

Species of mosquitoes present in Abruzzo and Molise and their possible role as vectors of Usutu and West Nile viruses

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Veterinaria Italiana 2022, **58** (4), 435-445. doi: 10.12834/VetIt.3046.20276.1

Accepted: 30.12.2022 | Available on line: 30.12.2022

Keywords

Mosquitoes,
Usutu virus,
West Nile virus,
Italy.

Summary

In 2019, entomological survey on mosquitoes was carried out in Abruzzo and Molise regions in central Italy to obtain data on local mosquito fauna. Collection sites were selected based on a previous ecoregion classification of the territory. From 2019 to 2021 virological surveillance for West Nile virus (WNV) and Usutu virus (USUV) on mosquitoes was carried out in the same regions, selecting ecoregions where virus circulation and vector presence were more likely, all mosquitoes were collected and identified, and the female mosquitoes were sorted in 3046 pools and tested for the presence of WNV and USUV by Real-time PCR. All pools tested negative for WNV, while USUV was detected in 7 pools of *Aedes caspius* collected in Molise region, 17 pools of *Culex pipiens* s.l. (2 collected in Molise, 15 in Abruzzo), and 1 pool of *Culiseta longiareolata* collected in Molise. These results suggest the presence of an USUV enzootic cycle, maintained by *Culex pipiens* s.l. and *Aedes caspius* in both Italian regions, as well as providing a useful picture in terms of species presence and abundance for both regions. Ecoregions proved to be a very valuable tool in determining high risk areas for vector borne diseases.

Introduction

West Nile (WNV) and Usutu viruses (USUV) are mosquito-borne arboviruses belonging to the *Flaviviridae* family within the Japanese encephalitis virus complex (Calisher *et al.* 1989). Both viruses share a similar natural enzootic cycle that involves mosquitoes, primarily those of the *Culex* genus, and many species of resident and migratory birds (Barzon 2018, Roesh *et al.* 2019). Mammals, amphibians and reptiles may become infected but generally do

not show clinical signs. Horses and humans may develop severe neurological signs sometimes fatal. Because of the low and transitory viremia, they, however, are not able to infect the vectors and for this reason, they are considered as incidental or dead-end hosts. Conversely, because of the length and titer of their viraemia, many bird species may act as amplifying reservoir hosts. Mosquitoes of the *Culex* spp. generally represent the main vectors for these viruses (Nikolay 2015).

Please refer to the forthcoming article as: De Ascentis *et al.* 2022. Species of mosquitoes present in Abruzzo and Molise and their possible role as vector of Usutu and West Nile viruses. *Vet Ital.* doi: 10.12834/VetIt.3046.20276.1.

WNV was first isolated in Uganda in 1937 (Smithburn *et al.* 1940), since then the virus kept spreading, becoming the most widespread arthropod-borne virus infection in the world (Rizzoli *et al.* 2015). The geographical spread has been causing sporadic cases and outbreaks in Europe since 1950s (Hubalek and Halouzka 1999). In recent years, more and more cases of WNV outbreaks have been reported in Europe (Nikolay 2015, Vilibic-Cavlek *et al.* 2019), reaching a peak in 2018 with 2,083 human cases and 285 cases in equids, being by far the season with the highest number of WNV cases (ECDC 2018). In Europe, the principal vector species, other than *Culex pipiens s.l.*, are *Culex perexiguus* and *Culex modestus* (Balenghien *et al.* 2007, Engler *et al.* 2013, Tamba *et al.* 2011, Calzolari *et al.* 2012, Calzolari *et al.* 2015, Mancini *et al.* 2017, Calzolari *et al.* 2022, Mavridis *et al.* 2018, Vazquez *et al.* 2011, Cabanova *et al.* 2019). WNV has been detected in, but not limited to, these mosquito species in different European countries. In Italy, for instance, WNV has been detected also in *Aedes caspius* while in Serbia also in *Aedes vexans*, and *Culiseta annulata* (Petric *et al.* 2016).

USUV was first identified in South Africa in 1959 (Wodall 1964). In the following years, as for WNV, it also spread into many geographical areas outside Africa, emerging for the first time in Europe, Italy, in 1996 (Weissenböck *et al.* 2013). Sharing the same ecological niche, the geographical areas where USUV circulate, overlap those of WNV.

Regarding vectors, the main USUV vector species in Europe is considered to be *Culex pipiens s.l.*, even if the virus was also found in other mosquito genera within the *Culicidae* family (Nikolay 2015). USUV was also found in *Aedes albopictus*, *Aedes caspius*, *Aedes detritus* and *Anopheles maculipennis s.l.* (Calzolari *et al.* 2022, Calzolari *et al.* 2017, Calzolari *et al.* 2013, Mancini *et al.* 2017, Bakonyi *et al.* 2014, Eiden *et al.* 2018, Kemenesi *et al.* 2018).

In Italy, WNV first appeared in horses in Tuscany region in 1998 (Autorino *et al.* 2002), in the same region where two years earlier an outbreak caused by USUV was first described in balckbirds (Weissenböck *et al.* 2013).

Following these first outbreaks, in 2002, a National Surveillance Plan involving humans, horses, birds and mosquitoes was implemented in Italy. Initially, the plan included only WNV but, after USUV was associated to two cases of meningoencephalitis in human patients in 2009 (Pecorari *et al.* 2009, Cavrini *et al.* 2009), the surveillance plan was rescheduled and USUV was also included. Currently, the surveillance plan is part of the National Plan for Arbovirus Prevention, Surveillance and Response (PNA) 2020-2025. It monitors WNV and USUV circulation in the country through an integrated human, animal (equid and bird) and entomological

surveillance. Based on virus circulation and eco-climatic conditions, Italy has been divided into high, low and minimum risk areas. A very close monitoring activity including active and passive surveillance is recommended in the high and low risk areas whereas passive surveillance is requested for the minimum risk areas (Piano Nazionale di prevenzione, sorveglianza e risposta alle Arbovirosi (PNA) 2020–2025).

According to this risk based division, provinces (Chieti, Pescara and Teramo) of Abruzzo and both provinces (Isernia and Campobasso) of Molise have been included amongst the low-risk areas for virus circulation while L'Aquila, the fourth province of Abruzzo, within the minimum risk areas.

Concerning the circulation of Flaviviruses in the two regions, WNV was detected once in Molise in 2011 while USUV was found in *Culex pipiens s.l.*, *Culiseta annulata* and *Aedes detritus* mosquitoes collected from both regions (Mancini *et al.* 2017). To comply with what recommended by the PNA and to monitor whether WNV and USUV vectors are present and whether these viruses have circulated and/or are circulating in both regions, a comprehensive entomological surveillance was implemented and the results presented in this paper.

For the surveillance, the mosquito collection sites were selected according to the ecoregion classification. Ecoregions are defined as areas which present "associations of interacting biotic and abiotic features" (Bailey 2004), usually classified using both abiotic (such as climate, orography and geological factors), and biotic characteristics (for example vegetation).

Within these homogenous land units, natural communities and species interact with the physical elements of the environment (Blasi *et al.* 2014).

A previous work by Ippoliti *et al.* (2019) adopted a data-driven spatial clustering approach to characterise the Italian territory into similar areas from a climatic and environmental point of view.

The seven variables considered (standard deviation of altitude, mean daytime land surface temperature, mean amplitude and peak timing of the annual cycle of land surface temperature, mean and amplitude of the annual cycle of greenness value, and daily mean amount of rainfall) were chosen as relevant for a broad set of human and animal vector-borne diseases.

The approach classified the Italian territory into 22 ecoregions at 250 meters spatial resolution. The surveillance based on the ecoregion approach also gave us the opportunity to investigate on the possible influence of ecoregions on mosquito fauna composition and USUV/WNV circulation.

Materials and methods

To obtain data on the mosquito fauna composition in different ecoregions, the entire territory of Abruzzo and Molise was parcelled in ecoregions based on the previous study by Ippoliti *et al.* (2019). According to this classification, 22 ecoregions were identified.

The ecoregions located in areas with altitude higher than 600 meters on sea level were excluded from the mosquito monitoring activity as mosquitoes are not present above this altitude.

Thus, the ecoregions selected for the mosquito surveillance were reduced to 16. In addition, to guarantee harmonization and representativeness of the data originated from the entomological surveillance, the two regions were divided in grids of 400 km² (20x20 km). In each grid, at least one mosquito collection site was placed (Figure 1).

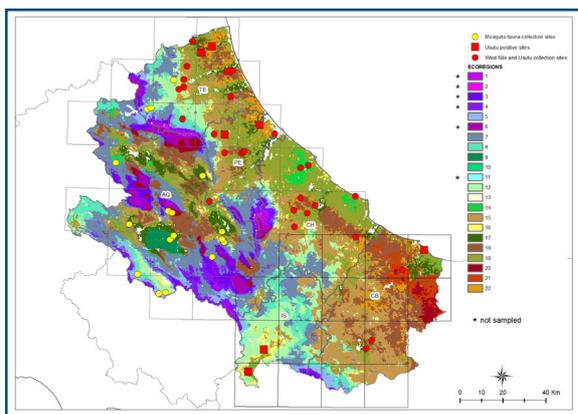


Figure 1. Collection sites selected in Abruzzo and Molise regions (Italy) according to ecoregions classification. Collection sites used for WNV and USUV monitoring are highlighted with a red dot, while yellow dots represent sites used to obtain data on local mosquito fauna. Each ecoregion is identified with a different colour. TE= Teramo province; PE= Pescara province; AQ= L'Aquila province; CH= Chieti province; IS= Isernia province; CB= Campobasso province; *= ecoregion where the mosquito collections were not performed.

In 2019, the entomological surveillance was then carried out on the 16 selected ecoregions.

These regions comprised all provinces of Abruzzo and Molise. To investigate presence of WNV and USUV circulation in the two regions and to evaluate the influence of abiotic and biotic characteristics on the circulation of these viruses, the entomological surveillance was restricted to six ecoregions only (number 14, 15, 17, 19, 21 and 22).

These regions were selected as they share the same climatic-environmental conditions to those located in other Italian regions in which WNV and USUV circulation has been reported. Therefore, in 2020 and 2021, also the entomological activity continued

only in the six ecoregions selected for WNV and USUV investigation (Figure 1).

Also for the virological monitoring, at least one collection site was placed in each grid.

Sampling was planned on a weekly basis in Abruzzo and every two weeks in Molise (from June to November 2019 and from May to November 2020/2021). The collections were performed by Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale" personnel in collaboration with the local veterinary services.

Mosquito collections were performed using CDC type traps baited with both light and dry ice. The traps were placed at about 1.5 m above ground, and activated just before sunset.

The following morning, collection bags were removed and labelled with site name and collection date, which was the day when the insects were removed.

Collected insects were transferred to the laboratory at refrigerated temperatures and euthanized by keeping them at -20°C for at least 15 minutes. After that, mosquitoes were counted and morphologically identified under a light stereomicroscope using the identification keys developed by Severini *et al.* (2009).

In case species identification was not possible due to their bad preservation state, the genus or subfamily was indicated.

For virological analyses, female mosquitoes were sorted by species, collection site and date in pools of up to 50 individuals.

Each pool was homogenated and tested for the presence of USUV and WNV RNA by using specific real-time RT-PCRs. Virus RNA of the sample's homogenates was extracted by using the MagMAX CORE Nucleic Acid Purification KIT (Applied Biosystem, Termofisher Scientific, Life technologies corporation, TX, USA) according to the manufacturer's instructions.

The presence of WNV and/or USUV RNA was detected by real time RT-PCR according to Del Amo *et al.* (2013), and Cavrini *et al.* (2010).

The results of mosquito testing were used to calculate the *Minimum Infection Rate (MIR)* for WNV and USUV, which is defined as the total number of positive pools /total number of mosquitoes sampled $\times 100$.

Results

To obtain data on local mosquito fauna, the entomological surveillance was performed in the sixteen previously selected ecoregions.

The excluded regions accounted for the 8.6% of the entire territory of the two regions (Figure 2).

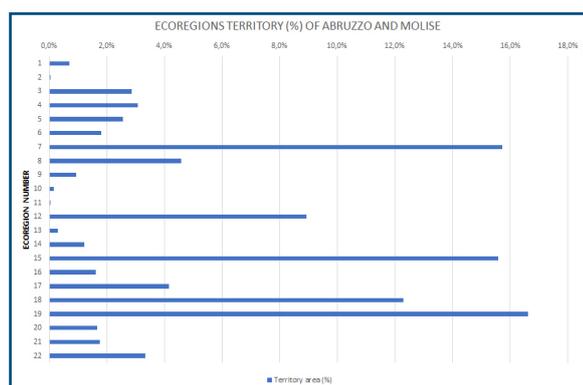


Figure 2. Portion (%) of Abruzzo and Molise territory occupied by each ecoregion.

In 2019, 1026 mosquito collections (957 in Abruzzo and 69 in Molise) were completed. This led to a total of 13408 mosquito specimens examined. Eighteen species, representing all seven Italian genera were identified (Table I). *Culex pipiens* s.l. was the most abundant species found, accounting for 77.75% of

Table I. Number of mosquito species collected in 2019 in Abruzzo and Molise (Italy), grouped by region.

Species	2019		Total
	Abruzzo	Molise	
<i>Aedes albopictus</i>	291	8	299
<i>Aedes caspius</i>	17	643	660
<i>Aedes geniculatus/sticticus</i>	433		433
<i>Aedes pulcritarsis</i>	3		3
<i>Aedes vexans</i>	380	1	381
<i>Aedes</i> sp.	132	12	144
<i>Anopheles claviger</i>	2		2
<i>Anopheles maculipennis</i> s.l.	21	6	27
<i>Anopheles plumbeus</i>	102	9	111
<i>Anopheles</i> sp.	8	6	14
<i>Coquillettidia richiardii</i>	297	215	512
<i>Culex pipiens</i> s.l.	9590	835	10425
<i>Culex mimeticus</i>	162	1	163
<i>Culex hortensis</i>	6		6
<i>Culex territans</i>	1		1
<i>Culex theileri</i>		2	2
<i>Culiseta annulata</i>	47	14	61
<i>Culiseta longiareolata</i>	25	1	26
<i>Culiseta</i> sp.	1		1
<i>Orthopodomyia pulcricarpis</i>	1		1
<i>Uranotaenia unguiculata</i>	8		8
Culicinae	128		128
TOTAL	11655	1753	13408

the total collected mosquitoes, followed by *Aedes caspius* (4.92%), *Coquillettidia richiardii* (3.82%), *Aedes geniculatus/sticticus* (3.23%), *Aedes vexans* (2.84%), *Aedes albopictus* (2.23%), *Culex mimeticus* (1.22%), *Anopheles plumbeus* (0.83%), *Culiseta annulata* (0.45%), *Culiseta longiareolata* (0.19%), *Anopheles maculipennis* (0.20%) and *Anopheles claviger* (0.01%). Finally, 2.30% of the examined mosquitoes belonged to less common species or were identified to genus or subfamily level only. Figure 3 shows the mosquito species abundance detected in each sampled ecoregion in 2019.

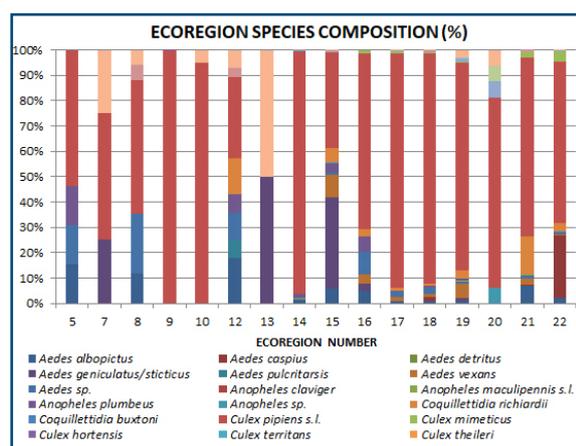


Figure 3. Species composition (%) of mosquitoes caught in each ecoregion of Abruzzo and Molise in 2019.

Nearly all sampled ecoregions evidenced a higher prevalence of *Culex pipiens* s.l., in fact, this species was the most abundant species found in all of sampled ecoregions except one where it was not found (ecoregion 13). Even if *Culex pipiens* s.l. was the most abundant species, many ecoregions showed a good mosquito species variety, in fact, if we don't consider *Culex pipiens* s.l., ecoregions number 15, 17, 18, 19, 21 and 22 showed the presence of nine or more mosquito species each (9, 10, 12, 14, 13 and 12 species, respectively) as displayed in Figure 4.

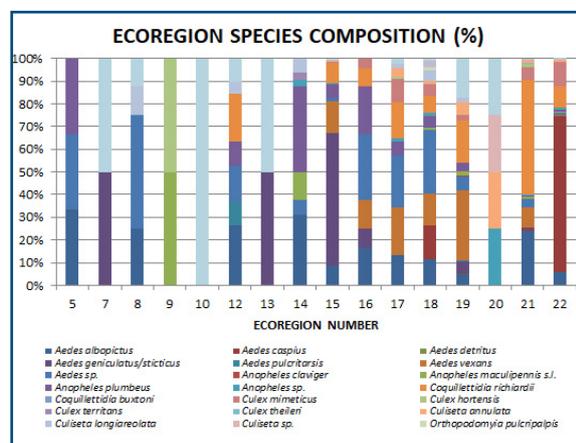


Figure 4. Species composition (%) of mosquitoes other than *Culex pipiens* s.l. caught in each ecoregion of Abruzzo and Molise in 2019.

During the three years covered by this study (2019-2021), the entomological surveillance was first (2019) carried out in the 16 selected areas whereas then (2020-2021) it was continued on the six areas selected according to their capacity of sustaining WNV/USUV circulation. The territory covered by these six areas was 42.7% of the entire territory of Abruzzo and Molise. Figure 5 details the species composition of these six ecoregions found in 2019 and 2020-2021. Each ecoregion showed a broad and different species composition. However, even if variations in the percentage of the collected species were evident in each selected ecoregion, no differences in the species composition present in these selected areas were found between the two periods examined. Similarly to what found in all

ecoregions, *Culex pipiens* s.l. was the most abundant species caught (Figure 5A, B).

For the virological analyses, 3046 pools were sorted and tested for the presence of WNV and USUV. Results of the tests and of the calculation of MIR are shown in Table II.

All pools were negative for WNV. In Molise, 10 pools were found positive for USUV. Eight were from Campobasso province (5 pools in 2019 and 3 pools in 2020), the remaining two from Isernia province (both in 2020). USUV was detected in pools of *Aedes caspius* (7 pools), *Culex pipiens* s.l. (2 pools) and *Culiseta longiareolata* (1 pool). No infected mosquitoes were collected in 2021. MIR for USUV infection ranged from 0.5 to 0.6% for *Aedes caspius*

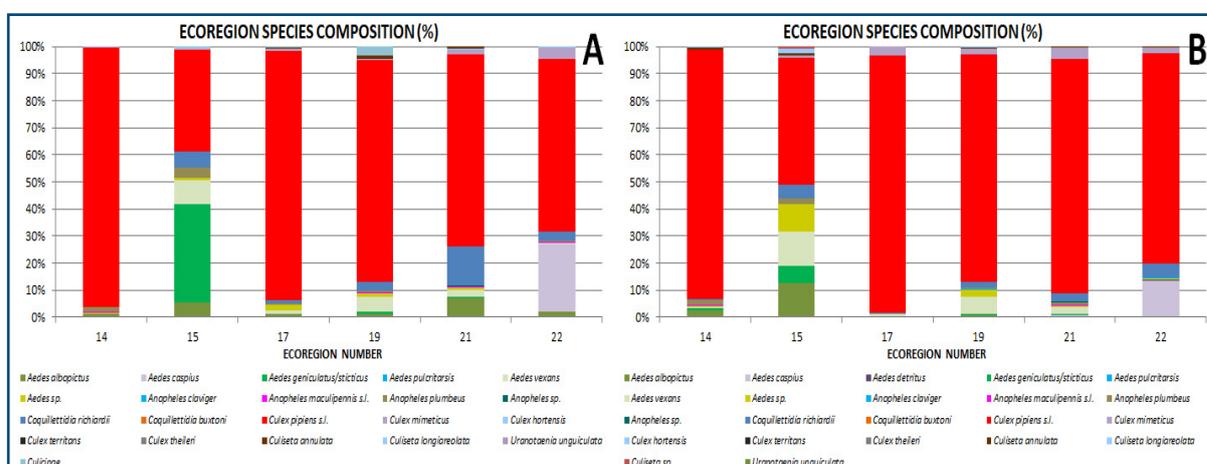


Figure 5. Species composition (%) of ecoregions selected for possible WNV and USUV circulation in Abruzzo and Molise regions (Italy) in 2019 (A), 2020 and 2021 (B).

Table II. Usutu virus minimum infection rate found in mosquito pools caught in Abruzzo and Molise regions (Italy) between 2019 and 2021.

Species	2019		2020		2021	
	N. of positive pools/ pools tested; N. of mosquitoes tested (USUTU MIR %)					
	ABRUZZO	MOLISE	ABRUZZO	MOLISE	ABRUZZO	MOLISE
<i>Aedes albopictus</i>	0/118; 269	0/7; 8	0/36; 74	0/2; 2	0/52; 76	0/9; 15
<i>Aedes caspius</i>	0/6; 17	4/26; 642 (0.62)	0/4; 7	3/26; 600 (0.50)	0/2; 2	0/24; 686
<i>Aedes geniculatus/sticticus</i>	0/26; 366	-	0/15; 55	-	0/4; 7	-
<i>Aedes vexans</i>	0/97; 374	0/1; 1	0/67; 515	0/3; 3	0/42; 127	0/1; 1
<i>Anopheles claviger</i>	0/2; 2	-	0/10; 29	0/3; 6	0/5; 5	0/3; 5
<i>Anopheles maculipennis</i> s.l.	0/10; 15	0/5; 6	0/7; 7	0/3; 3	0/6; 6	0/4; 4
<i>Anopheles plumbeus</i>	0/53; 91	0/7; 9	0/32; 45	0/2; 3	0/14; 18	0/3; 4
<i>Coquillettidia richiardii</i>	0/88; 291	0/15; 215	0/67; 312	0/6; 18	0/51; 391	0/4; 29
<i>Culex mimeticus</i>	0/53; 145	0/1; 1	0/68; 357	-	0/60; 211	0/1; 1
<i>Culex pipiens</i> s.l.	4/732; 7294 (0.05)	1/77; 811 (0.12)	4/350; 9747 (0.04)	1/23; 169 (0.59)	7/400; 10271 (0.07)	0/25; 64
<i>Culiseta annulata</i>	0/24; 39	0/8; 11	0/18; 29	0/1; 1	0/24; 28	0/4; 4
<i>Culiseta longiareolata</i>	0/12; 19	0/1; 1	0/18; 19	1/1; 1 (100)	0/9; 11	0/4; 5
Other	0/71; 143	0/11; 20	0/46; 255	0/10; 14	0/18; 53	0/8; 56
TOTAL	4/1292; 9065 (0.04)	5/159; 1725 (0.29)	4/738; 11451 (0.03)	5/80; 820 (0.61)	7/687; 11206 (0.06)	0/90; 874

-- data not available; MIR= minimum infection rate.

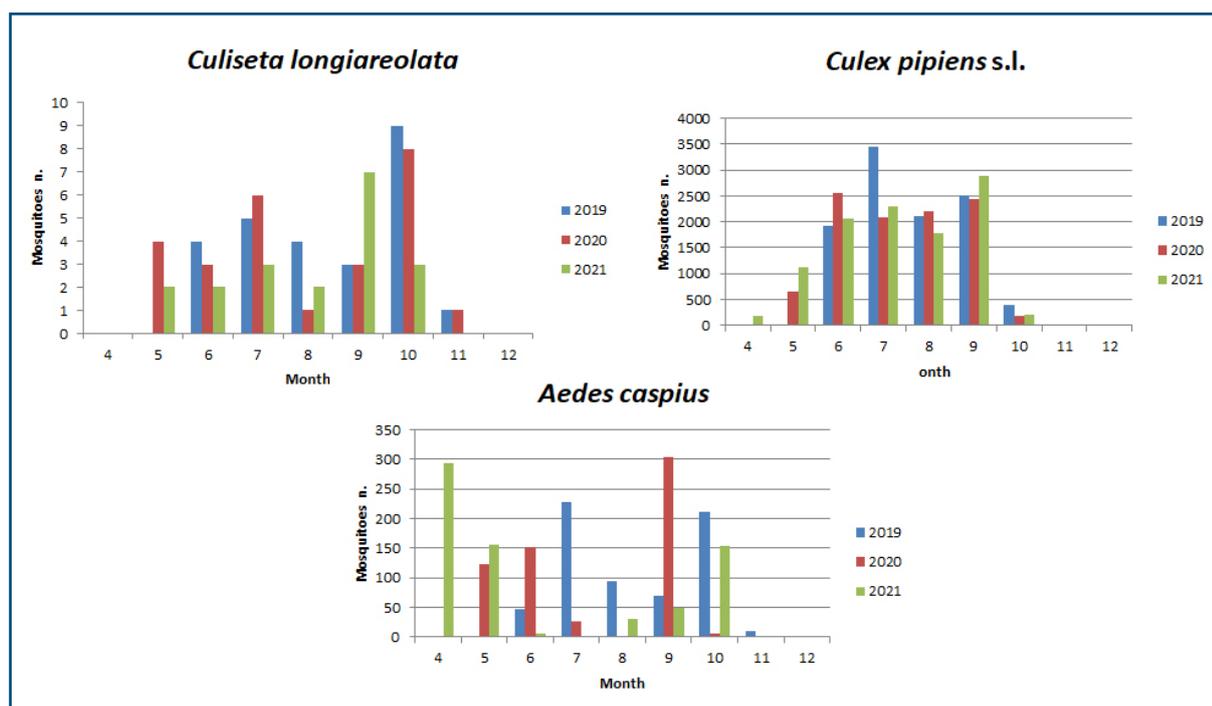


Figure 6. Seasonal trends of *Culex pipiens s.l.* (A); *Aedes caspius* (B) and *Culiseta longiareolata* (C) in Abruzzo and Molise between 2019 and 2021.

and from 0.04 and 0.59% for *Culex pipiens s.l.*

In Abruzzo, 15 pools were found positive to USUV between 2019 and 2021. Eleven were from Teramo province and 4 from Pescara province. *Culex pipiens s.l.* was the only mosquito species found infected with USUV.

The USUV positive originated from ecoregions number 14 (1 pool), 17 (2 pools), 19 (5 pools), 21 (4 pools) and 22 (13 pools) (Table III and Figure 1).

All USUV positive mosquitoes of both regions were collected between September and October (Table III).

The annual seasonal trends of the mosquito species found positive to USUV are shown in Figure 6.

Discussion

This comprehensive study on the presence and abundance of mosquito species in the Abruzzo and Molise regions completes the scanty information on this topic present in the literature (Toma *et al.* 2008, Mancini *et al.* 2017, Traversa *et al.* 2010) and provides a useful picture in terms of species present, abundance and seasonal trends for both regions. The 1026 catches with 13408 mosquitoes collected and identified, evidenced the presence of 18 different species, 17 in Abruzzo and 11 in Molise. Seven of these species were caught only in Abruzzo

Table III. Details of the mosquito pools found positive to Usutu virus caught in Abruzzo and Molise regions (Italy) between 2019 and 2021.

Species	Province	SAMPLE DATE	Ecoregion
<i>Aedes caspius</i>	CAMPOBASSO	01-oct-19	22
		23-sep-20	22
		01-oct-19	22
		10-sep-19	21
		27-sep-19	21
	TERAMO	11-oct-19	17
		10-sep-20	17
		17-sep-20	22
		17-sep-20	22
		17-sep-20	22
<i>Culex pipiens s.l.</i>		10-sep-21	21
		16-sep-21	21
		07-oct-21	22
	ISERNIA	13-oct-21	22
		16-sep-20	19
		18-sep-20	19
		26-aug-21	19
PESCARA	08-sep-21	19	
	22-sep-21	19	
<i>Culiseta longiareolata</i>	ISERNIA	16-sep-20	14

whereas *Culex theileri* was collected in Molise only. Overall, all seven Italian genera were found in both regions. (Table I). As observed in other Italian regions and in the great part of the world (Mancini *et al.*

2017, Vogels *et al.* 2016, Andreadis 2012), also in this study *Culex pipiens* s.l. was by far the most abundant species (77.75%) caught in both regions, followed by *Aedes caspius* (4.92%), *Coquillettidia richiardii* (3.82%), *Aedes geniculatus/sticticus* (3.23%) and *Aedes vexans* (2.84%) which was found only in Abruzzo. *Culex pipiens* s.l. was also the most prevalent species found in all, except one, of the identified ecoregions where the surveillance was carried out. Of the 22 ecoregions identified in Abruzzo and Molise, six were in fact not included in the surveillance as they were more than 600 metres high and mosquitoes are not present above this altitude (Figure 1). They cover only a minor part (8.6%) of the Abruzzo and Molise territory. Ecoregion classification was obtained using data from a previous study done by Ippoliti *et al.* (2019). Ecoregions could represent a very valuable tool as they consent to associate mosquito species composition to particular abiotic (such as climate, rainfall, orography and geological factors), and biotic characteristics (for example vegetation) (Table III, Figure 3 and 4). Interactions between climatic conditions/environment and mosquito distribution have already been proven (Kraemer *et al.* 2015, Rosà *et al.* 2014, Carrieri *et al.* 2014, Marini *et al.* 2016). In this study, differences in species compositions were found in the sampled ecoregions. Each ecoregion had its own species composition in term of presence and abundance. The species composition seems to be stable in the years while the abundance of each species may vary depending on the year of collection. Based on the presence of the abiotic and biotic characteristics capable of sustaining activity, survival and distribution of the vectors, ecoregions could also be important to characterize areas where vector borne pathogen might find the right conditions to establish and spread. Concerning the species composition identified in this study, it is possible to affirm that most of the territory of both regions have the right conditions to sustain vector survival and Usutu and West Nile virus replication. To investigate whether WNV and USUV were circulating in these regions, the virological monitoring was limited to six ecoregions. These areas were selected as having the same climatic-environmental conditions of other Italian ecoregions in which WNV and USUV circulation was reported. They cover a great part (42.7%) of both investigated regions. As said before, no variations have been demonstrated in the vector composition of the selected regions. In line with the data of the National surveillance plan, the virological surveillance performed in this study between 2019 and 2021 was not able to detect WNV in the collected mosquitoes. In contrast, Usutu virus was detected in pools of *Culex pipiens* s.l., *Aedes caspius* and *Culiseta longiareolata* collected from both regions.

Culex pipiens, with its two biotypes *Culex pipiens*

pipiens and *Culex pipiens molestus*, is one of the most widespread and abundant vector species of WNV and USUV in Italy and Europe. Its efficiency and competency in the transmission of USUV and WNV have been supported by laboratory trials. This species is probably one of the most important vectors of Flaviviruses (Calzolari *et al.* 2010, Monaco *et al.* 2010, Jöst *et al.* 2011, Calzolari *et al.* 2012). The fact that *Culex pipiens* s.l. was by far the most widespread species found in this survey and that infected *Culex pipiens* s.l. have already been found in Molise in previous investigations (Mancini *et al.* 2017), reinforces the hypothesis that *Culex pipiens* s.l. is the main vector of USUV in Abruzzo and Molise regions. This role is due more to its abundance than its infection rate.

Aedes caspius is a mammophilic and anthropophilic species. During periods of high abundance, it was occasionally found engorged with avian blood (Balenghien *et al.* 2006). Being often associated with coastal habitats, its distribution is limited compared to other species (Severini *et al.* 2009). Even if low distributed, seven pools of this species were found infected by USUV in this study. All of them were from a single collection site located in a coastal area in Molise region. *Aedes caspius* has been considered an inefficient vector of WNV at least under laboratory conditions (Balenghien *et al.* 2007). The findings of numerous positive pools, its high MIR, and the relative high abundance found in this study might suggest an important role in spreading USUV infection at least in certain areas near the coast. Because of their feeding behaviour, this species might also represent an important bridge vector in the USUV transmission cycle in both regions.

Culiseta longiareolata is mainly an ornitophilic species, which rarely bites humans (Severini *et al.* 2009). It is rather common in Italy. In this survey, one *Culiseta longiareolata* female, collected in Molise in 2020, was found positive to USUV. It is difficult to interpret this result. The vector role of this species has been poorly investigated (Martinet *et al.* 2019), but given its ornitophilic feeding behaviour, they can play a role in perpetuating the endemic cycle of USUV, however further studies are needed to better understand the epidemiological role as vector played by this species.

USUV circulation was found in five of the selected regions. This result confirms that these areas have the right conditions for USUV to establish which in turn implies that presence of pathogens and eco-climatic characteristics are likely to be connected (Ippoliti *et al.* 2019). Amongst the six selected regions, ecoregion 22 was the mostly infected region (n=17). It represents only the 3.3% of the territory under study. USUV was detected in pools of both, *Aedes caspius* and *Culex pipiens* s.l.. Few infected

pools were also detected in the other four selected areas, involving either *Culex pipiens* s.l. or *Culiseta longiareolata*. In this study, no flaviviruses were found in the ecoregion 15, which is the ecological region highly represented (15.6%) in Abruzzo and Molise. The selected ecoregions include plain or hilly-cropped areas characterised by temperature values and fluctuation ranges, on average, higher and greater, respectively, than those found at National level. Equally, in these areas mean daily rainfall is less than that observed in other regions the country. Regarding this last value, being the selected regions cropped areas, possible effects of continuous irrigations cannot be ignored. Looking at the USUV infected areas, it is worthy to note that they were not contiguous but distributed in two of the four provinces of Abruzzo and in both provinces of Molise (Table III). It means that USUV infection in these areas was probable the outcome of different and independent introductions.

As they are not staging areas for long distance migratory birds, it is likely that birds, along short migratory routes, were responsible for these new introductions, as already noted by other authors (Mencattelli *et al.* 2022). From the data of this study, unfortunately, it is not possible to assert whether USUV, once introduced, locally established and adapted to the diverse eco-climatic conditions and maintenance hosts.

In one collection site (ecoregion 22) in the province

of Teramo, USUV was continuously detected between 2019 and 2021, similarly in another site in the province of Pescara, USUV was detected between 2020 and 2021. In the other sites, the presence of USUV was not constantly detected.

Most infected mosquitoes were collected between September and October, concomitantly with the peaks of mosquito population abundance. In 2019, both *Culex pipiens* s.l. and *Aedes caspius* populations reached the peak of abundance in September and October, respectively (Figure 6). Similarly, in 2020, *Aedes caspius* and *Culex pipiens* s.l. reached the highest population abundance peak in September (Figure 6). In 2021, USUV was detected only in *Culex pipiens* s.l. in September, again in concomitance with the peak of population abundance. Interestingly, in the same year, USUV was not detected in *Aedes caspius* as in 2021 this species peak of abundance occurred in April (far earlier than previous years) and since then the population abundance rapidly decreased until the end of the summer. These findings again underline how important is each single factor in determining the occurrence of vector borne infections and within this scenario, ecoregions proved to be a very valuable tool in determining high risk areas for vector borne diseases. This peculiar association between presence of pathogens and ecoregions can be used to draw prevision model based on ecoregion classification of the territory (even at local level) and to provide a functional tool for monitoring WNV and USUV circulation.

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