with the Lajta-Hanság National Park. In Kőkút and Erdőkertes, the mosquito collection was not performed. WNV was found in mosquitoes collected in Jánossomorja, Szeged and Mórahalom, while, despite the human WNV infection, in Vác, only USUV was detected in the mosquito. This latter result might be explained by the information that the human patient was a refugee from Ukraine, so the infection might have been imported from abroad and not locally acquired.

Animal cases were reported in the same period in Tatabánya, Domonyvölgy, where horses were detected WNV positive; Győr, where a penguin at the Zoo was found WNV positive; and Tata, where a dead blackbird was sent to the laboratory that found to be USUV positive. As Tata is quite near to Tatabánya, the mosquito collection was conducted only in Tatabánya. Mosquito collection was conducted in Domonyvölgy despite the suspicion that the infection was imported from abroad, as the horse which showed clinical signs was transported from Italy about a week before. In samples collected in Győr Zoo, USUV was detected; in the mosquitoes sampled in Jánossomorja, WNV and USUV were detected. The mosquitoes collected in Tatabánya and Domonyvölgy flaviviruses were not detected. Altogether, we found that hot-spot sampling is important not only to have positive samples but also for the confirmation of the presence of the viruses in the vectors, thus providing data on the epidemiology of the investigated flaviviruses. However, for more detailed information, if possible, the sampling should be collected at the place where the human or animal infections were reported, and the timing is

important, i.e. within a few weeks after the report, the traps should be placed at the sites, especially considering the incubation time and the period necessary to the laboratory confirmation of the human or animal infection.

From the 1198 pools of mosquitoes from all around Hungary except the Kardoskút sampling site, 6 pools of positive samples for WNV lineage 2 and 9 pools of positive samples for USUV were found in 2022. From the 1018 pools of mosquito samples from Kardoskút, where the mosquitoes were collected exclusively from nesting places of common kestrel and red-footed falcon, 11 samples contained WNV lineage 2 and 9 samples contained USUV strain. Due to the endemic presence of WNV and USUV in the European Union, numerous European nations are prioritising the surveillance of arbovirus vector mosquitoes as a crucial measure to investigate viral activity. The reported WNV case numbers are summarised in Table 6.

Disease/year	2017	2018	2019	2020	2021	2022
West Nile Fever EU human	204	1605	463	336	164	1133
West Nile Fever HU human	21	215	36	3	7	14
West Nile Fever EU horse	127	285	93	183	43	101
West Nile Fever HU horse	3	91	7	1	3	3
West Nile Fever EU bird	n.d.	n.d.	54	2	8	323

VI. Table the number of West Nile Fever reported in EU/EAA countries and Hungary (HU). Data were retrieved from the ECDC website on 3 November 2023. (https://www.ecdc.europa.eu/en/mosquito-borne-diseases) The highest reported case numbers between 2017 and 2022 are indicated in red.

Current distribution of human and animal WNV cases reported in Europe is shown in Figure 11.

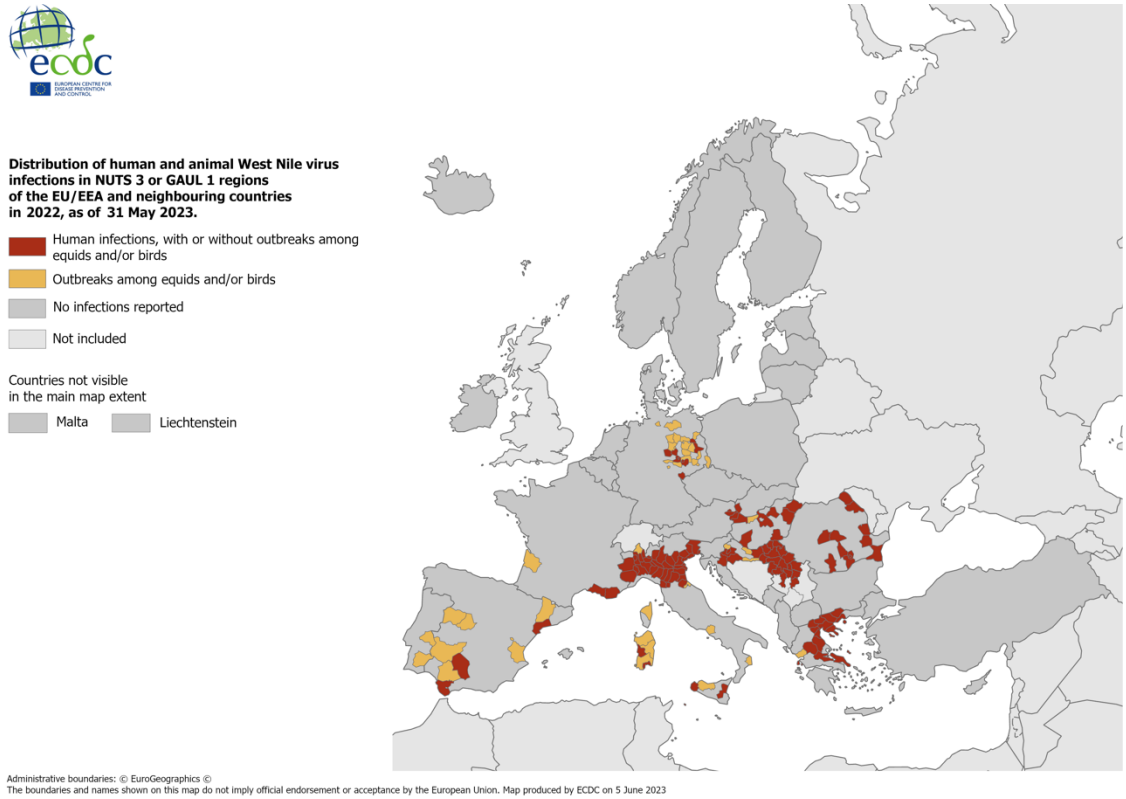

Figure 11.: Current distribution of human and animal West Nile Virus infections in Europe. Derived from ECD C on 04. 11. 2023.

In Northern Italy, where human cases of USUV were reported in 2009, a regional surveillance program for WNV was initiated in 2008. This program is primarily centred around monitoring mosquitoes and screening wild birds. Three polymerase chain reactions (PCR) tests were employed for Flavivirus, WNV, and USUV. Throughout the summer of 2009, the surveillance reported that, out of 1,789 mosquito pools tested, 56 pools (54 consisting of *Culex pipiens* and 2 of *Aedes albopictus*) were positive for USUV, while 27 pools (all *Culex pipiens*) tested positive for WNV. Furthermore, among the 1,218 wild birds tested, 44 were found to be WNV-positive, while 11 birds were USUV-positive [55].

In the Emilia-Romagna Region of Italy in 2010, researchers conducted surveys monitoring mosquito and bird samples. A total of 438,558 mosquitoes, organised into 3,111 pools, were examined, along with 1,276 birds, including 1,130 actively sampled birds and 146 from passive surveillance. The biomolecular analysis revealed the presence of WNV in three *Culex pipiens* pools, while USUV was identified in 89 *Culex pipiens* pools and 2 *Aedes albopictus* pools. In addition, two birds were confirmed as WNV-positive, and 12 were found to be USUV-positive. Notably, there were no reported human WNV cases in the region in 2010 [56].

In 2018, Italy, along with several other European countries, experienced a resurgence of viral circulation, resulting in an increased number of human cases of WNV. During this critical period, 385,293 mosquitoes belonging to 13 different species were sampled from 4 June to 25 October. The mosquitoes, primarily of the *Culex* genus, were organised into 2,337 pools for testing, revealing that 232 of these pools were positive for lineage 2 of WNV. It is worth noting that the majority of the tested pools, and particularly those that tested positive, were composed of *Culex pipiens*. A significant relationship was observed between the rate of infected mosquitoes collected in each province and the incidence of West Nile Neuroinvasive Disease (WNND) cases in the same province [57].

Within the central region of Italy, surveillance was conducted across four municipalities in 657 equine holdings. Through a combination of direct and indirect diagnostic methods, the investigation unveiled eight confirmed cases of WNV in horses, each originating from distinct equine establishments. These cases emerged from a total of 193 equids tested. Simultaneously, mosquito surveillance played a pivotal role in these investigations, where a total of 2,367 specimens of *Culex pipiens* mosquitoes were collected. These mosquitoes were categorised into 56 distinct pools and tested using reverse transcription-

polymerase chain reaction (RT-PCR) aimed at detecting the presence of WNV and USUV. Notably, among the 56 pools subjected to analysis, 17 gave positive test results for USUV, originating from three distinct collection sites. The Minimum Infection Rate (MIR) across the tested mosquito pools was 0.72%. However, WNV RNA was not detected within any of the tested mosquito pools. Additionally, during the same year, an analysis was conducted on a total of 4,611 and 4,278 human blood donations within the provinces of Roma and Latina, respectively. This collective analysis covered a broad region, totalling 31,970 individuals living in Lazio. The findings of this screening confirmed the presence of two asymptomatic donors who tested positive in the West Nile Virus Nucleic Acid Testing (NAT) screening examination [58].

Researchers conducted a serosurvey to evaluate the presence of WNV and USUV in captive birds and mammals within a zoo located in the southern region of France. This geographical area has been documented as a hot spot for the circulation of these two viruses. Over the course of 16 years, from 2003 to 2019, a dataset was put together, comprising a total of 411 samples obtained from 70 different species. Notably, the seroprevalence of USUV in birds exceeded that of WNV by a factor of ten, with respective rates of 14.59% and 1.46%. Within the avian population, species such as the greater rhea (*Rhea Americana*) and common peafowl (*Pavo cristatus*) had the highest USUV seroprevalence. The instances of infection were concentrated between the years 2016 and 2018, correlating with a period marked by heightened viral circulation across Europe [59].

In a parallel study, researchers evaluated WNV and USUV prevalence through a repeated cross-sectional investigation. This assessment incorporated serological and molecular analyses, taking samples from humans, dogs, horses, birds, and mosquitoes within the Camargue region, including the city of Montpellier. The study spanned the years from 2016 to 2020. The findings

showed active transmission of both viruses within the region, with notably higher prevalence rates of USUV observed in humans, dogs, birds, and mosquitoes. In contrast, WNV prevalence exhibited a greater occurrence among the equine population. Among the 500 human samples analysed, 15 were found to be positive for USUV, while 6 tested positive for WNV [60].

In the central region of the Netherlands, in August 2020, the first local detection of WNV in a common whitethroat (*Curruca communis*) was detected. The first human WNV case without a recent travel abroad record followed a few months later. Also, in July and August 2020, further investigation analysed the cases of unknown neuroinvasive diseases, which led to the detection of more human WNV cases in the Netherlands [61].

Analysing the previous investigations done in Italy, Southern France, and the Netherlands, a clear positive correlation is observed between the mosquito positivity to WNV strains with human (WNND) and horse outbreaks of WNV infections. The WNF outbreak in horses appears to have a relatively higher number of positive cases compared to the outbreaks in humans.

In Germany, a study collected over 600 dead birds from 2011 to 2013 to check the presence of USUV since the occurrence of USUV in wild birds in June 2011. Infected blackbirds (*Turdus merula)* were frequently found dead in southwest Germany, and other bird species in the same region were found affected. The study revealed 209 positive cases from the collected samples, and 88% of them were blackbirds [62].

Between 2014 and 2016, an extensive collection of more than 1,900 wild bird blood samples, representing 20 different orders and 136 distinct bird species, was assembled for WNV and USUV investigation. These samples underwent real-time PCR specific for WNV and USUV, coupled with differentiating virus

neutralisation tests. While WNV-specific RNA remained absent, there was a discovery of four wild bird blood samples tested positive for USUV-specific RNA. Furthermore, the year 2016 performed a surveillance of deceased birds, identifying 73 USUV-positive birds, while no instances of WNV-specific RNA were detected either in wild birds or mosquitoes during this period [63].

In the year 2018, Germany experienced an exceptionally hot and dry summer, ranking as the second hottest and driest on record. These extraordinary climatic conditions likely catalysed the expanded range and efficient spread of the zoonotic arthropod-borne WNV across numerous Southern, Southeastern, and even Central European nations. The markedly elevated temperatures in 2018 were instrumental in reducing the averaged extrinsic incubation period values for WNV in mosquitoes. This, in turn, facilitated accelerated virus amplification and increased the risk of transmission to vertebrate hosts within the German landscape. The climatic conditions of the year played a pivotal role in shaping the dynamics of this arboviral infection [64].

More than a seven-fold increase in the number of cases was observed in the EU and EU neighbouring countries in 2018 compared to 2017; this number exceeds the total number of WNV cases from the past seven years. Hungary was affected by the epidemic of 2018 as well. During the transmission season between 2014 and 2017, 80 human patient cases were reported, and 32 of them (40%) were positive. In 2018, a total of 225 cases were reported to ECDC, and 53 patients were positive using PCR. The positive cases were further investigated with RT-PCR and sequencing and confirmed that 46 (27.7%) cases were positive. From this, a 1.4-fold increase in PCR-positive cases is observed within a single transmission period compared to the total number of cases from the previous four years. These results stay in line with the serological data increasing by ninefold in the number of human cases [65].

In 2019, a research study was conducted in the Republic of Korea (ROK) to investigate the monitoring of arbovirus vector mosquitoes. The study found that *Aedes vexans nipponii* was the most frequently collected mosquito species, accounting for 56.5% of the specimens, followed by *Ochlerotatus dorsalis* (23.6%), *Anopheles spp.* (10.9%), and *Culex pipiens* complex (5.9%). In rural areas of Hwaseong, *Aedes vexans nipponii* had the highest population at 62.9%, followed by *Ochlerotatus dorsalis* (23.9%) and *Anopheles spp.* (12.0%). In another rural region in Incheon, which is a habitat for migratory birds, *Culex pipiens complex* was the most prevalent species, representing 31.4% of the population, followed by *Ochlerotatus dorsalis* (30.5%) and *Aedes vexans vexans* (27.5%). In urban regions, the *Culex pipiens complex* dominated with 84.7% of the mosquito population. Additionally, three out of the 2,683 pools tested positive for Culex flaviviruses (CxFV). These positive samples were obtained from *Culex pipiens pallens* collected at the habitats for migratory birds in Incheon [66].

Culex flavivirus (CxFV), distributed worldwide, is found in mosquitoes of the *Culex* genus. In acase-control study conducted in Chicago, United States, a notable connection was observed between CxFV and WNV infection. The research indicated that *Culex* mosquito pools that tested positive for WNV were approximately four times more likely to have CxFV when compared to the pools that were negative for WNV. In the research, 6 out of 15 (40%) individual mosquitoes that were confirmed as WNV-positive were also identified as CxFV-positive [67]. This observation highlights the potential for both viruses to co-infect mosquitoes within their natural habitats and poses greater significance in the findings of the positive cases of CxFV in 2019 in ROK, relating to the potential presence of WNV.

In 2020, another study focused on monitoring flavivirus infection in mosquitoes. The researchers collected a total of 67,203 mosquitoes at 36 collection sites

within 30 urban regions and migratory bird habitats. The predominant mosquito species were the *Culex pipiens* complex, *Armigeres subalbatus*, *Aedes albopictus, Aedes vexans*, and *Culex tritaeniorhynchus.* These mosquitoes were grouped into 4953 pools for the purpose of monitoring flavivirus infection. The study revealed a minimum infection rate of 0.01%. Notably, the Japanese encephalitis virus (JEV) was detected in seven pools of *Culex orientalis* from Sangju, and JEV was isolated from two of these pools. Phylogenetic analysis confirmed that all detected JEV belonged to genotype V, marking the first instance of genotype V JEV isolation from Culex orientalis in the Republic of Korea. To date, there have been no indigenous cases of other flaviviral diseases, such as West Nile or Yellow fever [68].

In European nations, including Hungary and Italy, *Culex pipiens* constituted the majority of the mosquito samples collected, with a smaller number of *Ae. albopictus* pools were also gathered for examination. Conversely, in the Republic of Korea, the composition of mosquito samples exhibited distinct variations based on location. In rural areas, *Aedes vexans* emerged as the dominant mosquito species, whereas in urban regions, *Culex pipiens complex* accounted for a significant 84% of the samples. Additionally, in the habitat of migratory birds, the *Culex pipiens complex* took 31% of the mosquito population.

In Italy and Hungary, WNV and USUV strains were primarily detected from *Culex pipiens* mosquitoes. Aligning with this data, in Korea, Culex flavivirus (CxFV) was detected in *Culex pipiens* collected from the migratory bird habitat, while JEV was identified in *Culex orientalis* mosquitoes. These findings pose significance in the diverse mosquito species and their role in the transmission of these arboviruses in different regions.

In the year 2022, a significantly high number of WNV cases were reported across several European countries, with Italy leading the count at 723 cases, followed by Greece with 283 cases. Romania reported 47 cases, while Germany had 16 cases, and Hungary reported 14 cases. Croatia recorded 8 cases, Austria had 6, France reported 6, in Spain 4 cases were detected, and in Slovakia 1 case was revealed, as reported to the European Centre for Disease Prevention and Control (ECDC).

Notably, Italy had the highest number of outbreak cases, followed by Greece. Hungary had a considerably low number of cases, but higher than Croatia, Austria, France, Spain, and Slovakia. The year 2022 was remarkable, with the highest number of WNV outbreaks reported since 2018. It is notable that in 2018, Hungary detected a substantial number of human and horse cases, aligning with the total number of outbreaks recorded in the EU. However, in 2022, Hungary's count of human and horse cases was comparatively lower despite the increased total number of outbreaks across the EU.

This study has provided valuable insights into the complex relationship between climate, the environment, and globalisation with respect to mosquito populations. It highlighted how alterations in temperature and humidity can drive mosquito migration to new regions. Given their role as key vectors for arboviruses, mosquitoes hold immense significance in the introduction, spread, and outbreak of these arbovirus diseases. Additionally, the movement of migratory birds and travelling humans plays an essential role in the dynamics of virus outbreaks. I understood the necessity of closely monitoring various factors such as global industry, trade, global warming, migrating birds, and the distribution of mosquito species. These components are intricately connected and collectively apply a profound influence on the outbreaks of diseases caused by arboviruses.

Conducting a comprehensive investigation of mosquito samples across Hungary, including the nesting places of migratory birds, was essential for gaining crucial data to monitor and predict WNV and USUV virus activity. Furthermore, an intensive collection of human blood donations, horse samples, and dead bird samples suggests a significant enhancement of the sensitivity of virus detection within the country. Organising further research and study on actively improving the investigation method for this subject cannot be overstated and, therefore, should be continuously supported by the responsible parties.

Summary

Globalization and climate change contributing to the migration of invasive mosquitoes into Europe highlights the importance of monitoring the mosquito borne arbovirus disease activity in the region. This study aimed to collect mosquitoes in several places in Hungary during the 2022 mosquito season, gathering information on the prevalence of invasive species and, by qRT-PCR, detect mosquito-transmitted viruses that are already known to be present in Europe.

The mosquito collection was conducted in Győr, where in the Zoo animal cases were detected and in Jánossomorja, with the Lajta-Hanság National Park. WNV was found in mosquitoes collected in Jánossomorja, Szeged and Mórahalom, while in Vác, only USUV was detected in the mosquito despite the human WNV infection. This may be explained with the information that the patient was from abroad, so the infection might have been imported instead of acquiring locally. From the 2216 pools of mosquito collected from Hungary, 17 pools of positive samples for WNV lineage 2 and 18 pools of positive samples for USUV were found in 2022.

The conclusion drawn from comparing our research results with those of other groups suggests the need for a more organised approach to mosquito trapping. It emphasises the importance of increasing sampling intensity. The surveillance efforts should not only focus on vectors but also include active monitoring in amplifying hosts and dead-end hosts. The One Health approach is recommended, encouraging collaboration among experts from various fields such as entomologists, ornithologists, veterinarians, medical doctors, hunters, and members of nature conservation organisations. Furthermore, international cooperation in conducting flavivirus mornitoring is recommended.

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References

1. Vogels CBF, Hartemink N, Koenraadt CJM (2017) Modelling West Nile virus transmission risk in Europe: effect of temperature and mosquito biotypes on the basic reproduction number. In: Scientific Reports. https://doi.org/10.1038/s41598-017-05185-4
2. Gomes B, Sousa CA, Novo MTLM, Freitas FB, Alves RR, Côrte-Real AR, Salgueiro P, Donnelly MJ, Almeida A, Pinto J (2009) Asymmetric introgression between sympatric molestus and pipiens forms of Culex pipiens (Diptera: Culicidae) in the Comporta region, Portugal. In: BMC Evolutionary Biology. https://doi.org/10.1186/1471-2148-9-262
3. Becker N, Petric D, Zgomba M, Boase C, Madon M, Dahl C, Kaiser A (2010) Mosquitoes and Their Control. https://doi.org/10.1007/978-3-540-92874-4
4. Dehghan H (2010) The Morphological Variations of Culex pipiens Larvae (Diptera: Culicidae) in Yazd Province, Central Iran. In: PubMed Central (PMC). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3385558/
5. Khoobdel M, Keshavarzi D (2020) Survey of Usutu Virus Potential Vectors and their Diversity in Iran: A Neglected Emerging Arbovirus. In: The Open Public Health Journal. https://doi.org/10.2174/1874944502013010114
6. Günay F, Alten B, Şimşek FM, Aldemır A, Linton Y (2015) Barcoding Turkish Culex mosquitoes to facilitate arbovirus vector incrimination studies reveals hidden diversity and new potential vectors. In: Acta Tropica. https://doi.org/10.1016/j.actatropica.2014.10.013
7. Boukraa S, Dekoninck W, Versteirt V, Schaffner F, Coosemans M, Haubruge E, Francis F (2015) Updated checklist of the mosquitoes (Diptera: Culicidae) of Belgium. Journal of Vector Ecology 40:398-407 . https://doi.org/10.1111/jvec.12180

8. Balenghien T, Vazeille M, Reiter P, Schaffner F, Zeller H, Bicout Dj (2007) Evidence of laboratory vector competence of *Culex modestus* for West Nile Virus. Journal of the American Mosquito Control Association 23:233–236

9. Golding N, Nunn MA, Medlock JM, Purse BV, Vaux AG, Schäfer SM (2012) West Nile virus vector Culex modestus established in southern England. Parasites & Vectors 5:. https://doi.org/10.1186/1756-3305-5-32

10. Molaei G, Andreadis TG (2006) Identification of Avian- and Mammalian-Derived Bloodmeals in Aedes vexans and Culiseta melanura (Diptera: Culicidae) and Its Implication for West Nile Virus Transmission in Connecticut, U.S.A. Journal of Medical Entomology 43:1088–1093 . https://doi.org/10.1093/jmedent/43.5.1088

11. Vaux AGC, Watts D, Findlay-Wilson S, Johnston C, Dallimore T, Drage P, Medlock JM (2021) Identification, surveillance and management of Aedes vexans in a flooded river valley in Nottinghamshire, United Kingdom. Journal of the European Mosquito Control Association 39:15–25 . https://doi.org/10.52004/jemca2021.0001

12. Kinley JR (2008) Studies on the vector competence of a Mid-western strain of Aedes vexans (Meigen) (Diptera: Culicidae) for West Nile Virus. https://doi.org/10.31274/rtd-180813-16551

13. Medlock JM, Vaux AGC (2015) Seasonal dynamics and habitat specificity of mosquitoes in an English wetland: implications for UK wetland management and restoration. Journal of Vector Ecology 40:90–106 . https://doi.org/10.1111/jvec.12137

14. Uusitalo R, Siljander M, Culverwell CL, Hendrickx G, Lindén A, Dub T, Aalto J, Sane J, Marsboom C, Suvanto MT, Vajda A, Gregow H, Korhonen EM, Huhtamo E, Pellikka P, Vapalahti O (2021) Predicting Spatial Patterns of Sindbis Virus (SINV) Infection Risk in Finland

Using Vector, Host and Environmental Data. International Journal of Environmental Research and Public Health 18:7064 . https://doi.org/10.3390/ijerph18137064

15. Marcell Sáringer-Kenyeres, Sándor Tóth, Zoltán Kenyeres (2018) Journal of the European Mosquito Control Association . Updated checklist of the mosquitoes (Diptera: Culicidae) of Hungary Vol. 36:

16. Sivan A, Shriram AN, Sunish IP, Vidhya PT (2015) Host-feeding pattern of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) in heterogeneous landscapes of South Andaman, Andaman and Nicobar Islands, India. In: Parasitology Research. https://doi.org/10.1007/s00436-015-4634-5

17. Hawley WA, Pumpuni CB, Brady RH, Craig GB (1989) Overwintering Survival of Aedes albopictus (Diptera: Culicidae) Eggs in Indiana. Journal of Medical Entomology 26:122–129 . https://doi.org/10.1093/jmedent/26.2.122

18. Roiz D, Vázquez A, Seco MPS, Tenorio A, Rizzoli A (2009) Detection of novel insect flavivirus sequences integrated in Aedes albopictus (Diptera: Culicidae) in Northern Italy. In: Virology Journal. https://doi.org/10.1186/1743-422x-6-93

19. Marini G, Arnoldi D, Baldacchino F, Capelli G, Guzzetta G, Merler S, Montarsi F, Rizzoli A, Rosà R (2019) First report of the influence of temperature on the bionomics and population dynamics of Aedes koreicus, a new invasive alien species in Europe. In: Parasites & Vectors. https://doi.org/10.1186/s13071-019-3772-5

20. Negri A, Arnoldi I, Brilli M, Bandi C, Gabrieli P, Epis S (2021) Evidence for the spread of the alien species Aedes koreicus in the Lombardy region, Italy. In: Parasites & Vectors. https://doi.org/10.1186/s13071-021-05031-7

21. Huber K, Pluskota B, Jöst A, Hoffmann KH, Becker N (2012) Status of the invasive speciesAedes japonicus japonicus(Diptera: Culicidae)

in southwest Germany in 2011. In: Journal of Vector Ecology. https://doi.org/10.1111/j.1948-7134.2012.00252.x

22. Bova J, Soghigian J, Paulson SL (2019) The Prediapause Stage of Aedes japonicus japonicus and the Evolution of Embryonic Diapause in Aedini. In: Insects. https://doi.org/10.3390/insects10080222

23. Cunze S, Koch L, Kochmann J, Klimpel S (2016) Aedes albopictus and Aedes japonicus – two invasive mosquito species with different temperature niches in Europe. In: Parasites & Vectors. https://doi.org/10.1186/s13071-016-1853-2

24. Milankov V (2009) Taxonomy, biology, genetic variability and medical importance of Ochlerotatus caspius (Pallas, 1771) and O. dorsalis (Meigen, 1830) (Diptera: Culicidae). https://www.semanticscholar.org/paper/Taxonomy%2C-biology%2C-genetic-variability-and-medical-Milankov-Petri%C4%87/609eca53451273f9c2ce6382e6ac09bf777681ac

25. Medlock JM, Snow KR, Leach S (2005) Potential transmission of West Nile virus in the British Isles: an ecological review of candidate mosquito bridge vectors. Medical and Veterinary Entomology 19:2-21 . https://doi.org/10.1111/j.0269-283x.2005.00547.x

26. Merdić E (2008) Distribution of the Annulipes group (Diptera, Culicidae) OF EASTERN CROATIA. https://www.semanticscholar.org/paper/distribution-of-the-annulipes-group-(Diptera%2C-OF-Merdi%C4%87-Jeli%C4%8Di%C4%87/6e842528fe74a53d546d330116ba946926e02488

27. Kemenesi G, Kurucz K, Kepner A, Dallos B, Oldal M, Herczeg R, Vajdovics P, Bànyai K, Jakab F (2015) Circulation of Dirofilaria repens, Setaria tundra, and Onchocercidae species in Hungary during the period 2011–2013. In: Veterinary Parasitology. https://doi.org/10.1016/j.vetpar.2015.09.010

28. Nikookar S (2010) Species Composition and Diversity of Mosquitoes in Neka County, Mazandaran Province, Northern Iran. In: PubMed Central (PMC). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3385557/

29. Nikookar SH (2015) Fauna and Larval Habitat Characteristics of Mosquitoes in Neka County, Northern Iran. In: PubMed Central (PMC). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4662797/

30. Mercier A, Shukullari A, Gantier J-C, Reiter P (2014) Ochlerotatus geniculatus, a native European mosquito with a high vector competence for Chikungunya virus. In: ResearchGate. https://www.researchgate.net/publication/279845466_Ochlerotatus_geniculatus_a_native_European_mosquito_with_a_high_vector_competence_for_Chikungunya_virus

31. Bakran-Lebl K, Jerrentrup H, Daroglou E, Pfitzner WP, Fuehrer H, Allerberger F (2022) First records of Aedes pulcritarsis (Rondani, 1872) (Diptera: Culicidae) in Austria. In: Parasitology Research. https://doi.org/10.1007/s00436-022-07430-w

32. Ibáñez-Justicia A, Cianci D (2015) Modelling the spatial distribution of the nuisance mosquito species Anopheles plumbeus (Diptera: Culicidae) in the Netherlands. In: Parasites & Vectors. https://doi.org/10.1186/s13071-015-0865-7

33. Bueno-Marí R, Jiménez-Peydró R (2011) Anopheles plumbeus Stephens, 1828: a neglected malaria vector in Europe. In: Malaria reports. https://doi.org/10.4081/malaria.2011.e2

34. Akiner MM (2016) Impact of environmental factors on Anopheles maculipennis complex (Diptera: Culicidae) populations in three localities of Turkey. https://www.semanticscholar.org/paper/Impact-of-environmental-factors-on-Anopheles-in-of-Akiner-Eksi/3ad93ea45001a73eacc43921641cf654c0c93b12

35. Ponçon N, Balenghien T, Toty C, Ferré JB, Thomas C, Dervieux A, L'Ambert G, Schaffner F, Bardin O, Fontenille D (2007) Effects of Local Anthropogenic Changes on Potential Malaria VectorAnopheles hyrcanusand West Nile Virus VectorCulex modestus,Camargue, France. Emerging Infectious Diseases 13:1810–1815 . https://doi.org/10.3201/eid1312.070730

36. Robert MA, Stewart-Ibarra AM, Estallo EL (2020) Climate change and viral emergence: evidence from Aedes-borne arboviruses. Current Opinion in Virology 40:41–47 . https://doi.org/10.1016/j.coviro.2020.05.001

37. Hayes, C.G., (2001). West Nile virus: Uganda, 1937, to New York City, 1999. Annals of the New York Academy of Sciences, 951(1), pp.25-37.

38. Gray TJ, Webb CE. (2014) A review of the epidemiological and clinical aspects of West Nile virus. Int J Gen Med; 7:193–203.

39. Erdogan Bamac, O., Cizmecigil, U.Y., Mete, A., Yilmaz, A., Aydin, O., Tali, H.E., Tali, B.H., Yilmaz, S.G., Gurel, A., Turan, N. and Ozsoy, S., (2021). Emergence of West Nile Virus Lineage-2 in Resident Corvids in Istanbul, Turkey. Vector-Borne and Zoonotic Diseases.

40. Ziegler U, Seidowski D, Globig A, Fereidouni SR, Ulrich RG, Groschup MH. (2010) Sentinel birds in wild-bird resting sites as potential indicators for West Nile virus infections in Germany. Arch Virol. Jun;155(6):965-9. doi: 10.1007/s00705-010-0618-z. Epub 2010 1 May. PMID: 20437062.

41. Calistri P, Giovannini A, Hubalek Z (2010) Epidemiology of west nile in europe and in the mediterranean basin. Open Virol J.;4:29-37. doi:10.2174/1874357901004020029

42. Brugman VA, Hernández-Triana LM, England ME, Medlock JM, Mertens PPC, Logan JG (2017) Blood-feeding patterns of native mosquitoes and insights into their potential role as pathogen vectors

in the Thames estuary region of the United Kingdom. Parasit Vectors.10(1):163.

43. Petersen LR, Brault AC, Nasci RS. (2013) West Nile virus: review of the literature. JAMA. ;310(3):308-15.

44. Sambri V, Capobianchi M, Charrel R, Fyodorova M, Gaibani P, Gould E (2013). West Nile virus in Europe: emergence, epidemiology, diagnosis, treatment, and prevention. Clin Microbiol Infect;19(8):699-704.

45. El Garch, H., Minke, J.M., Rehder, J., Richard, S., Toulemonde, C.E., Dinic, S., Andreoni, C., Audonnet, J.C., Nordgren, R. and Juillard, V., (2008). A West Nile virus (WNV) recombinant canarypox virus vaccine elicits WNV-specific neutralising antibodies and cell-mediated immune responses in the horse. Veterinary immunology and immunopathology, 123(3-4), pp.230-239.

46. Venter, M., van Vuren, P.J., Mentoor, J., Paweska, J. and Williams, J., (2013). Inactivated West Nile Virus (WNV) vaccine, Duvaxyn WNV, protects against a highly neuroinvasive lineage 2 WNV strain in mice. Vaccine, 31(37), pp.3856-3862.

47. Pecorari, M., Longo, G., Gennari, W., Grottola, A., Sabbatini, A.M., Tagliazucchi, S., Savini, G., Monaco, F., Simone, M.L., Lelli, R. and Rumpianesi, F., (2009). First human case of Usutu virus neuroinvasive infection, Italy, August-September. Eurosurveillance, 14(50), p.19446.

48. Clé M, Beck C, Salinas S, Lecollinet S, Gutierrez S, Van de Perre P, Baldet T, Foulongne V, Simonin Y. (2019) Usutu virus: A new threat? Epidemiol Infect ;147:e232. doi: 10.1017/S0950268819001213. PMID: 31364580; PMCID: PMC6625183.

49. Gaibani P, Rossini G. (2017) An overview of Usutu virus. Microbes Infect.;19(7-8):382-387. doi: 10.1016/j.micinf.2017.05.003. Epub PMID: 28602915.

50. Roesch, F., Fajardo, A., Moratorio, G. and Vignuzzi, M., (2019). Usutu virus: an arbovirus on the rise. Viruses, 11(7), p.640.

51. Vilibic-Cavlek T, Petrovic T, Savic V, Barbic L, Tabain I, Stevanovic V, Klobucar A, Mrzljak A, Ilic M, Bogdanic M, Benvin I, Santini M, Capak K, Monaco F, Listes E, Savini G. (2020) Epidemiology of Usutu Virus: The European Scenario. Pathogens. 26;9(9):699. doi: 10.3390/pathogens9090699. PMID: 32858963; PMCID: PMC7560012.

52. Walker PJ, Siddell SG, Lefkowitz EJ, Mushegian AR, Adriaenssens EM, Dempsey DM, Dutilh BE, Harrach B, Harrison RL, Hendrickson RC, Junglen S, Knowles NJ, Kropinski AM, Krupovic M, Kuhn JH, Nibert M, Orton RJ, Rubino L, Sabanadzovic S, Simmonds P, Smith DB, Varsani A, Zerbini FM, Davison AJ. Changes to virus taxonomy and the Statutes ratified by the International Committee on Taxonomy of Viruses (2020). Arch Virol. 2020 Nov;165(11):2737-2748. PMID: 32816125.

53. Del Amo J., Sotelo, E., Fernández-Pinero, J., Gallardo, C., Llorente, F., Agüero M., Jiménez-Clavero, M.A.: A novel quantitative multiplex real-time RT-PCR for the simultaneous detection and differentiation of West Nile virus lineages 1 and 2, and of Usutu virus. J Virol Methods. 2013 May;189(2):321-7. https://doi.org/10.1016/j.jviromet.2013.02.019

54. Cavrini, F., Della Pepa, M.E., Gaibani, P., Pierro, A.M., Rossini, G., Landini, M.P., Sambri, V.: A rapid and specific real-time RT-PCR assay to identify Usutu virus in human plasma, serum, and cerebrospinal fluid. J Clin Virol. 2011 Mar;50(3):221-3. https://doi.org/10.1016/j.jcv.2010.11.008

55. Tamba M, Bonilauri P, Bellini R, Calzolari M, Albieri A, Sambri V, Dottori M, Angelini P (2011) Detection of Usutu Virus Within a West Nile Virus Surveillance Program in Northern Italy. Vector-Borne and

Zoonotic Diseases 11:551–557 . https://doi.org/10.1089/vbz.2010.0055

56. Calzolari M, Gaibani P, Bellini R, Defilippo F, Pierro A, Albieri A, Maioli G, Luppi A, Rossini G, Balzani A, Tamba M, Galletti G, Gelati A, Carrieri M, Poglayen G, Cavrini F, Natalini S, Dottori M, Sambri V, Angelini P, Bonilauri P (2012) Mosquito, Bird and Human Surveillance of West Nile and Usutu Viruses in Emilia-Romagna Region (Italy) in 2010. PLoS ONE 7:e38058 . https://doi.org/10.1371/journal.pone.0038058

57. Calzolari M, Angelini P, Bolzoni L, Bonilauri P, Cagarelli R, Canziani S, Cereda D, Cerioli MP, Chiari M, Galletti G, Moirano G, Tamba M, Torri D, Trogu T, Albieri A, Bellini R, Lelli D (2020) Enhanced West Nile Virus Circulation in the Emilia-Romagna and Lombardy Regions (Northern Italy) in 2018 Detected by Entomological Surveillance. Frontiers in Veterinary Science 7: . https://doi.org/10.3389/fvets.2020.00243

58. Scaramozzino P, Carvelli A, Bruni G, Cappiello G, Censi F, Magliano A, Manna G, Ricci I, Rombolà P, Romiti F, Rosone F, Sala MG, Scicluna MT, Vaglio S, De Liberato C (2021) West Nile and Usutu viruses co-circulation in central Italy: outcomes of the 2018 integrated surveillance. Parasites & Vectors 14: . https://doi.org/10.1186/s13071-021-04736-z

59. Constant O, Bollore K, Clé M, Barthelemy J, Foulongne V, Chenet B, Gomis D, Virolle L, Gutierrez S, Desmetz C, Moares RA, Beck C, Lecollinet S, Salinas S, Simonin Y (2020) Evidence of Exposure to USUV and WNV in Zoo Animals in France. Pathogens 9:1005 . https://doi.org/10.3390/pathogens9121005

60. Constant O, Gil P, Barthelemy J, Bolloré K, Foulongne V, Desmetz C, Leblond A, Desjardins I, Pradier S, Joulié A, Sandoz A, Amaral R, Boisseau M, Rakotoarivony I, Baldet T, Marie A, Frances B, Reboul

Salze F, Tinto B, Van de Perre P, Salinas S, Beck C, Lecollinet S, Gutierrez S, Simonin Y (2022) One Health surveillance of West Nile and Usutu viruses: a repeated cross-sectional study exploring seroprevalence and endemicity in Southern France, 2016 to 2020. Eurosurveillance 27: . https://doi.org/10.2807/1560-7917.es.2022.27.25.2200068

61. de Bellegarde de Saint Lary C, Kasbergen LMR, Bruijning-Verhagen PCJL, van der Jeugd H, Chandler F, Hogema BM, Zaaijer HL, van der Klis FRM, Barzon L, de Bruin E, ten Bosch Q, Koopmans MPG, Sikkema RS, Visser LG (2023) Assessing West Nile virus (WNV) and Usutu virus (USUV) exposure in bird ringers in the Netherlands: a high-risk group for WNV and USUV infection? One Health 16:100533 . https://doi.org/10.1016/j.onehlt.2023.100533

62. Ziegler U, Jöst H, Müller K, Fischer D, Rinder M, Tietze DT, Danner K-J, Becker N, Skuballa J, Hamann H-P, Bosch S, Fast C, Eiden M, Schmidt-Chanasit J, Groschup MH (2015) Epidemic Spread of Usutu Virus in Southwest Germany in 2011 to 2013 and Monitoring of Wild Birds for Usutu and West Nile Viruses. Vector-Borne and Zoonotic Diseases 15:481–488 . https://doi.org/10.1089/vbz.2014.1746

63. Michel F, Fischer D, Eiden M, Fast C, Reuschel M, Müller K, Rinder M, Urbaniak S, Brandes F, Schwehn R, Lühken R, Groschup M, Ziegler U (2018) West Nile Virus and Usutu Virus Monitoring of Wild Birds in Germany. International Journal of Environmental Research and Public Health 15:171 . https://doi.org/10.3390/ijerph15010171

64. Ziegler U, Lühken R, Keller M, Cadar D, van der Grinten E, Michel F, Albrecht K, Eiden M, Rinder M, Lachmann L, Höper D, Vina-Rodriguez A, Gaede W, Pohl A, Schmidt-Chanasit J, Groschup MH (2019) West Nile virus epizootic in Germany, 2018. Antiviral Research 162:39–43 . https://doi.org/10.1016/j.antiviral.2018.12.005

65. Zana B, Erdélyi K, Nagy A, Mezei E, Nagy O, Takács M, Bakonyi T, Forgách P, Korbacska-Kutasi O, Fehér O, Malik P, Ursu K, Kertész P, Kepner A, Martina M, Süli T, Lanszki Z, Tóth GE, Kuczmog A, Somogyi B, Jakab F, Kemenesi G (2020) Multi-Approach Investigation Regarding the West Nile Virus Situation in Hungary, 2018. Viruses 12:123 . https://doi.org/10.3390/v12010123

66. Bahk YY, Park SH, Kim-Jeon M-D, Oh S-S, Jung H, Jun H, Kim K-A, Park JM, Ahn SK, Lee J, Choi E-J, Moon B-S, Gong YW, Kwon MJ, Kim T-S (2020) Monitoring Culicine Mosquitoes (Diptera: Culicidae) as a Vector of Flavivirus in Incheon Metropolitan City and Hwaseong-Si, Gyeonggi-Do, Korea, during 2019. The Korean Journal of Parasitology 58:551-558 . https://doi.org/10.3347/kjp.2020.58.5.551

67. Newman CM, Cerutti F, Anderson TK, Hamer GL, Walker ED, Kitron UD, Ruiz MO, Brawn JD, Goldberg TL (2011) Culex Flavivirus and West Nile Virus Mosquito Coinfection and Positive Ecological Association in Chicago, United States. Vector-Borne and Zoonotic Diseases 11:1099-1105 . https://doi.org/10.1089/vbz.2010.0144

68. Seo M-G, Lee HS, Yang S-C, Noh B-E, Kim T-K, Lee W-G, Lee HI (2021) National Monitoring of Mosquito Populations and Molecular Analysis of Flavivirus in the Republic of Korea in 2020. Microorganisms 9:2085 . https://doi.org/10.3390/microorganisms9102085

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