Mosquito species involved in the circulation of West Nile and Usutu viruses in Italy

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Veterinaria Italiana 2017, **53** (2), 97-110. doi: 10.12834/VetIt.114.933.4764.2
Accepted: 11.03.2016 | Available on line: 30.06.2017

Keywords
Italy,
Mosquitoes,
Overwintering,
Polymerase chain reaction,
Usutu virus,
West Nile virus.

Summary
Usutu (USUV) and West Nile (WNV) are mosquito-borne Flavivirus emerged in Italy in 1996 and 1998, respectively, and reappeared 10 years later. The aim of this work is to review the Italian mosquito species found positive for WNV and USUV between 2008 and 2014. Moreover, the role of mosquitoes in promoting the overwintering of these viruses is discussed, as a result of the mosquito collections performed in Molise region between September 2010 and April 2011. Overall 99,000 mosquitoes were collected: 337 and 457 mosquito pools tested positive by real time reverse transcriptase polymerase chain reaction (real time RT-PCR) for WNV and USUV, respectively. West Nile virus was detected in pools of *Culex pipiens s.l.* (329), *Ochlerotatus caspius* (4), *Culex modestus* (2), and *Culex* spp. (2). Positive USUV pools were from *Cx. pipiens s.l.* (435), *Aedes albopictus* (12), *Oc. caspius* (5), *Culex* spp. (2), *Anopheles maculipennis s.l.* (1), *Culiseta annulata* (1), and *Ochlerotatus detritus* (1). In Molise region, 1,694 mosquitoes were collected, and USUV was identified in *Cx. pipiens s.l.*, *Cs. annulata*, and *Oc. detritus* pools. This paper shows that *Cx. pipiens s.l.* is the mosquito species most involved in the WNV and USUV circulation in Italy, although other species would also support the spread of both the viruses during Winter.

Specie di zanzare coinvolte nella circolazione dei virus della West Nile e Usutu in Italia

Parole chiave
Italia,
Overwintering,
Reazione a catena della polimerasi in tempo reale,
Virus Usutu,
Virus della West Nile,
Zanzare.

Riassunto
Il virus Usutu (USUV) e il virus della West Nile (WNV) sono virus tramessi da zanzare (Culicidae), appartenenti al genere *Flavivirus* e comparsi per la prima volta in Italia rispettivamente nel 1996 e nel 1998. Dopo essere stati silenti per dieci anni, i due virus sono ricomparsi nel territorio italiano nel 2007 e nel 2008, causando infezioni, forme cliniche e decessi in uccelli, cavalli e uomo. Obiettivo del presente lavoro, è quello di elencare e descrivere le specie di culicidi in cui sono stati identificati USUV e WNV negli anni 2008-2014. In particolare, si riporta il ritrovamento del virus della West Nile in specie di zanzare a seguito di apposite catture entomologiche effettuate in aziende sede di focolaio, in quattro regioni italiane. Inoltre, specifiche catture entomologiche sono state condotte tra Settembre 2010 e Aprile 2011 nella regione Molise, al fine di valutare quale ruolo potrebbero svolgere le zanzare nel promuovere l'*overwintering* dei due virus. In totale sono state catturate e analizzate 99.000 zanzare. Tra il 2008 e il 2014 sono risultati positivi alla reazione a catena della polimerasi in tempo reale (real time RT-PCR) per la ricerca del genoma di WNV e USUV rispettivamente 337 e 457 pools. Il virus della West Nile è stato rilevato in 329 pools di *Culex pipiens s.l.*, in 4 pools di *Ochlerotatus caspius*, in 2 pools di *Culex modestus* e in 2 pools di *Culex* spp. Infine 435 pools di *Cx. pipiens s.l.*, 12 pools di *Aedes albopictus*, 5 pools di *Oc. caspius*, 2 pools di *Culex* spp., 1 pool di *Anopheles maculipennis s.l.*, 1 pool di *Culiseta annulata* e 1 pool di *Ochlerotatus detritus* hanno reagito positivamente alla ricerca del virus Usutu. Al termine delle catture entomologiche effettuate nella regione Molise sono state collezionate e analizzate 1.694 zanzare; 1 pool di *Cx. pipiens s.l.*, 1 pool di *Cs. annulata* e 1 pool di *Oc. detritus* sono risultati

positivi alla ricerca dell'USUV. Il presente lavoro evidenzia come *Cx. pipiens s.l.* risulti la specie più coinvolta nella circolazione del virus della West Nile e del virus Usutu in Italia, sebbene altre specie catturate e risultate positive alla ricerca dei due virus potrebbero favorirne la diffusione, anche durante i mesi invernali.

Introduction

West Nile (WNV) and Usutu (USUV) viruses are mosquito-borne *Flavivirus*, belonging to the Japanese encephalitis virus complex. They circulate in an enzootic cycle between birds and mosquitoes (Diptera; Culicidae) and eventually in an epizootic cycle in mammals. *Culex* species mosquitoes act as vectors, while birds act as amplifying reservoir hosts. Humans, horses, and other mammals are considered incidental or "dead-end" hosts, which can be infected by mosquito species acting as "bridge vectors" (Komar 2000).

In Europe, WNV isolations have been attempted from mosquitoes of the genus *Culex*, namely *Culex pipiens s.l.* (Linnaeus, 1758) (in Bulgaria, Czech Republic, Romania, and Italy), *Culex modestus* (Ficalbi, 1889) (in France, Russia and Czech Republic), and *Culex perexiguus* (Theobald, 1903) (in Spain) (Hannoun *et al.* 1964, Hubálek and Halouzka 1999, Calzolari *et al.* 2010, Vázquez *et al.* 2011, Rudolf *et al.* 2014). Other sporadic isolations were obtained from *Anopheles maculipennis s.l.* (Meigen, 1818), *Ochlerotatus cantans* (Meigen, 1818), *Ochlerotatus caspius* (Pallas, 1771), *Ochlerotatus excrucians* (Walker, 1856), *Aedes vexans* (Meigen, 1830), and from *Coquillettidia richiardii* (Ficalbi, 1899) (Fernandes *et al.* 1998, Hubálek and Halouzka 1999).

At the same time, USUV genome has been detected in different European mosquito species, however the virus isolation has been obtained only from pools of *Cx. pipiens s.l.* in Germany and in Italy (Jöst *et al.* 2011, Calzolari *et al.* 2013).

In Italy, the first outbreak of WNV occurred in Tuscany region in 1998, among horses close to the Padule di Fucecchio marshes. In that occasion, *Cx. pipiens s.l.*, *Oc. caspius*, and *Culex impudicus* (Ficalbi, 1890) resulted the most abundant potential vectors (Autorino *et al.* 2002, Romi *et al.* 2004). The same region had experienced also the first epidemic of USUV among wild birds in 1996, as demonstrated by a retrospective study (Weissenböck *et al.* 2013).

Few years after this first WNV outbreak, in 2002, a National Surveillance Plan started in Italy. This plan – which is still in place and includes sentinel animals (horses and chickens) and mosquito collections – has been strengthened by extensive Regional Surveillance Plans, in Emilia-Romagna (since 2008), in Veneto (since 2009), Friuli Venezia-Giulia (since 2011), and Lombardy (since 2013) regions. Since 2009, mosquitoes collected within the West Nile Disease (WND) surveillance have also been tested for the presence of USUV.

In 2007 and 2008, USUV and WNV reappeared in the North-Eastern regions of Italy causing death and clinical signs in birds, horses, and humans (Manarolla *et al.* 2010, Monaco *et al.* 2010, Savini *et al.* 2012). Afterward they were found in many other Italian regions, including the major islands of Sardinia and Sicily (Calistri *et al.* 2010a, Bagnarelli *et al.* 2011, Monaco *et al.* 2011, Savini *et al.* 2011, Monaco *et al.* 2015). In particular, repeated incursions of West Nile virus have been reported between 2009 and 2014 in Italy. The reoccurrence of WNV in some areas could have been caused by the establishment of endemic cycles through hosts and vectors, allowing the virus to survive during the cold season (Calzolari *et al.* 2010, Monaco *et al.* 2011, Capelli *et al.* 2013).

To maintain an endemic cycle during Winter, the stratagems employed by arboviruses include the hibernation (Winter diapause) of adult infected mosquito females, the transovarial transmission (Farajollahi *et al.* 2005), and the viral persistence in vertebrate hosts (Kuno 2001).

The aim of this work is to review the Italian mosquito species with a possible role in WNV and USUV circulation and transmission between 2008-2014, as resulting from National and Regional surveillance plans.

In particular, we report the species found positive for WNV as a result of 4 *ad hoc* entomological investigations performed on affected farms, in 2008 in Emilia Romagna, following the first case of WND diagnosed in a racehorse, and in 2011 in Friuli Venezia-Giulia, Sardinia, and Sicily.

Finally, the possible role of mosquitoes in promoting the overwintering of WNV and USUV is discussed, as a result of the mosquito collections performed in Molise region between September 2010 and April 2011.

Materials and methods

The entomological surveillance was carried out from March 2008 until October 2014 as a part of

the West Nile Virus National Surveillance Plan and Regional WNV Surveillance Plans. The collection sites were selected according to the epidemiological situations, namely in areas with WNV circulation or wetlands considered at risk for WNV introduction through migratory birds. Insects were collected using different methods:

i) Centre for Disease Control (CDC) miniature light-traps (John W. Hock Company, Gainesville, Florida, United States), CDC traps baited with dry ice (IMT, Italian Mosquito Trap, Cantù, Italy), and modified CDC traps baited with CO_2 (Bellini *et al.* 2002) to collect host-seeking adult female mosquitoes of various species operating from sunset to sunrise, placed at about 1.5 meters from ground and kept working for 2 consecutive nights;

ii) BG-Sentinel traps (Biogents AG, Regensburg, Germany), placed on the ground in protected sites by animal attacks and operating for 48 hours;

iii) gravid mosquito traps (BioQuip Products, Rancho Dominguez, California, United States) selective mostly for gravid female mosquitoes of the genus *Culex* spp., operating in the same conditions for BG-Sentinel trap;

iv) electric aspirators (Watkins & Doncaster, Leominster, United Kingdom) to catch engorged mosquitoes in their resting sites, (*i.e.*, wall of horse boxes and animal shelters) during morning;

v) natural and artificial potential larval breeding sites close to the mosquito traps were visited for larval collections, made with a standard 500mL enamel dipper to improve the monitoring of mosquito species.

Within the national surveillance activities, targeted mosquito collections were performed on WNV affected farms in order to investigate the mosquito species composition and to identify the possible vector species. In particular in 2008, following the first case of WND diagnosed in a racehorse in Emilia-Romagna, mosquito collections were performed in Ferrara and Bologna provinces; whereas in 2011, collections were carried out in Friuli Venezia-Giulia (Gorizia, Pordenone and Udine provinces), Sardinia (Oristano province), and Sicily (Messina province).

Furthermore, following the first detection of WNV in sentinel chickens in Molise region in 2010, 3 farms (farm 1: 14.911858°E-41.884086°N; farm 2: 15.023589°E-41.882753°N; farm 3: 14.999015°E-41.89776°N) were selected to evaluate the possible contribution of the mosquitoes in the overwintering of the virus (Figure 1). These farms are located in proximity of Guardalfiera Lake (Molise region, Italy), a wetland area considered at risk for WNV introduction and characterised by the presence of a significant number of water fowl, including

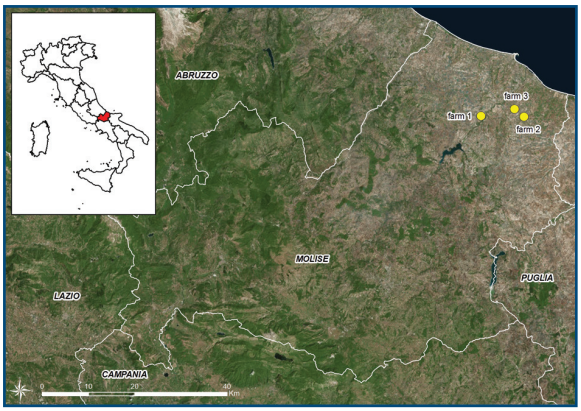

Figure 1. *Map of the Molise region, Italy, showing the mosquito collection sites for overwintering study, performed between September 2010 and April 2011.*

species of migratory birds. Larval sampling and adult mosquito collections were performed between the 16th of September 2010 and the 30th of April 2011, using CDC miniature light-traps, BG-Sentinel traps, gravid mosquito traps, and electric aspirators. Land Surface Temperature Night and Land Surface Temperature Day were extracted from MOD11A2 NASA product (1 km spatial resolution, temporal resolution 8 days) for the 2008-2011 period. Data were downloaded from the Land Processes Distributed Active Archive Center (LP DAAC) service at NASA website[1].

In all cases, adult mosquitoes collected were transported on dry ice to the laboratory, whereas larvae were preserved in 70% ethanol. The insects were identified according to Severini and colleagues (Severini *et al.* 2009) and Romi and colleagues (Romi *et al.* 1997) and divided in pools according to collection site, time, trapping method, and species, with a maximum number of 50 individuals per pool. Adults were stored at -80°C and larvae in 70% ethanol until virological analyses. In Emilia-Romagna and Lombardy, adult mosquitoes were pooled with a maximum of 200 insects per pool (Sutherland and Nasci 2007). All pools were tested for WNV and USUV by real time reverse transcriptase polymerase chain reaction (real time RT-PCR) (Cavrini *et al.* 2010, Del Amo *et al.* 2013). Males, freshly engorged, and non-engorged females were pooled and tested separately.

Results

Figure 2 and Table I show the relative abundance of the mosquito species collected in Italy, based on a total of 99,000 mosquitoes identified within the

[1] http://e4ftl01.cr.usgs.gov/MOLT.

national surveillance program between 2008 and 2014. Besides the low number of insects collected, further 2,648,878 mosquitoes from Regional Surveillance Plans were sorted and tested for WNV and USUV (Table II).

Overall, 337 mosquito pools of mosquitoes collected in 8 regions (Emilia-Romagna, Friuli Venezia-Giulia, Liguria, Lombardy, Piedmont, Sardinia, Sicily, and Veneto) and 26 provinces resulted positive for WNV in Italy, while USUV genome was found in 457 mosquito pools collected in 11 Italian regions (Emilia-Romagna, Friuli Venezia-Giulia, Liguria, Lombardy, Marche, Molise, Piedmont, Sardinia, Tuscany, Umbria, and Veneto) and involving 29 provinces.

Figure 3 shows the geographical distribution of the mosquito species resulted positive to WNV and USUV positive pools and the overall distribution of the 2 viruses in Italy. West Nile virus was detected for the first time in *Cx. pipiens s.l.* and *Oc. caspius* pools in 2008. Among all the positive pools, 329 of *Cx. pipiens s.l.* were from Sardinia and Sicily and from 6 Northern Italian regions: Emilia-Romagna, Friuli Venezia-Giulia, Liguria, Lombardy, Piedmont, and Veneto. In addition, positive pools of *Oc. caspius* (4 pools), *Cx. modestus* (2 pools), and *Culex* spp. (2 pools) were also found among those collected in Emilia-Romagna, Lombardy, and Sardinia. In particular, Table III shows the species composition of mosquitoes collected on WNV affected farms in Emilia-Romagna (2008), and in Friuli Venezia-Giulia, Sardinia, and Sicily (2011). This table also shows tested and WNV positive species as well.

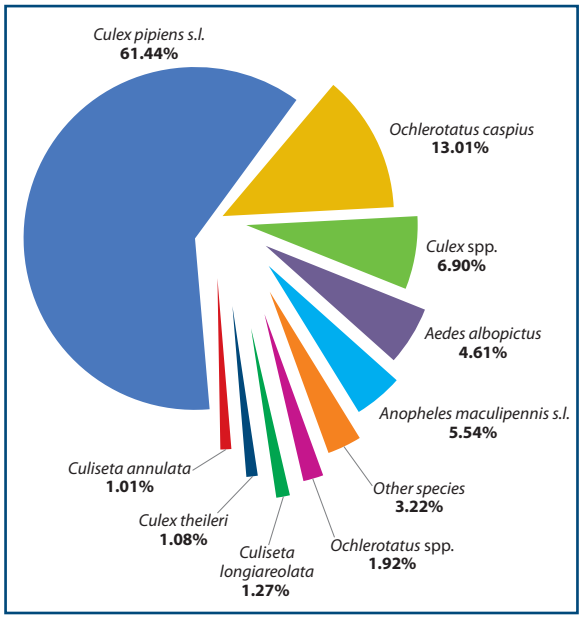

Figure 2. *Relative abundance of mosquito species collected between 2008 and 2014 in Italy within the National Surveillance Plan for West Nile Disease (total mosquitoes 99,000).*

Usutu virus was first detected in *Cx. pipiens s.l.* and in *Aedes albopictus* (Skuse, 1897) pools in 2009 in Emilia-Romagna. Four-hundred and thirty-five pools of *Cx. pipiens s.l.* were found positive in all the 11 regions affected by USUV. *Ochlerotatus caspius* was found positive in Veneto (1 pool) and Emilia-Romagna (4 pools). Finally, positive pools of *Ae. albopictus* (12 pools), *An. maculipennis s.l.* (1 pool), and *Culex* spp. (2 pools) were found among those collected in Emilia-Romagna and *Ochlerotatus detritus* (Haliday, 1833) (1 pool) and in *Culiseta annulata* (Shrank, 1776) (1 pool) collected in Molise.

During the overwintering study conducted in Molise, 301 mosquito collections were performed and 1,694 mosquitoes belonging to 5 genera and 13 species were collected and identified (Table IV). The most abundant species resulted *Cx. pipiens s.l.* (68.24 %) and *Cs. annulata* (16.23 %), which were collected during all Winter months, followed by *Oc. detritus* (5.79 %) and *Oc. caspius* (3.13%). Land Surface Temperature Night and Land Surface Temperature Day, recorded from September 2010 to April 2011 in the study area, are shown in Figure 4.

A total of 315 pools were sorted and tested for WNV and 83 pools for USUV, respectively (Table V); USUV genome was detected in 1 pool of *Cx. pipiens s.l.*, 1 pool of *Cs. annulata*, and 1 pool of *Oc. detritus*. None of the positive mosquitoes were blood engorged and all the mosquitoes were collected on April 2011. Conversely, WNV RNA was not detected in the tested samples.

Discussion

Although suggesting viral infection of mosquitoes, the detection of viral genome in arthropod does not imply vector competence. According to WHO (WHO 1967), 4 criteria have to be satisfied to consider an arthropod as competent vector for arbovirus:

i) detection of the virus from field collected insects not freshly engorged;

ii) demonstration of the ability to become infected by feeding on a viraemic vertebrate host or on an artificial substitute;

iii) demonstration of the ability to transmit biologically by biting the pathogen to a susceptible host;

iv) accumulation of field evidence confirming the significant association of the infected arthropods with the appropriate vertebrate population in which the disease or infection is occurring.

In our study, WNV was detected in 3 Italian mosquito species belonging to 2 genera: *Cx. pipiens s.l.*, *Cx. modestus*, and *Oc. caspius*. *Culex pipiens s.l.* and *Oc. caspius* resulted also positive for USUV, which was

Table I. *Mosquito species collected in Italy in the period 2008-2014, in the frame of West Nile Virus National Surveillance Plan.*

Species	2008	2009	2010	2011	2012	2013	2014	Total
Aedes albopictus (Skuse, 1897)	46	317	1,053	1,449	1,179	324	197	4,565
Aedes spp.	18	N.D.	2	1	32	1	1	55
Aedes vexans (Meigen, 1830)	2	3	16	16	40	2	N.D.	79
Anopheles algeriensis (Theobald, 1903)	N.D.	N.D.	1	N.D.	N.D.	N.D.	N.D.	1
Anopheles claviger/petregnani	1	3	16	16	13	40	16	105
Anopheles hyrcanus (Pallas, 1771)	N.D.	N.D.	N.D.	N.D.	6	175	N.D.	181
Anopheles maculipennis s.l. (Meigen, 1818)	228	658	755	2,675	661	266	239	5,482
Anopheles plumbeus (Stephens, 1828)	63	8	54	213	2	28	16	384
Anopheles spp.	12	15	16	9	10	11	31	104
Anopheles superpictus (Grassi, 1899)	N.D.	N.D.	N.D.	3	4	7	1	15
Coquillettidia richiardii (Ficalbi, 1899)	4	5	10	60	16	113	12	220
Coquillettidia spp.	N.D.	N.D.	N.D.	N.D.	N.D.	20	N.D.	20
Culex brumpti (Galliard, 1931)	N.D.	1	N.D.	3	N.D.	N.D.	N.D.	4
Culex hortensis (Ficalbi, 1889)	1	1	31	38	5	2	8	86
Culex impudicus (Ficalbi, 1890)	7	N.D.	1	N.D.	N.D.	N.D.	N.D.	8
Culex laticinctus (Edwards, 1913)	N.D.	N.D.	6	N.D.	N.D.	41	5	52
Culex mimeticus (Noè, 1899)	1	1	1	N.D.	N.D.	5	N.D.	8
Culex modestus (Ficalbi, 1889)	2	12	2	22	N.D.	N.D.	N.D.	38
Culex pipiens s.l. (Linnaeus, 1758)	5,004	12,487	6,933	12,635	15,448	2,088	6,231	60,826
Culex spp.	45	135	233	1,426	1,048	2,770	1,170	6,827
Culex territans (Walker, 1856)	N.D.	1	6	N.D.	3	N.D.	N.D.	10
Culex theileri (Theobald, 1903)	N.D.	14	422	142	15	196	279	1,068
Culex univittatus (Theobald, 1901)	N.D.	68	126	2	N.D.	N.D.	N.D.	196
Culiseta annulata (Schrank,1776)	78	25	158	250	132	172	187	1,002
Culiseta litorea (Shute, 1928)	18	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	18
Culiseta longiareolata (Macquart, 1838)	55	56	278	392	112	12	356	1,261
Culiseta morsitans (Theobald, 1904)	N.D.	N.D.	N.D.	N.D.	N.D.	3	N.D.	3
Culiseta spp.	4	5	N.D.	1	N.D.	6	15	31
Culiseta subochrea (Edwards, 1921)	N.D.	N.D.	N.D.	1	N.D.	N.D.	N.D.	1
Ochlerotatus atropalpus (Coquillet, 1902)	N.D.	N.D.	1	N.D.	N.D.	N.D.	N.D.	1
Ochlerotatus caspius (Pallas, 1771)	1,561	1,775	1,586	1,673	1,603	600	4,077	12,875
Ochlerotatus communis (De Geer, 1776)	214	1	48	109	45	3	2	422
Ochlerotatus detritus (Haliday, 1833)	430	10	N.D.	177	42	102	71	832
Ochlerotatus dorsalis (Meigen, 1830)	1	N.D.	3	N.D.	N.D.	N.D.	N.D.	4
Ochlerotatus echinus (Edwards, 1920)	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	2
Ochlerotatus geniculatus (Olivier, 1971)	N.D.	N.D.	5	1	6	N.D.	N.D.	12
Ochlerotatus rusticus (Rossi, 1790)	72	1	1	1	1	1	N.D.	77
Ochlerotatus spp.	280	198	10	644	233	227	312	1,904
Ochlerotatus zammitii (Theobald, 1903)	187	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	187
Uranotaenia unguiculata (Linch Arribalzaga, 1891)	7	6	9	5	7	N.D.	N.D.	34
Total	8,343	15,806	1,1783	21,964	20,663	7,215	13,226	99,000

N.D. = Data not available

detected in other 4 species, namely *Ae. albopictus*, *An. maculipennis s.l.*, *Oc. detritus*, and *Cs. annulata*. For each species biological traits and vector status are reported below.

As in other European Countries, *Cx. pipiens s.l.* resulted the most important vector of WNV and USUV in Italy. Widespread and abundant all over the Country, as emerged also in the 4 WND epidemics investigated, *Cx. pipiens s.l.* was repeatedly found infected by the 2 viruses (Figure 2, 3; Table I, III, V). Laboratory trials demonstrating its high efficiency and competency in viral transmission strengthened the hypothesis suggesting that *Cx. pipiens s.l.* is one of the major *Flavivirus* vectors (Hubálek and Halouzka 1999, Busquets *et al.* 2008, Toma *et al.* 2008, Calzolari *et al.* 2010, Monaco *et al.* 2010, Vazquez *et al.* 2010, Jöst *et al.* 2011, Monaco *et al.* 2011, Savini *et al.* 2011, Calzolari *et al.* 2012, Capelli *et al.* 2013,

Table II. *Number of mosquitoes tested within the Regional Surveillance Plans in Italy for West Nile Virus and Usutu Virus, in period 2008-2014 with reference to sampling region.*

Species	Emilia-Romagna	Lombardy	Veneto	Friuli Venezia Giulia	Total
Aedes albopictus (Skuse, 1897)	9,433	365	2,816	1,698	14,312
Aedes cinereus (Meigen, 1818)	100	32	43	N.D.	175
Aedes koreicus (Edwards, 1917)	N.D.	N.D.	9	N.D.	9
Aedes/Ochlerotatus spp.	N.D.	N.D.	226	4	230
Aedes vexans (Meigen, 1830)	51,503	9,230	6,678	525	67,936
Anopheles claviger/petragnani	N.D.	N.D.	15	7	22
Anopheles maculipennis s.l. (Meigen, 1818)	2,274	6,696	3,245	36	12,251
Anopheles plumbeus (Stephens, 1828)	23	122	49	28	222
Anopheles spp.	9	N.D.	1	N.D.	10
Coquillettidia richiardii (Ficalbi, 1899)	464	3	330	16	813
Culex hortensis (Ficalbi, 1889)	N.D.	N.D.	1	N.D.	1
Culex modestus (Ficalbi, 1889)	5,427	742	869	N.D.	7,038
Culex pipiens s.l. (Linnaeus, 1758)	1,553,913	114,110	637,013	41,188	2,346,224
Culex spp.	15	N.D.	562	N.D.	577
Culex territans (Walker, 1856)	N.D.	N.D.	53	1	54
Culicidae	N.D.	N.D.	1	N.D.	1
Culiseta annulata (Schrank,1776)	4	9	215	28	256
Culiseta longiareolata (Macquart, 1838)	N.D.	N.D.	1	N.D.	1
Culiseta spp.	2	N.D.	N.D.	N.D.	2
Ochlerotatus annulipes (Meigen, 1830)	N.D.	N.D.	70	N.D.	70
Ochlerotatus berlandi (Seguy, 1921)	N.D.	N.D.	2	N.D.	2
Ochlerotatus cantans (Meigen, 1818)	N.D.	N.D.	102	5	107
Ochlerotatus caspius (Pallas, 1771)	130,969	9,212	54,036	2,631	196,848
Ochlerotatus detritus (Haliday, 1833)	81	N.D.	688	1	770
Ochlerotatus geniculatus (Olivier, 1971)	715	13	42	91	861
Ochlerotatus sticticus (Meigen, 1838)	N.D.	N.D.	83	3	86
Total	1,754,932	140,534	707,150	46,262	2,648,878

N.D. = Data not available

Fros *et al.* 2015 a, b). This species has 2 biological forms: a rural and an ornithophilic one. *Culex pipiens pipiens* and *Culex pipiens molestus* are urban and mainly anthropophilic; while *Culex pipiens* f. *pipiens* is anautogenous (the first oviposition requires a blood meal) and eurygamous (the mating occurs only in large open spaces). On the contrary, *Cx. pipiens* f. *molestus* can complete the first biological cycle without blood meal (autogenous) and it mates in limited spaces (stenogamous). Moreover, *Culex pipiens* f. *pipiens* is subjected to Winter diapause (heterodynamic), whereas *Cx. pipiens* f. *molestus* does not (homodynamic) (Vinogradova 2000). Therefore, the presence of both biological forms may promote the spread of WNV from enzootic circulation – mainly in rural or sylvatic areas – among insects and usually wetland birds (enzootic cycle), to widespread epidemics acting as "bridge vector", involving synanthropic or domestic birds, mammals and humans (epizootic cycle) (Hubálek and Halouzka 1999, Hamer *et al.* 2008). In both cycles, birds serve as amplifying hosts even if the role of some species (*i.e.*, magpies and rock pigeons) should be further investigated, both in relation to their capacity of supporting a WNV epidemic and to represent a possible link between rural and urban or suburban areas (Calistri *et al.* 2010b). Usually, birds do not show any symptoms when infected with WNV, although clinical signs and deaths were observed in Sardinia (Hubálek and Halouzka 1999, Monaco *et al.* 2015).

Culex modestus is another well-known vector of WNV in Europe as well in Italy (Hannoun *et al.* 1964, Fyodorova *et al.* 2006, Toma *et al.* 2008, Monaco *et al.* 2015). It occurs mainly along the coastal plains, the preimaginal stages can develop in slightly saline water (*i.e.,* irrigation channels, marshes, and rice fields) and the maximum density of the adult mosquitoes is in July and August, then the females may enter diapause. Although this mosquito is one of the most aggressive species against humans, able to bite also during daytime, it could be regarded as a bridge vector, since it also feeds on birds (Toma *et al.* 2008, Severini *et al.* 2009). Even though *Cx. modestus* resulted with a much lower relative abundance compared to other species (Figure 2, Table I), WNV genome was identified in

Italy, particularly in Sardinia and Emilia Romagna. In particular, during the Sardinian WNV epidemic in 2011, despite the small number of tested samples (310 mosquitoes), the presence of the viral genome was confirmed in 1 pool of *Cx. modestus* composed by 3 individuals (Table III). Our results do not provide a proof of the involvement of this species in the WNV transmission. However, *Cx. modestus* is very high competent laboratory vector of WNV (Balenghien *et al.* 2007, Balenghien *et al.* 2008).

Ochlerotatus caspius is characterized by mammophilic and anthropophilic blood feeding preference. Nonetheless, during periods of very

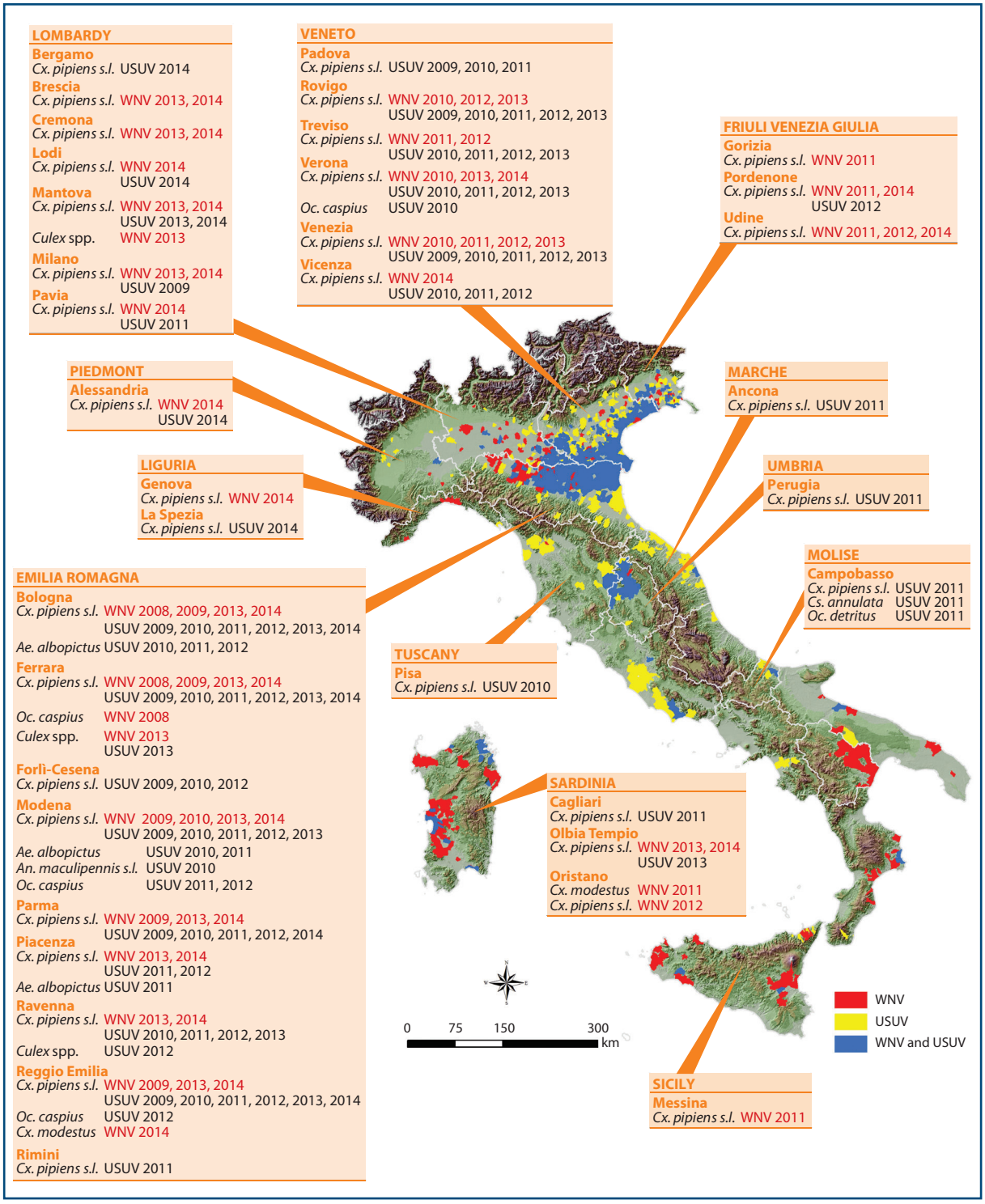

Figure 3. *West Nile and Usutu viruses circulation in Italy (1998-2014) and mosquito species resulted positive to real time reverse transcriptase polymerase chain reaction (real time RT-PCR) (2008-2014).* Red spot: area with West Nile virus circulation; yellow spot: area with Usutu virus circulation; blue spot: area with West Nile and Usutu virus circulation.

high abundance of this mosquito species, it has been occasionally found engorged with avian blood (Balenghien *et al.* 2006). Eggs generally hatch in Spring, depending on temperature as well as on the availability of water, while adult activity usually stops at the beginning of autumn (Romi *et al.* 1997). Together with *Cx. pipiens s.l.*, *Oc. caspius* resulted the most spread and abundant species (Figure 2, Table I), as previously reported by Toma and colleagues (Toma *et al.* 2008). Opposite to *Cx. modestus*, its competence for WNV resulted low under laboratory conditions (Balenghien *et al.* 2008), but its abundance could improve its vectorial capacity, as confirmed by our findings, with only 4 positive pools

Table III. *Collected and tested mosquitoes on West Nile Virus affected Italian farms in Emilia-Romagna (2008), Friuli Venezia-Giulia (2011), Sardinia (2011), and Sicily (2011).* Details related to West Nile Virus positive pools have been reported for each region.

Species	Emilia-Romagna		Friuli Venezia-Giulia		Sardinia		Sicily	
	N.	Pp/Tp	N.	Pp/Tp	N.	Pp/Tp	N.	Pp/Tp
Aedes albopictus	1	0/1	32	0/5	N.D.	N.D.	29	0/6
Aedes vexans	N.D.	N.D.	1	0/1	N.D.	N.D.	N.D.	N.D.
Anopheles claviger/petregnani	N.D.	N.D.	3	0/3	N.D.	N.D.	N.D.	N.D.
Anopheles maculipennis s.l.	25	0/11	61	0/4	117	0/11	4	0/4
Anopheles plumbeus	N.D.	N.D.	5	0/2	N.D.	N.D.	N.D.	N.D.
Coquillettidia richiardii	N.D.	N.D.	N.D.	N.D.	2	0/2	N.D.	N.D.
Culex modestus	N.D.	N.D.	N.D.	N.D.	4	1/2	N.D.	N.D.
Culex pipiens s.l.	428	3/54	130	1/14	92	0/11	278	1/27
Culex spp.	N.D.	N.D.	38	0/3	N.D.	N.D.	3	0/1
Culex theileri	N.D.	N.D.	N.D.	N.D.	14	0/4	N.D.	N.D.
Culiseta annulata	N.D.	N.D.	N.D.	N.D.	5	0/2	1	0/1
Culiseta longiareolata	8	0/6	1	0/1	7	0/2	7	0/2
Culiseta spp.	N.D.	N.D.	1	0/1	N.D.	N.D.	N.D.	N.D.
Ochlerotatus caspius	1,263	4/80	253	0/14	69	0/7	N.D.	N.D.
Ochlerotatus communis	N.D.	N.D.	7	0/3	N.D.	N.D.	N.D.	N.D.
Ochlerotatus spp.	N.D.	N.D.	42	0/6	N.D.	N.D.	N.D.	N.D.
Total	1,725	7/152	574	1/57	310	1/41	322	1/41

N.D. = Data not available; N. = number of collected and tested mosquitoes; Pp = positive pools; Tp = tested pools.

Table IV. *Number of mosquitoes collected from September 2010 to April 2011 in the study area of the Molise (Italy) grouped according to the collection method.*

Collection methods	CDC miniature light-trap	BG-Sentinel	Gravid trap	Electric aspirator	Larval collection	Total
Number of samplings	**133**	**106**	**36**	**48**	**45**	**368**
Species						
Aedes albopictus	1	5	N.D.	1	N.D.	7
Anopheles maculipennis s.l.	2	8	1	2	N.D.	13
Anopheles plumbeus	8	4	N.D.	8	N.D.	20
Culex hortensis	N.D.	N.D.	N.D.	5	N.D.	5
Culex modestus	N.D.	N.D.	N.D.	1	N.D.	1
Culex pipiens s.l.	330	90	402	37	297	1,156
Culex spp.	N.D.	1	24	N.D.	N.D.	25
Culex theileri	2	N.D.	N.D.	N.D.	N.D.	2
Culex univittatus	13	1	N.D.	1	3	18
Culiseta annulata	242	17	5	11	N.D.	275
Culiseta longiareolata	7	1	2	1	N.D.	11
Ochlerotatus caspius	50	1	1	N.D.	1	53
Ochlerotatus communis	10	N.D.	N.D.	N.D.	N.D.	10
Ochlerotatus detritus	67	11	1	19	N.D.	98
Total	732	139	436	86	301	1,694

N.D. = Data not available.

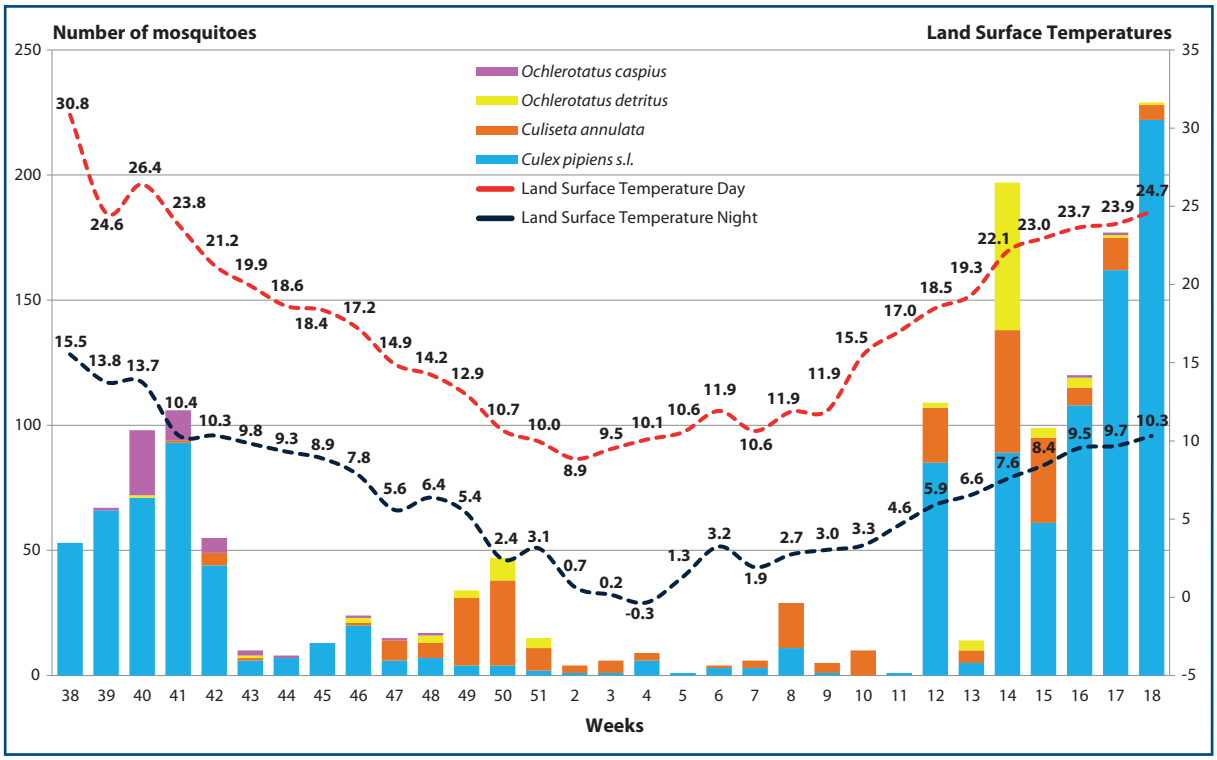

Figure 4. *Pattern of abundance of the 4 predominant species* Culex pipiens s.l., Culiseta annulata, Ochlerotatus detritus, Ochlerotatus caspius, *collected weekly in Molise (Italy) from September 2010 to April 2011.* The graph displays the Land Surface Temperature Night and Land Surface Temperature Day extracted from MOD11A2 NASA product.

(Emilia Romagna 2008) (Table III). These data would suggest that this species could be infected when the level of virus circulation is relatively high, as during an outbreak, and when the peak of abundance of *Oc. caspius* occurs. Similarly, few pools were positive for USUV (5 pools in Northern Italy), and although the vector competence for this virus is unknown, its wide distribution and its feeding behaviour make it a potentially important species in the USUV transmission cycle and a bridge vector for WNV.

Aedes albopictus, known as the 'Asian tiger mosquito', is an invasive mosquito, arrived in Italy in 1990 and nowadays it is widespread all over the country (Sabatini *et al.* 1990, Scholte and Schaffner 2007). This diurnal species is an opportunistic feeder even if it shows a strong anthropophilic behaviour and a preference for urban and peri-urban areas (Scholte and Schaffner 2007, Severini *et al.* 2009). The trapping sites, located mainly in rural areas of Italy, and the types of traps operating from sunset to sunrise (CDC miniature light-traps, CDC traps baited with dry ice and modified CDC traps baited with CO_2) may explain the lack of WNV detection from this species, and the low relative abundance (Figure 2; Table III, IV).

Nevertheless, USUV was repeatedly detected in Emilia-Romagna in *Ae. albopictus* (Figure 3), where it caused an epidemic of Chikungunya, a human disease transmitted by anthropophilic mosquitoes (Rezza *et al.* 2007). Although the mosquito activity spans from March to October, depending on rainfall and latitude, the species is able to overwinter through the production of diapausing eggs, and in temperate regions adults can fly throughout the year (Toma *et al.* 2003). The biological and ecological characteristics, including the continue trophic activity thorough Winter and the demonstrated laboratory competence for many arboviral infections, makes it suitable as bridge vector for WNV and USUV, also during the coldest months (Gratz 2004).

Anopheles maculipennis s.l. occurs as a complex and in Italy it includes 7 sibling species, namely: *Anopheles atroparvus* (Van Thiel, 1927), *Anopheles labranchiae* (Falleroni, 1926), *Anopheles maculipennis s.s.* (Meigen, 1818), *Anopheles melanoon* (Hackett, 1934), *Anopheles messeae* (Falleroni, 1926), *Anopheles sacharovi* (Favre, 1903), and *Anopheles subalpinus* (Hackett & Lewis, 1935) (Toma *et al.* 2008). Widespread in Italy, this species complex overwinters as adult mosquito in animal shelters or in warehouses (Romi *et al.* 1997). It is known to be a zoophilic species, attacking animals and humans and is considered a competent WNV vector, as demonstrated by virus isolation from field collections and laboratory competence studies. Therefore, its public health importance should

Table V. *Results from the real time reverse transcriptase polymerase chain reaction (real time RT-PCR) for West Nile Virus and Usutu Virus on mosquito pools collected from September 2010 to April 2011 in Molise (Italy).*

Species	Number of positive/Tested pools	
	WNV	USUV
Aedes albopictus	0/5	N.D.
Anopheles maculipennis s.l.	0/12	0/4
Anopheles plumbeus	0/14	0/5
Culex hortensis	0/3	N.D.
Culex modestus	0/1	N.D.
Culex pipiens s.l.	0/131	1/37
Culex spp.	0/3	0/3
Culex theileri	0/2	N.D.
Culex univittatus	0/11	N.D.
Culiseta annulata	0/70	1/21
Culiseta longiareolata	0/8	0/2
Ochlerotatus caspius	0/24	0/3
Ochlerotatus communis	0/3	0/1
Ochlerotatus detritus	0/28	1/7
Total	0/315	3/83

N.D. = Data not available.

be considered (Higgs *et al.* 2004). As shown in Figure 3, in Italy WNV has never been detected in *An. maculipennis s.l.*, whereas a single USUV positive pool was found in 2011 (Calzolari *et al.* 2012).

Culiseta annulata is common and widely spread across Italy, being considered as a bridge vector for WNV due to its blood feeding on birds, humans, and domestic animals (Higgs *et al.* 2004, Severini *et al.* 2009). We report for the first time the positive detection in Italy of USUV in *Cs. annulata* (Figure 3; Table V). This species was also found positive for USUV in Austria (Weissenbock *et al.* 2007). *Culiseta annulata* can overwinter in the larval and adult stages and the diapause is very short, because females can produce a generation also during Winter (Toma *et al.* 2008) as shown by the data displayed in Figure 4.

Finally, in 2011 in Molise we recorded, for the first time, 1 positive pool of *Oc. detritus* for USUV (Figure 3; Table V). This species is very common in the Italian coastal areas, being highly anthropophilic and considered a bridge vector since it also bites birds (Higgs *et al.* 2004, Severini *et al.* 2009). Adults of this species peak in Spring and Autumn and overwintering takes place as larva (Toma *et al.* 2008). *Ochlerotatus detritus* is considered a competent vector for the Japanese Encephalitis virus, the prototype virus of the homonym serocomplex, which includes WNV and USUV (Mackenzie-Impoinvil *et al.* 2015). Our results showed that this species could promote the overwintering of WNV and USUV, since we detected adults during Winter in Molise (Figure 4).

Considering the overwintering study, despite low abundance, an almost constant presence of adult mosquitoes was observed also during Winter marked by low temperatures with the minimum temperature close to 0°C in the period ranging from the 9th of December to the 20th of January. *Culex pipiens s.l.* was the most abundant species followed by *Cs. annulata* and *Oc. detritus*. If on one hand, the presence of *Cx. pipiens s.l.* was predictable, due to its ability to overwinter at temperate latitudes as adult females, on the other hand the finding of *Cs. annulata* and *Oc. detritus* was less expected. The former can overwinter in larval or adult stages, since females may lay eggs and produce a generation during the coldest month of the Winter (Toma *et al.* 2008). The latter shows 2 peaks in abundance, in Autumn and in Spring, as it can be observed in Figure 4, and it overwinters in the larval stages (Severini *et al.* 2009). So, the few specimens collected in the cold months could be the product of larvae hatched in late Autumn.

It is well known that weather conditions have direct effects on vectors. Many determinants (*i.e.*, temperature, rainfall, tide heights, wind speed, relative humidity, and photoperiod) influence the mosquito abundance. Previous studies reported that increased environmental temperatures and rainfall may enhance mosquito population by increasing metabolic rates, reproductive output, and host-seeking behaviour, although temperatures that are too high become unfavourable (Shone *et al.* 2006). The effects of temperature are complex due to the fact that the climate-mosquito abundance relationship is species-specific and time-dependent, and long data series are needed to continue the study of these trends (Roiz *et al.* 2014). Instead the weather effects have a much more direct and tangible impact on daily life. In our study, we extracted the Land Surface Temperature Night and Land Surface Temperature Day, which can be assumed as the minimum and maximum temperatures, to evaluate at which temperature mosquito collections were positive. In the study area at the end of November 2011 (48th week), an increase of the local air temperature and a concomitant increase of the adult mosquitoes were recorded in the 49th and 50th weeks (Figure 4). When tested for WNV and USUV, none of the mosquito pools collected during Winter resulted positive. However, during the Spring 2011, USUV was detected from *Oc. detritus* and *Cs. annulata* collected in the same farm, on April the 8th and on April the 19th, respectively. Later in the same month, USUV was detected from *Cx. pipiens s.l.* collected on the 26th April in another farm about 3 kilometres away. Although these findings confirm the circulation of USUV in the endemic mosquito fauna, they do not clarify how the pathogen was (re)introduced in the area. Maybe, the virus could have been (re) introduced by migrating birds, which have already been implicated in spreading flaviviruses in Europe

(Malkinson and Banet 2002). Otherwise, the (re) introduction of the pathogen could be the result of virus transmission among non-migratory local birds via indigenous mosquitoes. The establishment of a transmission cycle between local birds and vector population and the ability of USUV to overwinter in local mosquitoes have been already suggested in European Countries, including Italy (Weissenböck *et al.* 2002, Savini *et al.* 2011).

In fact, in Austria, Usutu virus genome was detected in overwintering *Cx. pipiens s.l.* pools by real time RT-PCR (Pfeffer and Dobler 2010). Ornithophilic mosquitoes of the genera *Culex*, *Coquillettidia*, and *Mansonia* are suspected to be the major vectors in Africa; whereas *Cx. pipiens s.l.* is considered the main vector of USUV in Europe (Weissenböck *et al.* 2007, Pfeffer and Dobler 2010, Savini *et al.* 2011, Calzolari *et al.* 2012).

Mosquitoes belonging to *Culex* genera may serve as overwintering reservoir hosts for WNV. Positive pools of diapausing *Cx. pipiens s.l.*, always at low rates, were collected during Winter in United States, usually after seasons with a high infection rate in mosquitoes (Nelms *et al.* 2013). Transovarial transmission was documented by the isolation of virus from field population of mosquito males and nulliparous females (Anderson *et al.* 2006). Moreover, laboratory tests demonstrated trans-stadial transmission from females to their offspring, thus providing the mechanism for persistence of the virus through cold Winters and re-emergence in the spring (Anderson and Main 2006, Anderson *et al.* 2008). Also diapausing immature stages of *Culex* genus resulted positive for WNV RNA by molecular tools (Unlu *et al.* 2010).

Based on the data collected, there is no evidence that USUV overwintered in hibernating mosquitoes. Nevertheless, this event cannot be excluded. Other mechanisms of viral overwintering likely occur, e.g. host birds could maintain the virus through chronic infections, although the duration of USUV infection in wild birds is still unknown, as reported by Tamba and colleagues (Tamba *et al.* 2011). In Italy, among the wild birds, the blackbird (Turdus merula) is one of the most involved species (Savini *et al.* 2011), even if USUV RNA has been detected in other bird species (Manarolla *et al.* 2010, Calzolari *et al.* 2012). In domestic chickens (*Gallus gallus*), USUV infection had different outcomes. Inconsistent viraemia or viral excretion and seroconversions were observed following experimental infection conducted by Chvala and colleagues (Chvala *et al.* 2005), whereas high titres of neutralizing antibodies were detected after natural infection in sentinel chickens (Lelli *et al.* 2008).

In conclusion, according to WHO definition, *Cx. pipiens s.l.* is confirmed to be the most frequently involved species in the WNV and USUV circulation in Italy. *Cx. pipiens s.l.* is one of the most abundant species collected, together with *Oc. caspius* (Figure 2, 3; Table I, III, IV). This result can be expected because this species is known to be the main vector for both viruses in Europe. However, we cannot ignore that the trapping activities were concentrated mainly in farming areas, thus selecting the livestock associated mosquitoes potentially involved in the epidemic cycle as bridge vectors, rather than bird feeders involved in the endemic circulation of the viruses. The types of traps and their location could also explain the scarce number of *Ae. albopictus* collected, since this species is very abundant in Italy in urban and peri-urban environments.

Furthermore, although the monitoring of Usutu virus derived from the National Surveillance Plan for West Nile Disease, USUV has been found in 6 species belonging to 5 genera, and in 11 Italian regions, suggesting a broad virus circulation in Italy and a high adaptability to different vectors. Four mosquito species, namely *Cx. pipiens s.l.*, *Cs. annulata*, *Oc. detritus*, *Oc. caspius*, all potential vectors of arboviruses, have been found active during a whole Winter season, when temperature reached almost 0°C. Even though mosquito activity decreases and stops with cold temperatures, in Italy the endemisation of these flaviviruses in hosts and vectors, which would permit the overwintering viruses, has been proposed. During Winter, very low temperatures inhibit and stop the extrinsic replication of WNV in the newly infected mosquitoes. Nevertheless, mosquitoes infected earlier could transmit the pathogens to the birds before that temperatures drop down. In addition, *Cx. pipiens* f. *molestus* does not diapause but it feeds on both human and avian blood, serving as a potential bridge vector. Finally, when the virus keeps its latency on insects during Winter, persistent infection in organs of infected birds may represent an alternative mechanisms of viral overwintering, in particular when combined with ingestion by predatory birds (Komar *et al.* 2003; Reisen *et al.* 2006). Given the biological traits described above, it is possible to conclude that these species could sustain both endemic and epidemic cycles of WNV and USUV also during winter. Further investigations are warranted to delve into this conclusion.

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