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Is it time to consider west Nile and Usutu viruses endemic in central Italy?

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ABSTRACT

West Nile (WNV) and Usutu (USUV) viruses co-circulated in a region of Central Italy (Lazio) in 2018, as evidenced by the detection of WNV in the nervous tissues of symptomatic horses and USUV in blood donors and mosquito pools. To assess whether these viruses were endemic in the region, we analysed: 1) diapausing *Culex pipiens* mosquitoes collected during the winter seasons 2022–2023 and 2023–2024, 2) *Cx. pipiens* mosquitoes collected during the adult activity period from April to November in 2022 and 2023 across 4 provinces, and 3) sera from 52 horses and tissues from 537 birds. Field-collected *Cx. pipiens*, including both diapausing and non-diapausing individuals, were tested in pools for WNV and USUV using real-time RT-PCR. Serum samples from horses were tested with two WNV ELISA assays, IgM and IgG, while bird tissues were tested for both viruses via real-time RT-PCR. A total of 18,834 *Cx. pipiens* females were collected, including 9,812 mosquitoes during the winter seasons and 9,022 during the adult activity periods. Mosquitoes were tested in 623 pools, with all pools of diapausing mosquitoes testing negative for both viruses and 12 pools of non-diapausing mosquitoes positive to USUV. The WNV IgG positivity of 7 horse sera, which were negative at the beginning of the study period, was not confirmed by the virus neutralization test. All tissue samples were negative for WNV and USUV. Since WNV and USUV were not detected in diapausing mosquitoes, there was no evidence of the two viruses endemicity in the study area.

1. Introduction

West Nile virus (WNV) belongs to the genus *Orthoflavivirus*, and is the most widely distributed arbovirus, posing a serious threat to public health worldwide (Chancey et al., 2015; Reiter, 2010). First identified in Uganda in 1937, WNV reached Europe in the 1950s, causing large outbreaks in European countries since then, with the largest reported so far in 2018, when lineages 1 and 2 co-circulated (Bardos et al., 1959; Koch et al., 2023; Smithburn et al., 1940). In eastern, Central and southern European countries, human cases of infection with WNV have been reported annually over the last decade (P. Calistri et al., 2010; Sambri et al., 2013; ECDC 2023). In Italy, two large epidemics occurred in 2018 and 2022, counting almost 600 confirmed human cases each year (Istituto Superiore di Sanità 2023), mainly affecting endemic regions of northern Italy, where human cases have been reported every year since 2010 (Riccò et al., 2022; Rizzo et al., 2016; Santi et al., 2012).

WNV is maintained in a mosquito-bird-mosquito transmission cycle, whereby mammals, particularly humans and horses, infected through mosquito bites, represent dead-end hosts for the virus and can be severely affected, with clinical manifestations ranging from mild febrile illness to severe neurological disease (Ahlers and Goodman, 2018; Barzon, 2018; Campbell et al., 2002). Among birds, species of the orders Passeriformes and Charadriiformes show greatest viremia in terms of magnitude and duration (Komar, 2003). In Europe, the main mosquito vector is represented by the widely distributed species *Culex pipiens* (Turell, 2012; Engler et al., 2013), occurring in two biological forms, *pipiens* and *molestus*, which can produce hybrids (Ciota et al., 2013). Although morphologically identical and both vectors of WNV, the two forms are genetically distinguishable and differ in their ecology and behaviour (Byrne and Nichols, 1999; Kent et al., 2007; Mattingly, 1952; Vinogradova, 2000). Hybrids are of great epidemiological importance due to their role of bridge-vector for WNV, showing a feeding strategy that includes mammals and birds (Huang et al., 2009; Fonseca et al., 2004; Kilpatrick et al., 2007; Spielman, 2001).

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Following the first detection of WNV in Italy, the Italian Ministry of Health issued an integrated plan for WNV surveillance, including the following activities: i) entomological surveillance, ii) serological surveillance in sentinel animals (horses and domestic birds), iii) passive surveillance in wild birds, iv) passive clinical surveillance in humans and horses, and v) active surveillance in synanthropic birds (*Pica pica, Garrulus glandarius, Corvus corone*) (Autorino et al., 2002; Ministero della Salute 2002; Barzon et al., 2013). The WNV surveillance plan, revised every year depending on the epidemiological situation, converged in the National Plan for Prevention, Surveillance and Response to Arboviruses 2020–2025 (PNA 2020–2025) (Ministero della Salute 2019). Since 2017, surveillance of Usutu virus (USUV), another mosquito borne *Orthoflavivirus* that shares the same amplifying hosts and vectors of WNV, has been added to the surveillance plan.

In 2018, the co-circulation of WNV and USUV was reported in Rome and Latina provinces (Lazio region, Central Italy), based on the detection of WNV in serological samples and nervous tissues from horses and USUV in mosquito pools and antibody to this virus in asymptomatic blood donors (Scaramozzino et al., 2021). Furthermore, in 2020, WNV lineage 2 was detected in a Eurasian magpie (*P. pica*) in the province of Latina, Southern Lazio region. In the decade preceding the data reported in this study (2012–2021), Lazio region was the area of Central Italy most frequently affected by WNV circulation, with cases reported in horses from three provinces of the region in 2012, 2016, 2017, and 2018 (Istituto Superiore di Sanità 2023). Given the recurrence of cases and the documented co-circulation of WNV and USUV (Scaramozzino et al., 2021), the Lazio region was considered the most epidemiologically relevant area for assessing the potential endemicity of both viruses.

In recent years, the possible WNV endemization by means of virus overwintering within diapausing *Cx. pipiens* females of the *pipiens* biological form has been investigated in several European areas and in the United States (Blom et al., 2023; Dörge et al., 2020; Reisen et al., 2006). In temperate climate zones, females of this biological form enter diapause at the end of their activity period (late autumn), suspending blood feeding until early spring (Foster and Walker, 2019). Hibernation sites (*hibernacula*) are generally caves, abandoned buildings or underground sites such as tunnels, basements, water systems and cellars, where females remain motionless during the cold season (Buffington and Zar, 1968; Nasci et al., 2000; Zittra et al., 2019). Recent studies have detected the presence of WNV in diapausing *Cx. pipiens* within their *hibernacula* (Koenraadt et al., 2019; Kampen et al., 2021; Rudolf et al., 2017).

The overall aim of the present study was to assess the potential endemicity of WNV and USUV in Central Italy and investigate their circulation during two consecutive vector activity seasons. Based on a previous research conducted in the study area (Scaramozzino et al., 2021), which highlighted the need to integrate surveillance components to detect USUV and WNV, we collected and tested horse sera, bird organs and pools of *Cx. pipiens*. In particular, mosquito sampling was intensified beyond the scheduled PNA 2020–2025 activities, by implementing winter collections and an augmented summer sampling within a restricted area affected by viral co-circulation. Samples of *Cx. pipiens* mosquitoes, collected during both winter diapause and the vector activity season, were tested for WNV and USUV using real-time RT-PCR, horse sera were analysed using WNV and USUV ELISA assays and bird tissues were tested for both viruses via real-time RT-PCR.

2. Methods

2.1. Mosquito collections

Mosquitoes were collected during the adult activity period from mid-April to mid-November, in 2022 and 2023 at 4 provinces in the Lazio region: Viterbo, Roma, Latina and Frosinone. The provinces cover approximately 16,500 km^2, extending from the Tyrrhenian coast (west) to the Apennine Mountains (east), within a latitude range of 41°09′N to 42°48′N and a longitude range of 11°20′E to 13°40′E. The study area is characterized by a Mediterranean climate along the coast and a continental climate in the Apennines. Mosquitoes collections were performed using CDC light traps, with dry ice as a source of CO_2, according to the PNA 2020–2025 (Ministero della Salute 2019). The traps were placed on farms and operated for one night on a fortnightly basis. A total of 603 mosquito collections were performed at 25 different sites: 22 in 2022 and 23 in 2023. Of these, 20 sites were sampled in both years, while the remaining sites were unique to a single year (Table 1A, Fig. 1A). Besides surveillance activities foreseen by the PNA 2020–2025, further mosquito samplings were performed in two municipalities of the Latina province, within the area interested by the 2018 WNV and USUV circulation (Table 1A, Fig. 1A). In these municipalities, 6 farms were selected, based on the presence of positive WNV horses in 2018 and of suitable mosquito larval habitats. Sixty samplings were carried out, placing two CDC—CO_2 light traps and one Gravid trap on each farm, which operated between the afternoon and the following late morning, on a fortnightly basis, from mid-May to mid-November 2022. Gravid traps were baited with a mixture of water, hay, guinea pig faeces and urine.

To detect the presence of WNV and USUV in diapausing *Cx. pipiens*, mosquito collections involved 30 samplings of overwintering female of this species using portable aspirators during the 2022–2023 and 2023–2024 winter seasons. Overwintering sites consisted in natural caves, artificial cavities, abandoned buildings and ancient ruins, in Rome and Latina provinces (Fig. 1B).

2.2. Mosquito identification and pooling

Collected mosquitoes were transported to the laboratory and stored at −20 °C for 30 min to ensure their death and then sorted and identified following the morphological key for the Italian Culicidae fauna (Severini et al., 2022). *Culex pipiens* females were divided into pools according to the date and location of collection. Pools consisted of not <4 individuals and up to 100 during the activity season and up to a maximum of 30 specimens, to increase virus detection sensitivity, during winter season. This strategy followed a more conservative approach than the PNA 2020–2025 (Ministero della Salute 2019), which allows pools of up to 200 individuals, while ensuring a balance between practical feasibility and detection sensitivity. All pools were stored at −80 °C until tested for WNV lineage 1 and 2 and for USUV.

2.3. Synanthropic birds collection

The active and passive surveillance on synanthropic birds was performed following the guidelines of the PNA 2020–2025. The active surveillance was carried out by means of scheduled removal (by shooting) of *C. corone, P. pica and Corvus* sp., from March 2022 to June 2024, in the provinces of Latina and Viterbo. Passive surveillance was carried out by collecting birds found dead of the above-mentioned species, whenever their presence was reported to public health authorities. These surveillance activities were carried out in the specified provinces, designated as WNV high-risk transmission areas by the PNA 2020–2025, following reports of WNV infections in equids in Viterbo (2017) and in Latina (2018) (Istituto Superiore di Sanità 2023). A total of 537 shot or found-dead birds were collected in 25 municipalities and were necropsied to obtain organs (spleen, kidney, brain or myocardium) to be tested separately or as organs' pools. Organs were stored at −80 °C until testing.

2.4. Molecular testing of mosquito pools and synanthropic birds for WNV and USUV

Cx. pipiens pools were suspended in 0.9 ml of ATL buffer (Animal Tissue Lysis buffer, Qiagen) and a 5 mm stainless steel bead. Single organs or organs pools were prepared as follows: 0.1 g of each sample was

Table 1
Summary of the entomological activities performed during the vector activity season (A) and during two consecutive winter seasons (B).

A)	Mosquito sampling activity (period)	Year	Province (municipality N° or name)	Total catches	Total *Cx. pipiens* female (N° tested)	Total pools N°	Positive Pools			MIR for USUV by catch/site (date and pool size)
							West Nile lineage 1	West Nile lineage 2	Usutu	
	PNA 2020–2025 (mid-April to mid-November)	2022	Viterbo (7)	84	78 (36)	3	0	0	0	NA
			Roma (8)	137	1773 (1522)	41	0	0	3	8.33 (20th July, N° = 12) 1.05 (20th July, N° = 108) 2.86 (3rd August, N° =36)
			Latina (4)	57	703 (647)	28	0	0	1	3.23 (28th September, N° = 36)
			Frosinone (1)	13	128 (118)	3	0	0	1	1.28 (20th July, N° = 80)
		2023	Viterbo (7)	97	118 (38)	4	0	0	0	NA
			Roma (8)	151	1712 (1555)	35	0	0	0	NA
			Latina (4)	64	911 (838)	33	0	0	1	20.00 (3rd November, N° = 6)
	Mosquito samplings in selected farms (mid-May to mid-November)	2022	Latina (Aprilia)	32	1683 (1672)	41	0	0	1	1.04 (13th July, N° = 96)
			Latina (Cisterna di Latina)	28	1920 (1909)	44	0	0	5	0.60 (3rd August, N° = 166) * 0.24 (31st August, N° = 419) 25.00 (31st August, N° = 4) 1.05 (14th September, N° = 95)
B)	**Mosquito sampling activity (period)**	**Winter season**	**Province (*hibernacula* N°)**	**Total catches**	**Total *Cx. pipiens* female (N° tested)**	**Total pools N**	**Positive Pools**			**MIR for USUV by catch/site (date)**
							West Nile lineage 1	**West Nile lineage 2**	**Usutu**	
	Mosquito samplings in *hibernacula* (December-March)	2022–2023	Latina (12)	17	8052 (8052)	253	0	0	0	NA
			Roma (2)	2	131 (131)	14	0	0	0	NA
		2023–2024	Latina (10)	11	1630 (1629)	124	0	0	0	NA

* Two pools of *Cx. pipiens* (total N° = 332) collected the same day at the same site resulted positive to USUV.

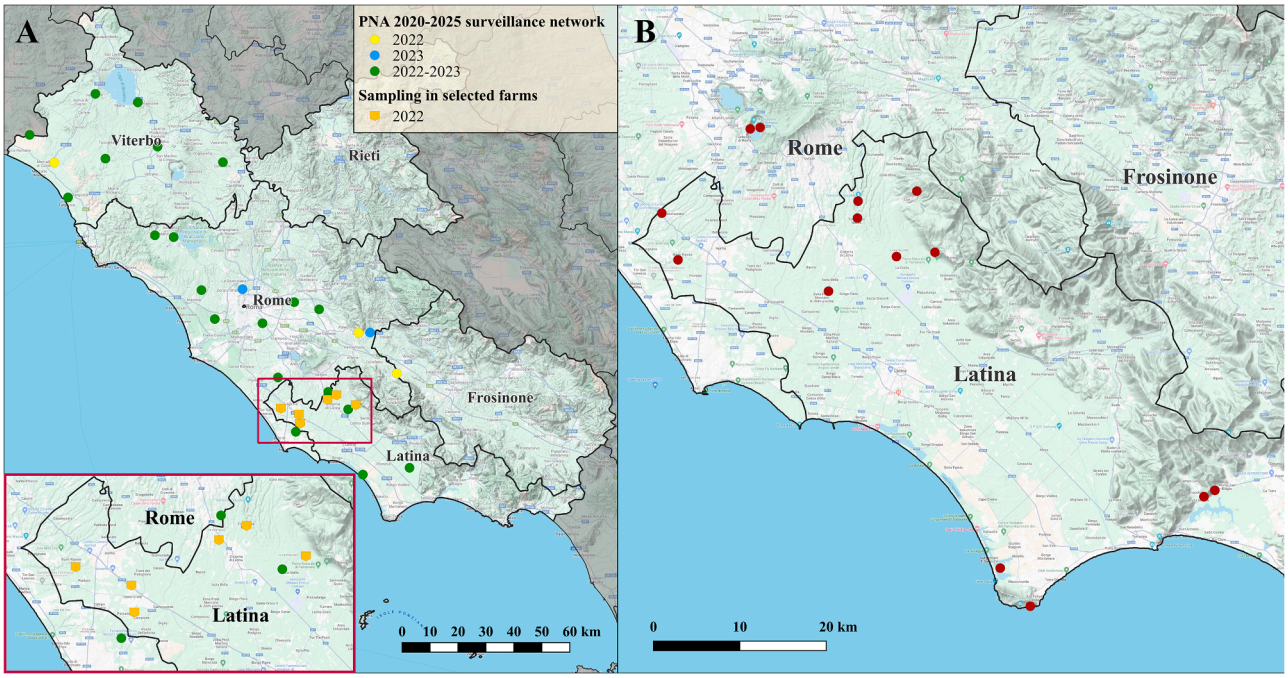

Fig. 1. Distribution of the entomological sampling sites. In (A), the sites were samplings were carried out following the national surveillance plan: circles correspond to single CDC—CO_2 trap, orange shields represent sites where two CDC—CO_2 traps and one gravid trap were used. In the bottom left rectangle, the area interested by WNV and USUV co-circulation in 2018, where further mosquito samplings were performed in 6 selected farms. In (B), red points indicate the 14 *hibernacula* where diapausing mosquitoes were collected in two winter seasons (2022–2023 and 2023–2024).

added to 0.9 ml of buffer ATL (tissue lysis buffer, Qiagen tissue lysis buffer) plus a 5 mm stainless steel bead. All samples were homogenized by Tissue Lyser II (QIAGEN, GmbH, Hilden, Germany), followed by a centrifugation at 16,000 g. A volume of 400 µl from the supernatant was used for the nucleic acid extraction using the automatic extractor QIAsymphony (DSP Virus/Pathogen Kit, Qiagen, GmbH, Hilden, Germany) according to the manufacturer instructions.

All samples were then subjected to a direct detection procedure of WNV-L1, WNV-L2 and USUV by real-time RT-PCR as previously described (Del Amo et al., 2013; Cavrini et al., 2011).

2.5. Serological sampling in horses

Twelve equine holdings were selected and sampled in three municipalities of the Latina province: Cisterna di Latina (N° = 8), Aprilia (N° = 2) and Cori (N° = 2). Horses with previous reported WNV vaccination and frequent movement for sport events were excluded. A total of 63 animals were selected and sampled at the first time-point (T_0). Horses whose blood serum tested positive for IgG against WNV were excluded, reducing the number of animals to 52. Blood samples were then collected at three subsequent time-points (T_1, T_2 and T_3), before and after the vector activity season in two consecutive years (2022 and 2023). If any of the 52 selected animals were moved or vaccinated for WNV during the study period, no samples were taken at T_1, T_2 and T_3. Horses' sera were tested for the presence of IgM and IgG against WNV and IgG against USUV using the ELISA kit ID Screen® Flavivirus Competition (IDvet, Grabels, France). Positive samples were sent to the National Reference Centre for Foreign Animal Diseases (CESME) for diagnosis confirmation with Virus Neutralisation (VN) test.

3. Results

3.1. Entomological sampling from April to November

A total of 663 mosquito collections were performed during the vector activity period (April to November) of two consecutive years (Table 1), collecting 10,801 mosquitoes through both catches on selected farms and as part of the entomological surveillance carried out according to PNA guidelines. If not specified, numbers hereafter reported refer to female mosquitoes. Mosquitoes samplings performed in selected farms of the Latina province yielded to the collection of 3992 individuals of Culicidae of which 3862 were *Cx. pipiens* (96.74 %): 3603 females and 259 males. The remaining 130 mosquitoes belonged to the following species: *Aedes albopictus* (74, 1.85 %), *Aedes caspius* (32, 0.80 %), *Culiseta annulata* (7, 0.18 %), *Culiseta longiareolata* (7, 0.18 %), *Anopheles plumbeus* (5, 0.13 %), *Aedes geniculatus* (2, 0.05 %), *Anopheles maculipennis* s.l. (2, 0.05 %) and *Culex modestus* (1, 0.02 %). Regarding the entomological activity for the PNA surveillance, 6781 Culicidae were collected of which 5489 were *Cx. pipiens* (80.95 %): 5423 females and 66 males. The remaining mosquitoes belonging to the following species: *Ae. albopictus* (510, 7.52 %), *Ae. caspius* (426, 6.28 %), *Aedes* sp. (156, 2.30 %). *Ae. detritus* (139, 2.05 %), *An. plumbeus* (16, 0.24 %), *Culiseta subochrea* (15, 0.22 %), *Cs. longiareolata* (13, 0.19 %), *An. maculipennis* s.l. (9, 0.13 %), *Ae. geniculatus* (4, 0.06 %), *Cs. annulata* (3, 0.04 %) and *Culiseta* sp. (1, 0.02 %).

3.2. Entomological sampling of diapausing mosquitoes

A total of 30 mosquito catches were performed in the selected 14 *hibernacula* (Table 1, Fig. 1B), mainly artificial (6) or natural caves (4), with few exceptions consisting in an ancient Roman cistern and three abandoned buildings in rural environment. Overall, 9879 Culicidae were sampled inside the *hibernacula*, 9832 being *Cx. pipiens* (99.52 %): 9813 females and 19 males. Remaining specimens belonged to the following species: *Culex hortensis* (35 females and 1 male, 0.36 %), *An. maculipennis* s.l. (7, 0.07 %) and *Cs. annulata* (4, 0.04 %).

3.3. Molecular analyses on mosquito and synanthropic birds

A total of 18,839 *Cx. pipiens* females were collected (Table 1). Taking into account the pooling procedure and the limited number of *Cx. pipiens* collected on certain sampling occasions, 232 pools were generated from 8335 specimens collected during the vector activity period and 391 pools from 9812 specimens collected in *hibernacula* (Table 1). All *Cx. pipiens* pools from active and diapausing mosquitoes tested negative for the presence of WNV lineage 1 and 2. None of the pools from diapausing mosquitoes tested positive for the presence of USUV. Twelve pools of *Cx. pipiens* collected from July to September in three Lazio provinces in 2022 (11 pools) and 2023 (1 pool), tested positive for the presence of USUV: 8 from Latina, 3 from Rome and 1 from Frosinone (Table 1) (Fig. 2A and B). The overall MIRs calculated for each province were as follows: Rome 0.20; Latina 0.17, Frosinone 0.85, for *Cx. pipiens* collected in 2022. In 2023, during the vector activity period, only one pool tested positive for USUV, in the province of Latina, whose overall MIR was 0.12. A total of 537 birds, belonging to the species *C. corone* (N = 519), *P. pica* (N = 13) or identified as *Corvus* sp. (N = 5), were collected in the provinces of Viterbo (northern Lazio) and Latina (southern Lazio) (Fig. 3). None of the analysed birds tested positive for the presence of WNV lineage 1 and 2 and USUV (Table 2).

3.4. Serological analyses in horses

Considering horses resulted negative al T_0, none seroconverted during the study period. All analysed sera tested negative to ELISA tests for WNV IgM and USUV IgG, while sera from 6 horses testing positive to WNV ELISA IgG at T_1 and one at T_3 were not confirmed at VN test (Table 3).

4. Discussion

The study was aimed at investigating the circulation and the possible endemization of West Nile and Usutu viruses in Central Italy, where their co-circulation was reported in recent years (Scaramozzino et al., 2021). To achieve these objectives, the different components of the integrated surveillance system worked in synergy, performing analyses on mosquitoes, horses and synanthropic birds. Furthermore, a targeted survey within *Cx. pipiens hibernacula* was performed to highlight the possible overwintering of WNV and USUV in diapausing mosquitoes. Our results highlighted that WNV did not circulate in the study area between spring 2022 and March 2024, as it was not detected in horse sera, bird tissues or in mosquitoes collected during the vector activity season or within *hibernacula*. These findings provide no evidence of WNV endemicity in the Lazio region. Indeed, as regards Central Italy, according to the National Epidemiological Bulletin on WN fever, only sporadic cases of infection in humans (N = 6) and horses, and 2 pools of WNV-positive mosquitoes were documented from 2018 to 2024 (Istituto Superiore di Sanità 2023). A markedly different epidemiological scenario is observed in northern Italy, where outbreaks with numerous human cases occur annually, reaching nearly 600 cases during the major outbreaks of 2022, while only three human cases were reported in Central Italy (northern Tuscany) during the same year (Riccardo et al., 2022; Young et al., 2019). As regards USUV, the detection of positive mosquito pools in both vector activity seasons could suggest an enzootic circulation of this virus in Central Italy, although the observed circulation pattern raises questions about the long-term stability of USUV in the area. While endemization cannot be ruled out, further longitudinal studies are needed to exclude fluctuating circulation patterns and other factors that may limit USUV's long-term establishment. Therefore, it could be hypothesized that the two viruses, although characterised by similar life cycles, have different circulation patterns.

However, the lack of WNV positive *Cx. pipiens* pools during the vector activity seasons may reflect the different sensitivity of the entomological surveillance to WNV and USUV. This result is in line with

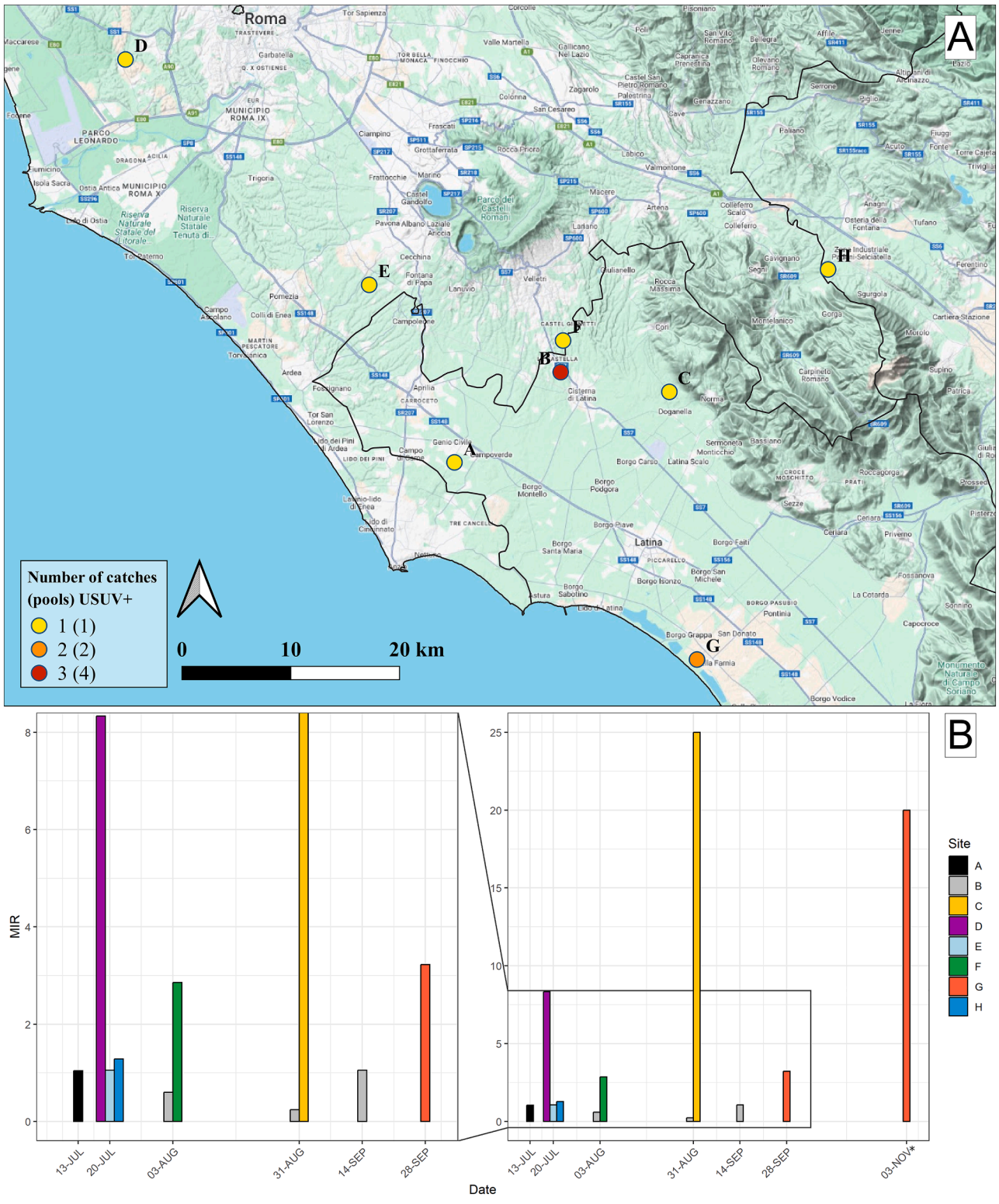

Fig. 2. Entomological sampling results. In (A), distribution of the 8 sites where pools of USUV positive mosquitoes were detected: 4 in the province of Latina (A, B, C and G), 2 in the province of Rome (E and F) and 1 in the province of Frosinone (H). In (B), minimum infection rate (MIR) calculated for each capture and site. All dates reported refer to 2022, except for the one indicated by the asterisk (*) which concerns 2023.

previous findings from northern Italy, where compared with 89 pools of *Cx. pipiens* positive for USUV, only three were positive for WNV (Calzolari et al., 2012). Differences in sensitivity between surveillance components were also reported by a previous study, where surveillance of horses revealed the presence of WNV at sites where mosquitoes were only positive for USUV (Scaramozzino et al., 2021). In fact, the importance of surveillance on equids has been repeatedly emphasised in recent years (Young et al., 2019; García-Carrasco et al., 2023). WNV was not detected in birds analysed during the study period, though a Eurasian magpie (*P. pica*) tested positive for the presence of WNV in the Latina province in November 2020.

Regarding winter sampling and analysis of diapausing mosquitoes,

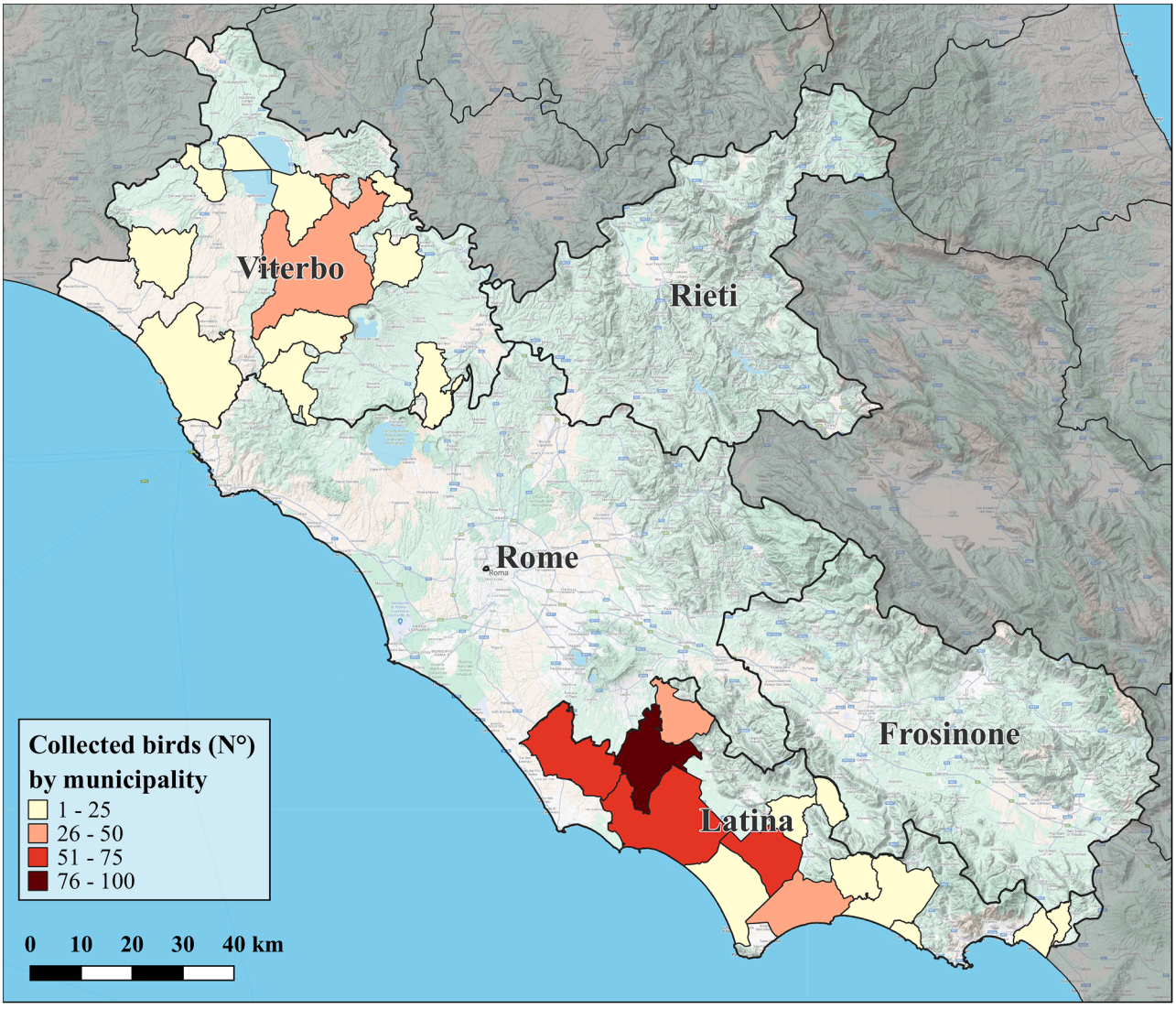

Fig. 3. Summary of the distribution of collected birds. The different shades of red indicate the number of culled or dead-found birds by municipality. None of the birds tested positive for the presence of WNV-L1, WNV-L2 and USUV.

Table 2
Summary of the results of the RT-PCR for Usutu and WN viruses on birds.

Year	Province (municipality N°)	Total bird collected	*Corvus corone*	*P. pica*	*Corvus* sp.	PCR results (N° positive/ N° tested birds)		
						West Nile lineage 1	West Nile lineage 2	Usutu
2022	Viterbo (1)	14	10	4	0	0/14	0/14	0/14
	Latina (12)	201	199	2	0	0/201	0/201	0/190
2023	Viterbo (11)	85	73	7	5	0/85	0/85	0/85
	Latina (7)	175	175	0	0	0/175	0/175	0/175
2024	Latina (5)	62	62	0	0	0/62	0/62	0/52

our results did not highlight an overwintering of WN and Usutu viruses in the study area. Nevertheless, despite the huge number of tested mosquitoes, it cannot be ruled out that they were infected at rates below our detection limit. In fact, no pool tested positive for the two orthoflaviviruses, despite the number of *Cx. pipiens* collected being comparable to previous studies, when over several thousand of collected *Culex* spp., just 5 pools of *Cx. pipiens* resulted positive to WNV and one pool of *Culex torrentium* for USUV (Koenraadt et al., 2019; Kampen et al., 2021; Rudolf et al., 2020; Sauer et al., 2023). It should be considered that not recording WNV and USUV in diapausing mosquitoes does not imply the complete absence of this overwintering route. Although massive captures of diapausing *Culex* spp. can be considered a useful method to demonstrate overwintering of orthoflaviviruses such as Usutu and West Nile, several factors could hinder their detection. As a matter of fact, though vertical transmission appears to be the mechanism by which the overwintering mosquitoes are infected, filial infection rates are generally low, suggesting that arbovirus persistence during cold season in diapausing mosquitoes occurs only sporadically (Zittra et al., 2019; Anderson et al., 2008; Anderson et al., 2012; Dohm et al., 2002). This raised questions about the cost-effectiveness of analysing diapausing mosquitoes for detecting arbovirus persistence, suggesting that other overwintering routes might be investigated (Blom et al., 2023).

Table 3
Summary of the results of the WNV ELISA IgG test on horse sera collected in the Latina province for each sampling period.

Municipality	Horse holdings	T_0 (April - May 2022)		T_1 (November - December 2022)		T_2 (March 2023)		T_3 (November - December 2023)	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Aprilia	Ap 01	0	8	0	4	0	3	0	2
	Ap 02	4	10	1	1	0	0	0	0
Cisterna di Latina	Ci 01	1	1	0	1	0	1	0	1
	Ci 02	0	9	0	5	0	4	1	2
	Ci 03	0	10	2	8	0	8	0	0
	Ci 04	0	2	0	1	0	1	0	1
	Ci 05	0	1	0	1	0	1	0	1
	Ci 06	2	3	2	0	0	0	0	0
	Ci 07	1	4	1	0	0	0	0	0
	Ci 08	2	3	0	1	0	1	0	0
Cori	Co 01	0	1	0	0	0	0	0	0
	Co 02	1	0	0	0	0	0	0	0
Total		**11**	**52**	**6***	**22**	**0**	**19**	**1***	**7**

* Results not confirmed by Virus Neutralisation test.

Two hypotheses can be drawn up to explain the occasional finding of the WN and Usutu viruses in the study area: a) the two viruses could be hypoendemic in the study area, with a continuous circulation cycle; b) repeated introductions by mean of long-distance migratory trans-Saharan birds (Calistri et al., 2010; Lu et al., 2024) or the re-occurrence of previously circulating strains as consequence of internal birds' movements between and within EU countries, as more recently suggested (Beck et al., 2020; Mencattelli et al., 2022; Zecchin et al., 2021). The relevance of the intra-Palearctic migratory route in the spread of WNV and USUV has been supported by the genetic similarities between the strains circulating in Italy and those circulating in Europe (Zehender et al., 2017; Mancuso et al., 2022).

5. Conclusions

In conclusion, there is no evidence to suggest the endemicity of WN and Usutu viruses in the study area. However, the surveillance results indicate that the environmental conditions required for USUV circulation are present. In contrast, the conditions necessary for the circulation of WNV appear to be only sporadically present (Istituto Superiore di Sanità 2023; Scaramozzino et al., 2021), suggesting that the conditions required for its establishment as endemic are lacking. Nonetheless, the possibility of a low-level hypoendemicity for WNV, undetectable by the current surveillance system, cannot be entirely excluded. The ease of sampling overwintering *Cx. pipiens* females, diapausing motionless and in high abundances in *hibernacula*, allows to speculate about the effectiveness of eventual control strategies focused on this phase of their life cycle. Indeed, in *hibernacula* it may be easy to eliminate large numbers of females on which the start of the new vector season relies, thus hindering the population from thriving (Liu et al., 2016; Kobayashi et al., 2012).

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Ethics approval and consent to participate

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Federico Romiti: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Teresa Scicluna:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Francesco Censi:** Resources, Investigation. **Florindo Micarelli:** Supervision, Resources. **Silvia Puccica:** Investigation, Data curation. **Andrea Carvelli:** Writing – review & editing, Validation, Formal analysis, Data curation. **Marcello Giovanni Sala:** Validation, Supervision, Conceptualization. **Irene Del Lesto:** Investigation, Formal analysis, Data curation. **Riccardo Casini:** Investigation, Formal analysis, Data curation. **Claudio De Liberato:** Writing – review & editing, Writing – original draft, Validation, Conceptualization. **Silvia Tofani:** Writing – original draft, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships:

Federico Romiti reports financial support, equipment, drugs, or supplies, and travel were provided by Italian Ministry of Health. Silvia Tofani reports equipment, drugs, or supplies and travel were provided by Italian Ministry of Health. Marcello Giovanni Sala reports article publishing charges and travel were provided by Italian Ministry of Health. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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