

Original Contribution

Transmission Dynamics of the West Nile Virus in Mosquito Vector Populations under the Influence of Weather Factors in the Danube Delta, Romania

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Abstract: Mosquitoes were collected in the Danube Delta during the active seasons of 2011–2013. For *Culex* spp. mosquitoes, the abundance was calculated. *Culex pipiens* (sensu lato), (*s.l.*) and *Culex modestus* pools were tested for the presence of West Nile virus (WNV) genome, and the maximum likelihood of the infection rate was established. Mean daily temperatures and precipitation were obtained for the closest meteorological station. A negative binomial model was used to evaluate linkages between the temperature/precipitation and mosquito population size. A zero-inflated negative binomial model was used to test the relationship between the temperature and the infection rate. A single complex model for infection rate prediction was also used. The linkages were calculated for lag 0 and for 10 days earlier (lag 1), 20 days earlier (lag 2), and 30 days earlier (lag 3). Significant positive linkages ($P < 0.001$) were detected between temperature and mosquito population size for lag 1, lag 2, and lag 3. The linkages between temperature and infection rates were positive and significant for lag 2 and lag 3. Negative significant ($P < 0.001$) results were detected between precipitation and infection rates for lags 1, 2, and 3. The complex model showed that the best predictors for infection rate are the temperature, 20 days earlier (positive linkage) and the precipitation, 30 days earlier (negative linkage). Positive temperature anomalies in spring and summer and rainfall decrease contributed to the increase in the *Culex* spp. abundance and accelerated the WNV amplification in mosquito vector populations in the following weeks.

Keywords: West Nile virus, mosquitoes, infection rate, Danube Delta, weather factors, climate change

INTRODUCTION AND PURPOSE

West Nile virus (WNV) is an emerging pathogen of public health importance. The virus is transmitted by mosquitoes

and belongs to the Japanese encephalitis (JE) serocomplex of the genus *Flavivirus*, *Flaviviridae* family (Paz 2015). The enzootic cycle is driven by continuous virus transmission to susceptible bird species through adult mosquito blood-meal feeding which results in virus amplification (Dohm et al. 2002). Wild birds are both enzootic and amplifying hosts and are involved in the dispersal and introduction of the virus into new areas (Rappole et al. 2000). *Culex* species

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mosquitoes (family *Culicidae*) are the main enzootic and/or epizootic vectors (Zeller and Schuffenecker 2004). The virus may be transferred to close-by human communities where it is amplified in *Culex* spp. mosquitoes-peridomestic bird cycles, and may generate outbreaks in secondary hosts (i.e., humans and horses) (Paz and Semenza 2013).

In Europe and Eurasia, WNV circulation is mainly confined to two land-use patterns: first, the circulation of WNV exists in urban areas where mosquitoes feed both on birds and on humans and thus act as bridge vectors (e.g., Platonov et al. 2008). The second and more frequent environment is found in rural regions where wetlands serve as bird-nesting areas that provide ideal conditions for establishing WNV endemic cycles (Hubálek and Halouzka 1999). These ecosystems include river deltas and floodplain areas where bird-feeding mosquitoes thrive and the bird-mosquito cycle is propagated (Paz and Semenza 2013). Bodies of open water are attractive biotopes for resting and nesting birds, and are semi-permanent larval habitats for mosquito species (e.g., *Cx. modestus*) in wet areas (Leblond et al. 2007). Therefore, wetlands which support large bird populations (García-Bocanegra et al. 2012) and serve as habitats for the development of stable mosquito vector populations provide adequate environmental and biotic conditions for the establishment, maintenance, and amplification of the WNV. Indeed, most WNV outbreaks in Europe and Eurasia have occurred in wetland areas such as the Rhone delta in southern France, the Volga delta in southern Russia, and the Danube Delta in Romania (Paz and Semenza 2013).

A major WNV fever outbreak occurred in humans in Romania in 1996, with 393 serologically confirmed or probable cases, of which 352 were neuroinvasive infections (Tsai et al. 1998). This was the first significant epidemic in Europe as well as the first occurring in urban settings in the continent, and was caused by a virus strain belonging to the WNV genetic lineage 1, the outbreak vector being *Culex pipiens* (*sensu lato*), (*s.l.*) (Savage et al. 1999). The affected area in 1996 was the southeast of the country and there was no evidence of transmission in Transylvania, suggesting that the Carpathian Mountains represented a barrier against further spread (Tsai et al. 1998). Since then, scattered human cases have been recorded every year in southeastern Romania (ECDC 2011). An expansion of the virus activity beyond the Carpathian Mountains in Transylvania, was detected in 2008 (Ceianu, unpublished). Another outbreak, with 54 cases of WNV neuroinvasive disease in humans, occurred in 2010, and was caused by a strain of the genetic

lineage 2 of WNV, similar (99%) to the strain that caused a human outbreak in the Volga Delta in 2007 (Sirbu et al. 2011). Molecular evidence for the persistence of this lineage 2 strain in Southeastern Romania (including the Danube Delta region) for 4 years was obtained both from mosquito vectors and patients with WNV fever (Dinu et al. 2015; Sirbu et al. 2011). These findings suggest that across two decades at least two introduction events of WNV strains belonging to different genetic lineages occurred in southeastern Romania, a region exposed to virus introduction by birds, as it is crossed by a main migration flyway connecting Africa to Europe (Paz et al. 2013).

Among other drivers, climatic conditions (specifically ambient temperature and precipitation) have direct and indirect influences on WNV transmission: Temperature plays an important role in viral replication rates and the transmission of WNV by affecting the length of extrinsic incubation and the seasonal phenology of mosquito host populations (Reisen et al. 2006; Kilpatrick et al. 2008; Ruiz et al. 2010; Paz et al. 2013; Paz 2015). When the temperature rises, it causes an upsurge in the growth rates of vector populations, decreases the interval between blood meals, shortens the incubation time from infection to infectiousness in mosquitoes, and accelerates the virus evolution rate (e.g., Ruiz et al. 2010; Paz 2015). The role of precipitation in WNV transmission is more indirect and less consistent, depending on differences in the ecology of mosquito vectors. On the one hand, above-average precipitation may lead to a higher abundance of mosquitoes and increased potential for disease outbreaks (Landesman et al. 2007), on the other, below-average precipitation can facilitate population outbreaks of some species of mosquitoes since wetlands drying disrupts the aquatic food-web interactions that limit larval mosquito populations (Chase and Knight 2003; Roehr 2012).

The importance of climatic factors as drivers in the epidemiology of WNV is increasing under conditions of climate change (Paz 2015). Indeed, the changing climate has impacts on the emergence of vector-borne diseases such as WNV (Lafferty 2009; Ostfeld 2009; Parham et al. 2015). According to the WHO (2015), climate change is likely to alter the geographic range of such diseases, to lengthen their transmission seasons and to affect beyond their current seasonal patterns. Recent studies showed that climatic changes, particularly the increase in ambient temperature and fluctuation in rainfall amounts, have contributed to the endemization process of WNV in various locations around the world (Paz and Semenza 2013; Paz 2015). Conse-

quently, it is important to analyze the effect of weather anomalies on WNV activity. Recent observations for Eastern Europe showed more temperature extremes, less summer precipitation, more river floods in winter, and higher water temperatures (IPCC 2013). The linkage between WNV outbreaks in Romania and severe heatwaves has been shown in a few studies. Paz et al. (2013) observed that the WNV outbreak of summer 2010 in Romania was preceded by extremely hot spells with deviations of 5°C above the 30-year mean average. Strong significant positive correlations were detected between the number of cases in humans in southeastern Romania, at lags of 1–3 weeks between the rise in temperature and the eruption of the disease in the city of Bucharest, an area with a continental climate, and a lag of 2–4 weeks in the Constanța area, on the Black Sea shore.

Although both mosquito and seroconversion in bird-based surveillance of WNV provided evidence of WNV activity before human cases were recorded, it was shown that mosquito surveillance performed better in terms of early WNV circulation detection (Unlu et al. 2009; Healy et al. 2015). Surveillance of WNV infection of mosquito vectors provides information on the level of its amplification prior to the emergence of disease in humans.

The purposes of the present study are to estimate mosquito abundance and infection rate in the wetland ecosystem of the Danube Delta during 2011–2013, as well as to analyze the influence of the two main weather factors, temperature and precipitation, on the abundance of the main *Culex* vector species in the area, and on the WNV infection rate in the vectors.

To the best of our knowledge, this is the first field study in Europe showing the weather impact on the WNV infection rate in mosquito vectors.

METHODS

Site Description

Mila 26 (Lat. 47°1794'N; Long. 29°0683'E) is located in the core of the fluvial Danube Delta between Sulina channel (S), Maliuc village (Fig. 1), the Crânjala canal, and the forest area of Păpădia. The site can be reached by boat only and includes an abandoned fish farm, with interconnected channels, which are flooded with water in the spring when the Danube level is high and the snow melts. During the summer, the region is not fed by water from the main

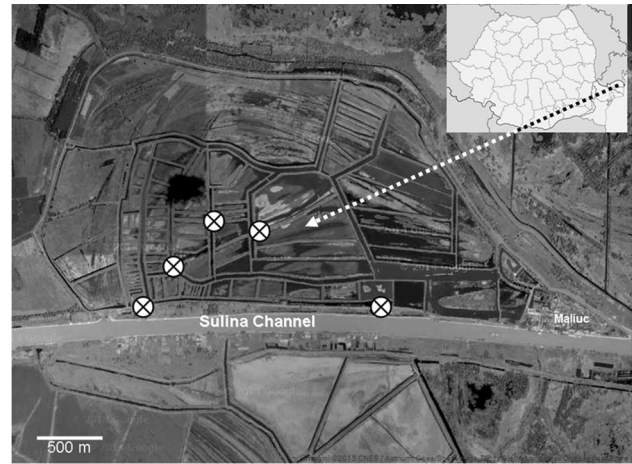


Figure 1. Map of the Danube Delta study site with the mosquito collection stations.

Danube river and, with the lack of precipitation, the water level decreases and some of the ponds even dry up by the end of the season. The ponds are characterized by extensive reed beds while between the channels the soil is sandy with xerophytic vegetation. The site serves as a passage and nesting area for many species of birds, mainly aquatic birds, together with many cows, pigs, and horses that roam freely in the area. The village of Maliuc (populated by fewer than 300 people) is situated in proximity to the study site along the Sulina canal; however, the anthropogenic pressure in the area is limited.

Mosquito Collections and Infection Rate Calculation

Vectors were collected during the active mosquito seasons, for one period of three to nine nights per month, from the beginning of May until the end of October over 3 years: 2011–2013. The mosquitoes were captured overnight in five locations, covering an area of about 0.6 sq km., three in the reed-bed area and two in the village (Fig. 1), using chicken-bait and rodent (laboratory mice or Guinea pig)-baited cylindrical box traps, with animal bait being protected from biting by mosquito nets. The traps in the village were set 2 km apart, while the traps in the reed bed were 0.3–0.8 km away from one another. The sites of capture in the village and those in the reed bed were 0.5–1.2 km apart. BG Sentinel traps with BG-Lure (Biogents AG, Germany) and Modified CDC backpack aspirators (John W. Hock Company, USA) were also used in 2012 and 2013, mainly in

reed beds. The insects were collected in the morning, placed in cryotubes and preserved and transported in dry nitrogen shippers to the laboratory, where they were kept in -60°C freezers till processing. For the virus detection, the mosquitoes were identified on a cold table, using a stereomicroscope and morphological key (Becker et al. 2010). Since both *Cx. pipiens s.l.* and *Cx. torrentium* are present in the fauna of Romania, and the females of these species are indistinguishable morphologically, the molecular identification of 18 individual females for each period of capture was performed using enzymatic restriction of fragment of mitochondrial cytochrome c oxidase subunit I (COI) gene following the protocol described in Hesson et al. (2010).

The abundance of *Culex* spp. mosquitoes for each of the *Culex* species—*Cx. modestus* and *Cx. pipiens*, was calculated for every interval of capture, as the average number of females collected per night in one bird-baited trap.

Mosquitoes were identified using entomological keys (Becker et al. 2010) and pooled by species, sex, physiological age, site, and date of capture, in pools that never exceeded 50 individuals. The pools were homogenized in 1 mL PBS (pH 7.2) supplemented 20% with fetal calf serum, 200 $\mu\text{g}/\text{mL}$ streptomycin, 200 U/mL penicillin, 1 $\mu\text{g}/\text{mL}$ amphotericin B, in a Sartorius Mikro-Dismembrator U (2000 rpm, 3 min.). The homogenate was centrifuged (14,000 rpm, 4°C) and 140 μL of supernatant was used for RNA extraction with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). For the detection of the WNV genome, a commercial TaqMan assay (Sacace Biotechnologies, Como, Italy) was performed.

For each interval of capture, the WNV infection rate (IR) index in mosquitoes was calculated for *Culex* spp. mosquitoes (cumulated number of *Cx. pipiens* and *Cx. modestus*), using the maximum likelihood estimate (MLE), an indicator which is more accurate than the minimum infection rate (MIR), mainly when infection rates are high (Gu et al. 2008). The MLE of WNV infection rate in mosquitoes was calculated using the PooledInfRate software (Biggerstaff 2009).

Weather Parameters

The mean daily temperatures and daily precipitation data for May–October in 2011–2013 were obtained from the National Administration for Meteorology of Romania, for the Gorgova meteorological station, which is situated eight km away from the study site and characterizes its weather conditions. The mean daily temperature and cumulated

daily precipitations were calculated for ten-day periods, (three ‘periods’ per month). These parameters were used in the statistical analysis. In addition, for a better understanding of the typical weather conditions in the period under study, the monthly temperature and precipitations anomalies from the monthly perennial averages of the period 1981–2010 were also obtained.

Statistical Analysis

The association between the mosquito abundance/mosquito infection rate (that showed non-Gaussian distribution) and the weather factors, was analyzed using Generalized linear models (GLM). A negative binomial Model (NB) appropriate for a counting mode, usually used for overdispersed count outcome variables (Bruin 2006), was used to evaluate possible linkages between the temperature/precipitation data and the mosquito population size. A zero-inflated negative binomial model (ZINB) for modeling count variables with excessive zeros, usually used for overdispersed count outcome variables (Bruin 2006), was used to test the relationship between the temperature/precipitation data and the infection rate to account for excess zeroes. The same model (ZINB) was used also to test associations between the mosquito population size and the infection rate. “MASS” package was used to fit the NB model (Venables and Ripley 2002) and R package “pscl” was used to fit the ZINB models (Zeileis et al. 2008; Jackman 2015). The infection rates were treated using the number of infected mosquitoes (as a count data) as an outcome and the total number of mosquitoes as a model offset. It is important to note that different models fit (Binomial, Poisson, NB, ZINB, Zero-Inflated Poisson) were compared by Akaike Information Criterion. ZINB model showed the best fit for infection rates data, and NB model—for the number of mosquitoes.

In addition, in order to integrate all predictor variables, a single complex model for the prediction of WNV infection rate was tested with the same statistical framework (ZINB model). Mosquito abundance, date (period), temperature, and precipitation were entered as potential predictor variables in hierarchical order. The model fit was evaluated using an Akaike Information Criterion (AIC). Variables, which did not contribute to the model, were omitted. The final model represents the model with the minimal AIC.

Based on previous studies that showed lag correlations between weather conditions and WNV outbreaks in humans in the following month (e.g., Soverow et al. 2009; Paz

et al. 2013), the linkages in the current study were calculated for real time (lag 0) and for three lag times of the weather parameters: 10 days earlier (lag 1), 20 days earlier (lag 2), and 30 days earlier (lag 3).

RESULTS

Mosquito Samples and Infection Rates

Three species of the genus *Culex* were captured: *Culex pipiens* (sensu lato) Linnaeus (59.38%), *Culex modestus* Ficalbi (40.61%), and *Culex martinii* Medschid (0.1%).

The identity of the *Cx. pipiens s.l.* species was confirmed by molecular testing; no *Cx. torrentium* Martini specimen was detected in the samples tested. The large majority of *Culex* spp. mosquitoes were captured by animal-baited traps: only 16.34 and 2.87% were captured by BG sentinel traps and backpack aspirator in 2012 and in 2013, respectively. The average number of captures per trap night during June-end of August/early September, for *Culex* spp., was 125.67 in 2011, 113.74 in 2012, and 108.50 in 2013. The abundance of the *Culex* spp. vectors expressed by the number of mosquitoes collected by bird-baited traps per night increased steadily in June–July, when the values ranged between a minimum of 54.13 to a maximum of 84.42 (both values recorded in July 2012), and reached a peak by the end of summer-early September (221.75 in 2011, 234.99 in 2012, and 184.82 in 2013). From mid-September, the captures by bird-baited traps suddenly decreased to below 20 individuals per night trap (Table 1; Fig. 2). *Culex pipiens* numerically exceeded *Cx. modestus* in most capture periods. It was clearly the dominant species from June until the end of August and represented 71.89–83.77% of the collected *Culex* spp. However, by the end of each transmission season an increase in the proportion of *Cx. modestus* was found (Table 1). The spatial distribution of *Cx. modestus* was significantly associated with the reed beds, while *Cx. pipiens* was distributed more widely, found in the whole area under investigation, and also collected in large numbers in the village.

WNV was detected by TaqMan assay in 82 of 302 *Cx. pipiens* and in 60 of 204 *Cx. modestus* pools tested.

Although the association between the mosquito population size and the infection rate was not significant, the seasonal dynamics of the WNV infection rate of the vectors, expressed by its maximum likelihood estimated (MLE) value per 1000 mosquitoes (%), showed that the virus amplification had a positive trend toward the end of the

summer. However, each transmission season had a different pattern: in 2011 the WNV-infected mosquitoes were detected only at the end of August and the infection rate was rather low (2.57‰). In 2012 the first infected mosquitoes appeared with a sudden high infection rate at the end of July (40.74‰), a warmer month than usual, then the WNV infection rate decreased by half by the end of August (20.22‰). In 2013 the earliest infected *Culex* spp. were detected at the end of June (4.08‰) and the WNV infection rate increased steadily until early September (16.62‰). After mid-September, with the weather cooling and shorter periods of daylight which are triggers of diapause induction, the mosquitoes did not show attraction to the hosts, the captures by bird-baited traps suddenly decreased, and no WNV-infected mosquitoes were found.

In each interval of capture in which a WNV presence was detected in mosquitoes, both *Cx. pipiens* and *Cx. modestus* were infected. In *Cx. pipiens*, the MLE of WNV infection rate per 1000 mosquitoes ranged from a minimum of 3.73 in late June 2013 to a maximum of 38.91 by the end of July 2012, with an average of 12.22. In *Cx. modestus*, it ranged between 1.31 recorded in August 2011 to 41.84 at the end of July 2012, with an average of 19.01.

Weather Conditions During the Study Period

Over the study period positive monthly temperature anomalies (from the monthly perennial averages of the standard period of 1981–2010) were observed each year from the spring to the fall, especially in 2012 which was warmer than usual from April to October, and less so in 2013 (Fig. 3). In 2012, from early July until August, the air temperature exceeded 25°C which is 3.1°C above the standard climatological norm for July. In 2011 a daily average temperature of 25.8°C was reached only for a short period by mid-July, while daily temperatures above 25°C were not recorded at all in 2013 (Fig. 4).

Precipitation deficits (Fig. 3) as compared to the monthly perennial averages occurred in the spring (March–April) each year, were severe from August 2011 to June 2013 and, despite partly compensated snowfall in winter, led to drought.

Linkages Between Weather Parameters and Mosquito Population Size/Infection Rate

Linkages between daily mean temperature and mosquito population size (both for ten-day periods) were significantly positive for lag 1 ($B = 0.25345$, $P < 0.001$), lag 2

Table 1. Average *Culex* spp Vector Captures by Bird-Baited Traps, Results of Tests for the Presence of West Nile Virus (WNV) Genome, and MLEs (Maximum Likelihood Estimates) of WNV Infection Rate in the Main WNV Vectors, *Cx pipiens* s.l. and *Cx modestus*, Danube Delta, 2011–2013.

| Year | Interval of capture | Mosquito species | Average no. of mosquitoes/night/trap | WNV positive pools/ no of mosquitoes tested | MLE of WNV infection rate/ 1000 <i>Culex</i> spp. (95% CI) |
|------|---------------------|------------------------|--------------------------------------|--|---|
| 2011 | June II | <i>Culex pipiens</i> | 48.52 | 0/194 | 0 |
| | | <i>Culex modestus</i> | 31.00 | 0/124 | 0 |
| | | Total <i>Culex</i> spp | 79.52 | 0/318 | 0 |
| | July II | <i>Culex pipiens</i> | 33.42 | 0/167 | 0 |
| | | <i>Culex modestus</i> | 42.34 | 0/211 | 0 |
| | | Total <i>Culex</i> spp | 75.76 | 0/378 | 0 |
| | August III | <i>Culex pipiens</i> | 98.75 | 2/426 | 4.96 (0.88–15.51) |
| | | <i>Culex modestus</i> | 123.00 | 1/775 | 1.31 (0.07–6.2) |
| | | Total <i>Culex</i> spp | 221.75 | 3/1201 | 2.57 (0.68–6.82) |
| 2012 | June II | <i>Culex pipiens</i> | 72.81 | 0/545 | 0 |
| | | <i>Culex modestus</i> | 8.62 | 0/113 | 0 |
| | | Total <i>Culex</i> spp | 81.43 | 0/658 | 0 |
| | July I | <i>Culex pipiens</i> | 41.32 | 0/381 | 0 |
| | | <i>Culex modestus</i> | 12.81 | 0/79 | 0 |
| | | Total <i>Culex</i> spp | 54.13 | 0/460 | 0 |
| | July III | <i>Culex pipiens</i> | 61.00 | 18/729 | 38.91 (23.96–62.50) |
| | | <i>Culex modestus</i> | 23.42 | 8/285 | 41.84 (20.42–84.72) |
| | | Total <i>Culex</i> spp | 84.42 | 26/1014 | 40.74 (27.34–60.29) |
| | August III | <i>Culex pipiens</i> | 207.25 | 24/1642 | 18.58 (12.27–27.39) |
| | | <i>Culex modestus</i> | 27.74 | 7/318 | 27.52 (12.53–55.32) |
| | | Total <i>Culex</i> spp | 234.99 | 31/1960 | 20.22 (14.08–28.44) |
| | September II | <i>Culex pipiens</i> | 9.00 | 0/100 | 0 |
| | | <i>Culex modestus</i> | 8.35 | 0/504 | 0 |
| | | Total <i>Culex</i> spp | 17.35 | 0/604 | 0 |
| 2013 | June III | <i>Culex pipiens</i> | 53.34 | 4/1116 | 3.73 (1.21–8.99) |
| | | <i>Culex modestus</i> | 7.14 | 1/160 | 6.11 (0.37–29.66) |
| | | Total <i>Culex</i> spp | 60.48 | 5/1276 | 4.08 (1.52–9.02) |
| | August I | <i>Culex pipiens</i> | 64.60 | 6/898 | 7.14 (2.97–14.83) |
| | | <i>Culex modestus</i> | 15.62 | 6/337 | 21.06 (8.89–44.24) |
| | | Total <i>Culex</i> spp | 80.22 | 12/1235 | 10.77 (5.39–18.26) |
| | September I | <i>Culex pipiens</i> | 102.22 | 32/2357 | 16.93 (11.85–23.70) |
| | | <i>Culex modestus</i> | 82.60 | 38/2924 | 16.25 (11.70–22.17) |
| | | Total <i>Culex</i> spp | 184.82 | 70/5281 | 16.62 (13.08–20.91) |
| | September III | <i>Culex pipiens</i> | 0.75 | 0/6 | 0 |
| | | <i>Culex modestus</i> | 4.00 | 0/24 | 0 |
| | | Total <i>Culex</i> spp | 4.75 | 0/30 | 0 |

The Roman numerals represent a ten-day period per each month (for example, June II is the second ten-day period of June).

($B = 0.19116$, $P < 0.001$), and lag 3 ($B = 0.21053$, $P < 0.001$), (Table 2). The relationships between temperature and infection rate were positive and significant for lag 2 ($B = 0.421804$, $P < 0.001$) and lag 3 ($B = 0.49826$, $P < 0.001$).

The linkages between the amounts of rainfall and the mosquito population size were not significant. However, significant negative relationships were detected between precipitation and infection rate in mosquitoes for lag 1 ($B = -0.08646$, $P < 0.001$), lag 2 ($B = -0.10994$,

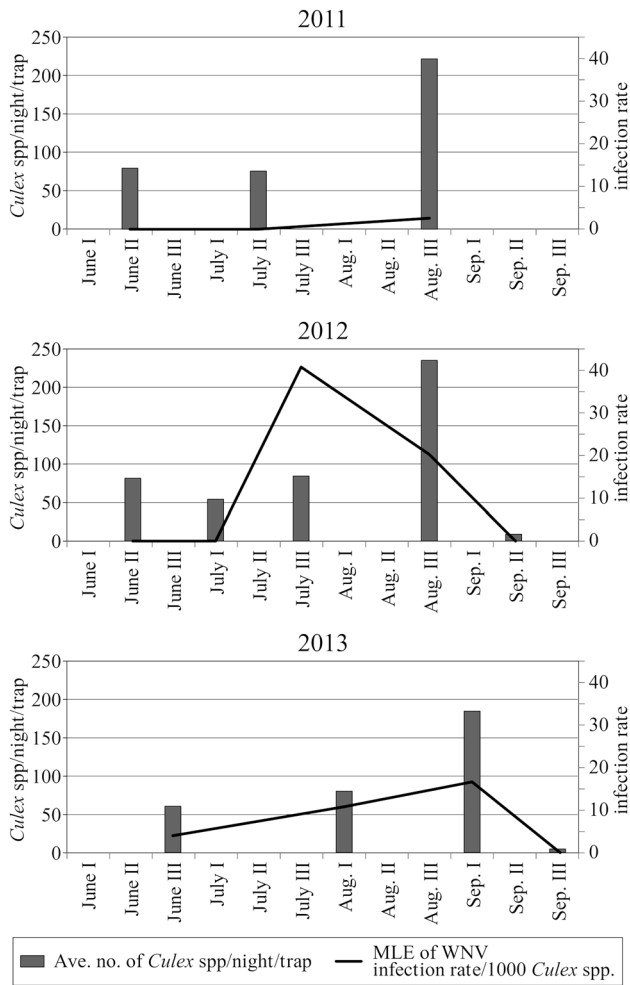


Figure 2. The abundance of West Nile virus (WNV) *Culex* spp. mosquito vectors, calculated as the average number of mosquitoes collected per night trap for each 10-day period of May–September (2011–2013), and the maximum likelihood estimate (MLE) of the WNV infection rate in *Culex* spp mosquitoes for the same period.

$P < 0.001$), and lag 3 ($B = -0.08446$, $P < 0.001$) (Table 2).

As was mentioned, a single complex model for prediction of WNV infection rate was tested. Since lag 2 and lag 3 of the temperature were highly correlated, we tested them in separate models and used only one of them, which contributed more to the model fit. The results of the final model after the process of variable selection are: for Temp_lag2: $B = 0.36287$; $P < 0.001$, for Rain_lag3: $B = -0.0917$; $P < 0.01$. It was found that the best predictors for the WNV infection rate are the temperature, 20 days earlier (lag 2) which shows positive linkage and the precipitation, 30 days earlier (lag 3) which has negative linkage.

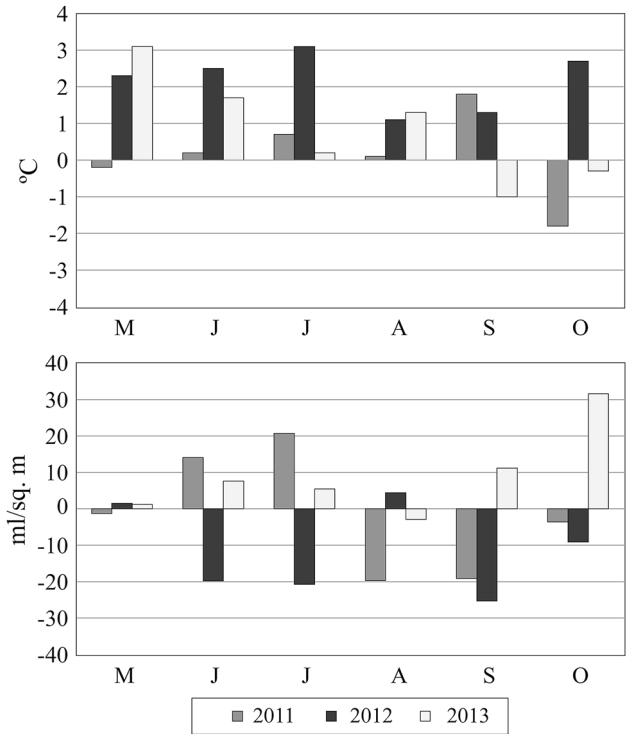


Figure 3. Monthly average temperature (*top*) and precipitation (*bottom*) anomalies of March–October 2011–2013, from the monthly perennial averages of the period 1981–2010.

DISCUSSION

The present data describe the WNV transmission mosquito vector populations, *Cx. pipiens s.l.* and *Cx. modestus*, in a wetland ecosystem, the Delta of the Danube River, under the influence of temperature and precipitation fluctuations.

WNV transmission in southeastern Romania in non-epidemic years appears very focal, with scattered small areas of transmission, and entomological surveillance may perform poorly because trap placement becomes of paramount importance (Ceianu et al. 2001). This site was chosen for investigation, despite the significant logistic challenges, since it had previously shown an active circulation of WNV revealed during three transmission seasons by higher infection rates in mosquitoes than those detected in other locations surveyed in Romania (Prioteasa L, unpublished). The Danube Delta is the final or intermediate station of migrating birds’ flyways which may introduce the virus and provide suitable conditions for WNV maintenance and amplification. The importance of this area as an endemic region for WNV activity is relevant to risk assessment in other areas on the same migrating route, as for example in Israel which is also a major stopover for

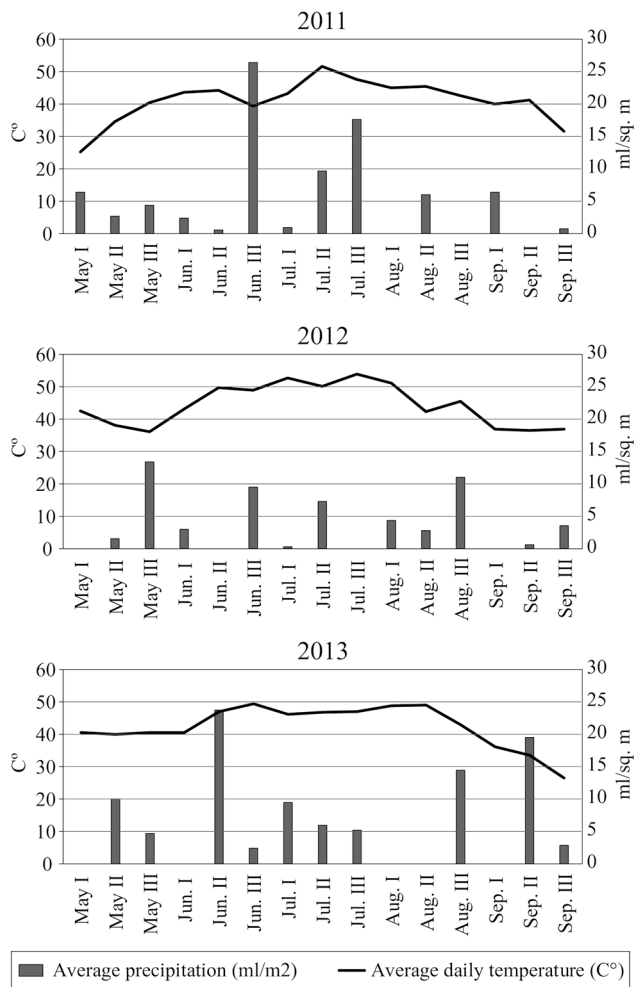


Figure 4. Average daily air temperature and precipitation amounts per 10-day period (I–III) per month for May–September, 2011–2013.

migrating birds. WNV detected in mosquitoes collected at this study site from 2011 to 2013 was sequenced and was 99% similar to the WNV lineage 2 that was involved in the Volgograd 2007 outbreak, and also closely related to the sub-Saharan isolates from South Africa and Congo (Dinu et al. 2015). Moreover, the molecular evidence for the maintenance of the same WNV genotype in the study site for three transmission seasons as well as the finding of that virus genotype, both in mosquitoes and human patients from other localities in southeastern Romania, is documenting endemic circulation in the area (Dinu et al. 2015).

Although the entomological investigation of 2011–2013 in the Danube Delta was not comprehensive and the mosquito captures were limited to periods of three to nine nights, as weather permitted, it still provides a reliable picture of the sylvatic cycle and transmission dynamics of WNV, under the impact of seasonal succession and weather

Table 2. Linkage Between Temperature/Precipitation and Mosquito Population Size/Infection Rate.

| | <i>B</i> | <i>P</i> value |
|---|-----------|----------------|
| Linkage between temperature and mosquito population size | | |
| lag 0 | 0.04377 | n.s. |
| lag 1 | 0.25345 | < 0.001 |
| lag 2 | 0.19116 | < 0.001 |
| lag 3 | 0.21053 | < 0.001 |
| Linkage between temperature and infection rate | | |
| lag 0 | 0.089513 | n.s. |
| lag 1 | 0.158855 | n.s. |
| lag 2 | 0.421804 | < 0.001 |
| lag 3 | 0.49826 | < 0.001 |
| Linkage between rainfall amounts and mosquito population size | | |
| lag 0 | 0.003309 | n.s. |
| lag 1 | −0.004395 | n.s. |
| lag 2 | −0.014508 | n.s. |
| lag 3 | 0.009264 | n.s. |
| Linkage between rainfall amounts and infection rate | | |
| lag 0 | −0.023007 | n.s. |
| lag 1 | −0.086456 | < 0.001 |
| lag 2 | −0.109940 | < 0.001 |
| lag 3 | −0.084455 | < 0.001 |

parameters. The seasonal population dynamics of *Cx. pipiens s.l.* and *Cx. modestus* found in this study site during 2011–2013 was similar to that reported by Török et al. (2016), for an area covering 160 sq km in the Danube Delta, in 2014, and is typical of the deltaic ecosystem. Every year the Delta mosquito habitat undergoes a landscape succession starting with spring floods and progressive water-level reduction, loss of contact with the main river channels, habitat shrinkage, and concentration of suspension and organic matter in the water by the end of the summer. This ecological succession favors the increase of *Cx. pipiens* abundance. In fact, *Cx. pipiens*, a species with well-documented bird-feeding preferences (Rizzoli et al. 2015), but also recognized as a bridge vector for various arboviruses (Farajollahi et al. 2011), was the dominant species in most of the collections. Indeed, urban *Cx. pipiens s.l.* has already been documented as the WNV epidemic vector in Romania (Savage et al. 1999). *Culex modestus*, the reed-bed mosquito, known as a WNV vector in the wetlands of southern Europe (Balenghien et al. 2006) was previously confirmed by our research group (Dinu et al. 2015) as a WNV vector for Romania for the first time. By its feeding behavior both on birds and mammals, it also

represents an excellent bridge vector (Balenghien et al. 2006; Radrova et al. 2013). The vector competence for WNV, as was shown in laboratory experiments (Balenghien et al. 2008), is significantly higher in *Cx. modestus*, as compared to *Cx. pipiens*, which was found to be only moderately competent.

In the site under investigation the *Cx. pipiens* population increased significantly in the summer of 2012 when high temperatures and drought affected the region and the WNV infection rate in mosquitoes also reached a peak, with high infection rates of 40.74/1000. In the transmission season of the following year, the WNV-infected mosquitoes were detected as early as June and it is reasonable to assume that this early amplification of WNV in 2013 was a consequence of a very active circulation of WNV in the previous season. Indeed, as was shown earlier, the identical genotype of WNV lineage 2, highly similar to the Volgograd 2007 WNV strain, was endemically established in southeastern Romania, being maintained over the winter by various mechanisms including vertically infected arthropods such as *Cx. pipiens* mosquitoes (Dinu et al. 2015) and *Hyalomma marginatum* ticks (Kolodziejek et al. 2014).

In the current research, significant positive linkages were found between the temperature and mosquito population size as well as with the infection rate, while the highest temperature anomalies (in July 2012) were followed by the highest infection rate (40.74/1000). Our study detected changes in the number of infected mosquitoes in the investigated populations, and found that temperature increases were correlated with higher rates of infected mosquitoes. These findings reinforce previous studies which showed that increased temperatures cause an upsurge in the growth rates of mosquito populations (Kunkel et al. 2006; Reisen et al. 2006; Jia et al. 2007; Kilpatrick et al. 2008; Ruiz et al. 2010). In an earlier study (Paz et al. 2013), significant lag correlations of up to four weeks between temperature and WNV eruptions in humans were demonstrated in southern and Eastern Europe (including Romania). The authors found that the mosquito population abundance and virus response followed increasing ambient temperature by several weeks while the strongest lag time correlations with human incidence obtained for Romania (and Russia) were in Bucharest at lags of 2–3 weeks and in Constanța at lags of 1–4 weeks. We assume that these strongest results for Eastern Europe may be a consequence of more sensitive temperature thresholds in regions with a temperate climate, which may have an im-

act on the virus epizootic/epidemic transmission. In such regions (including the current study area) an increase in temperature may lead to a stronger response in virus amplification than in warmer areas (Paz et al. 2013).

According to the National Meteorological Service of Romania (Meteo-Romania), the monthly average temperatures in the study area during the hot seasons of 2011–2013 were above the monthly perennial averages (of the period 1981–2010). The highest continuing positive anomalies were measured in 2012, with anomalies of 2.3–3.1°C from April to July. High anomalies were documented also in 2013 with 2–3.1°C above the monthly average in April–May and 1.7°C in June (Fig. 3). A previous study (Paz and Albersheim 2008) noted that extreme heat in the spring has a potential influence on the vector population increase and on the appearance of the disease in the human population weeks later.

We show that the early rise of temperature in the study area contributed to the increase in mosquito population size and accelerated the transmission of WNV in the vectors in the following weeks.

In the current study, significant negative linkages were detected between precipitation and infection rate in mosquitoes. Based on the documentation of Meteo-Romania, the monthly average precipitation in the study area during all the spring seasons of the years 2011–2013 were below the monthly perennial averages (of the period 1981–2010), with only 52% of the monthly average in March 2011, 22% in March 2012, and 27.4% in March 2013. April was also dry with 58% of the monthly mean in 2012 and only 25% in 2013. Dry conditions were documented also in the summer of 2012 with 56 and 42% of the monthly means in June and July, respectively.

A common dogma is that high amounts of precipitation increase the availability of standing water for the mosquito population and accelerate their abundance. However, the picture is more complex and the literature shows inconsistent results since the response might vary over different regions, depending on differences in the ecology of mosquito vectors (Paz 2015). Drought conditions may reduce the water flow and create stagnant water pools ideal for breeding mosquitoes. The linkage between a decrease in precipitation amounts and WNV transmission has been shown in several studies. Epstein and Defilippo (2001) noted that the outbreak in the Danube Valley in 1996 coincided with a prolonged drought from May to October. Regional trends showed that an earlier drought contributed to the initial WNV outbreak in the USA

(Hubálek 2000; DeGroot et al. 2008). Landesman et al. (2007) found evidence that human incidence in the western USA is associated with below-average rainfall in the previous year. More recently, Roehr (2012) noted that the WNV transmission in Texas (USA) in the summer of 2012 was attributed in part to drought conditions.

In the wetland ecosystem of the research area, the heat and drought conditions during the study period led to a shrinking of the water bodies and therefore the organic matter became more concentrated (eutrophication). Such conditions favor the *Cx. pipiens* species, also being attractive for several bird species. This might raise the bird-mosquito interaction (Paz 2015) and increase the infection rate potential (this can be seen in Table 1, for example for the third ten-day period of July 2012).

The results above show that the early rise of temperature and the decrease in rainfall amounts in spring contributed to the increase in vector population size and accelerated the transmission of WNV in the mosquito-bird cycles, leading to increased WNV infection rates in mosquito vectors in the following weeks.

CONCLUSIONS

The present study aimed to estimate mosquito abundance and infection rate in the wetland ecosystem of the Danube Delta during 2011–2013, as well as to analyze the influence of temperature and precipitation on the abundance of the main *Culex* vector species in the area, and on the WNV infection rate in the vectors.

It was found that higher temperatures in the early summer and drought conditions in the delta of the Danube River contributed to the increase of vector species abundance and WNV infection rate in the mosquitoes. Our results indicate a lag of 20–30 days from the temperature rise (the positive deviation from the standard mean) to the increased rate of WNV infection in mosquito vectors, a time which may be used to inform the possible exposed communities of the increased risk, and to intensify vector control operations.

A single complex model for prediction of WNV infection rate showed that the best predictors are the temperature, 20 days earlier (lag 2) which shows a positive linkage and the precipitation, 30 days earlier (lag 3) which has negative linkage.

The current study shows significant linkages between a rise in temperature, a decrease in precipitation and an in-

crease of vector species abundance, and WNV infection rate in the mosquitoes. Although this research deals with weather anomalies along short period, it provides another step toward a better understanding of the impact of weather variations on WNV transmission. Under conditions of climate change, for better preparedness, any assessment of future WNV transmission should take into consideration the impacts of weather fluctuations.

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