Seasonal Phenological Patterns and Flavivirus Vectorial Capacity of Medically Important Mosquito Species in a Wetland and an Urban Area of Attica, Greece

Stavroula Beleri 1,*, Georgios Balatsos 2, Vasilios Karras 2, Nikolaos Tegos 1, Fani Sereti 3, Georgios Rachiotis 4, Christos Hadjichristodoulou 4, Nikolaos Papadopoulos 5, Dimitrios Papachristos 2, Antonios Michaelakis 2 and Eleni Patsoula 3

1 Department of Public Health Policy, School of Public Health, University of West Attica, 115 21 Athens, Greece; ntegos@uniwa.gr (N.T.); epatsoula@uniwa.gr (E.P.)
2 Scientific Directorate of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 145 61 Attica, Greece; g.balatsos@bpi.gr (G.B.); v.karras@bpi.gr (V.K.); d.papachristos@bpi.gr (D.P.); a.michaelakis@bpi.gr (A.M.)
3 School of Public Health, University of West Attica, 115 21 Athens, Greece; sereti.fani@gmail.com
4 Department of Hygiene and Epidemiology, University of Thessaly Medical School, 412 22 Larissa, Greece; grachi@uth.gr (G.R.); xhatzi@uth.gr (C.H.)
5 Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, 38 446 Volos, Greece; nikopap@uth.gr
* Correspondence: smpeleri@uniwa.gr

Abstract: Seasonal patterns of mosquito population density and their vectorial capacity constitute major elements to understand the epidemiology of mosquito-borne diseases. Using adult mosquito traps, we compared the population dynamics of major mosquito species (Culex pipiens, Aedes albopictus, Anopheles spp.) in an urban and a wetland rural area of Attica Greece. Pools of the captured Cx. pipiens were analyzed to determine infection rates of the West Nile virus (WNV) and the Usutu virus (USUV). The data provided were collected under the frame of the surveillance program carried out in two regional units (RUs) of the Attica region (East Attica and South Sector of Attica), during the period 2017–2018. The entomological surveillance of adult mosquitoes was performed on a weekly basis using a network of BG-sentinel traps (BGs), baited with CO₂ and BG-Lure, in selected, fixed sampling sites. A total of 46,726 adult mosquitoes were collected, with larger variety and number of species in East Attica (n = 37,810), followed by the South Sector of Attica (n = 8916). The collected mosquitoes were morphologically identified to species level and evaluated for their public health importance. Collected Cx. pipiens adults were pooled and tested for West Nile virus (WNV) and Usutu virus (USUV) presence by implementation of a targeted molecular methodology (real-time PCR). A total of 366 mosquito pools were analyzed for WNV and USUV, respectively, and 38 (10.4%) positive samples were recorded for WNV, while no positive pool was detected for USUV. The majority of positive samples for WNV were detected in the East Attica region, followed by the South Sector of Attica, respectively. The findings of the current study highlight the WNV circulation in the region of Attica and the concomitant risk for the country, rendering mosquito surveillance actions and integrated mosquito management programs as imperative public health interventions.

Keywords: Culex; BG-sentinel; pathogens; fixed sampling site; RT-PCR

1. Introduction

Research on the distribution, abundance, and species composition of mosquitoes at a regional level is vital to estimate the risk of incidence of vector-borne diseases that are currently increasing in Europe, because of range expansion of native species and invasion events by alien species [1–4]. Factors, such as globalization of travel and trade, increasing land use and urbanization, high concentration of human populations, socioeconomics, and
climate change, enhance viral circulation. As a result, invasive species have expanded considerably their geographical and vectorial range, therefore increasing the risk of human exposure [5–7].

Entomological studies conducted in many regions of Greece [8–14] and in other European countries [15–17] demonstrate the importance of mosquito surveillance for transmitted viruses that can be a powerful tool as a part of an effective early-warning system [10,12,14,18,19]. Testing Culex mosquitoes for WNV, especially in high-risk areas is helpful for gaining insight into the virus circulation; it is a significant confirmation in cases where WNV was detected in mosquitoes before the symptoms’ onset in the human cases [14,20,21].

The emergence and resurgence of certain mosquito-borne diseases has led to the implementation of integrated programs, including entomological, veterinary, and human surveillance in several European countries. The goal is to prompt recognition and monitoring of arboviral activity; hence, the activation of proper control measures to prevent transmission [3,5,22–24].

Among several arboviruses being endemic in Europe and the Mediterranean Basin, two neurotropic mosquito-borne flaviviruses, the West Nile virus (WNV) and the Usutu virus (USUV), belonging to the Japanese encephalitis antigenic complex [25,26], cause sporadic cases of infection and outbreaks during the transmission seasons [5,13,14,27,28].

West Nile virus (WNV, Flaviviridae) is amongst the most widespread flavivirus in the world [29,30]. Since its discovery in 1937, it has spread beyond its original known geographic range and has caused human disease on every continent except Antarctica. It is continuously circulating in Europe with a recent increasing trend of incidence in several countries [3,31,32]. Greece is one of the most WNV-affected European countries with outbreaks of the virus being recorded since 2010 [13,14,18,30,33–36]; since then, the cases of WNV remain high on an annual basis [37–39].

The less renowned Usutu virus (USUV, Flaviviridae) is an African mosquito-borne flavivirus [40–42] that constitutes a worrisome threat to human and animal health worldwide. The virus was first detected in South Africa in 1959 [43,44], with the first cases in Europe dated in 1996 [45–47]. The first USUV outbreak was recorded in Austria in 2001 [27,48,49], and since then, the virus has spread throughout Europe [44,50,51], causing a considerable mortality among bird populations [50,52–54] and creating increasing concern for the potential zoonotic transmission to humans [40,55–61]. Usutu virus antibodies were first detected in Greece in 2010 in a dove [50,62], but no human cases have ever been recorded [10]. Nevertheless, targeted surveillance programs for vectors were not implemented; so far, both viruses share a similar enzootic transmission cycle, with birds as amplifying hosts and ornithophilic mosquitoes as vectors [5,50,51], and there are cases where the two viruses co-circulate in the same environment [17,51]. It has been reported that co-circulation of USUV and other related Flavivirus-like WNV may have an impact in terms of the respective epidemiological mode [28].

In this study, we present entomological and WNV/USUV detection data from two distinct regional units (RUs, NUTS3 level) of the Attica region (NUTS2 level) in Greece. The epidemiological profile based on previous transmission periods and the different environmental types, wetland and urban, were the reasons for the RUs selection for the surveillance program to be performed. The current study was part of the surveillance program in Attica region during the period 2017–2018.

The current program comprised of two axes with the following objectives:

(i) Monitoring and recording of mosquitoes’ species and population densities in the RUs under study; and

(ii) detection and monitoring of the circulation of WNV and USUV in collected Cx. pipiens s.l. for possible co-circulation.

This manuscript aimed to highlight the importance of implementing surveillance programs for the prompt detection of viruses’ circulation in mosquito populations and present, for the first time, surveillance data for USUV in mosquitoes.
2. Materials and Methods

2.1. Study Area

Two out of eight distinct RUs of Attica comprised the main study area, where traps were installed (Figure 1). The selection of the two RUs was based on the different environmental types, namely urban (UR) and wetland (WT) areas, as described in Table 1. Depending on the research coverage area in each RU, the corresponding number of traps was placed. Additionally, the epidemiological profile of the selected RUs of Attica played an important role concerning the risk for the residents, due to past WNV human infections in both RUs and previously recorded malaria cases in the East Attica Sector being reported to the NPHO since 2010 [38,63]. Data were collected from June 2017 to December 2018.

The region of Attica, “Attiki” in the Greek language (38.0° N 23.7° E; total area: 3808.10 km²; population: 3,828,434 inhabitants (2011 record data) [64], is the main metropolitan region of Greece, located on the eastern edge of the mainland, in Central Greece. The greater area of Attica region includes Athens (the capital of Greece) and Piraeus along with 62 other cities and settlements. It is bordered by the sea, to the east, including the south and southwest, while four mountains, Egaleo, Parnitha, Penteli, and Hymettus, delineate the hilly plain [65].

Figure 1. Geographical distribution of BGS traps with a schematic representation of the regional units participating in the research program, 2017–2018. (a) map of Greece with locations of surveyed Regional Unit Areas; (b) Regional Unit of Marathonas-Schinias (with red dots the sampling locations); (c) Regional Unit of Palaio Faliro (with yellow dots the sampling locations).
**Table 1.** Summary of data regarding sampling sites, catches, weeks, traps, collections of the RUs of the Attica region participated in the research program, 2017–2018.

| Attica Region (Abbreviation) and Period | Surveyed RU Areas and Period | BG-Sentinel Traps (BGs) CO₂ + BG-Lure | Sampling Location | GPS Coordinates (Decimal Degrees) | Microenvironment Description of Sampling Site | No. of Weeks | No. of Collections (Field/Problematic Collections) | Sampling Frequency |
|---------------------------------------|-----------------------------|---------------------------------------|-------------------|-----------------------------------|---------------------------------------------|-------------|-------------------------------------------------|-------------------|
| East Attica (EA)                      | Wetland Area (WT) Marathonas-Schinias (MS) 15/06/2017–28/12/2018 | MS1 Schinias | Private house/outdoor garden with a large number of trees and large green spaces, agricultural area (semi-urban area) | No. of Weeks | 80 | 74 (80/6) | Weekly |
|                                       |                             | MS2 Schinias | Private house/outdoor garden with a large number of trees and large green spaces, agricultural area (semi-urban area) | No. of Weeks | 80 | 69 (80/11) | Weekly |
|                                       |                             | MS3 Schinias | Private house/outdoor garden with a large number of trees and large green spaces, agricultural area (bordered by the marsh) | No. of Weeks | 59 | 59 (59/0) | Weekly |
|                                       |                             | MS4 Kato Souli | Private house/outdoor garden with a large number of trees and large green spaces, agricultural area (rural area) | No. of Weeks | 76 | 75 (76/1) | Weekly |
|                                       |                             | Total MS | 295 | 277 (295/18) | | |
| South Sector (SS)                     | Urban area (UR) Palaio Faliro (PF) 21/06/2017–27/12/2018 | PF1 Open Protection Centers for Elderly | Municipality building/outdoor garden with a large number of trees and large green spaces, urban area | No. of Weeks | 79 | 77 (79/2) | Weekly |
|                                       |                             | PF2 City Hall | Municipality building/outdoor garden with a large number of trees and large green spaces, urban area | No. of Weeks | 79 | 77 (79/2) | Weekly |
|                                       |                             | PF3 Rema Pikrodafnameis | Private house/outdoor garden with a large number of trees and large green spaces, urban area | No. of Weeks | 78 | 64 (78/14) | Weekly |
|                                       |                             | Total PF | 236 | 218 (236/18) | | |
The selected RUs are as follows:

(i) The RU of Marathonas-Schinias (MS): The wetland area (WT) sector of East Attica (EA) (38°00′ N 23°57′ E, total area: 1513 km²; population: 502,348 inhabitants [64]) covers the eastern part of the urban agglomeration of Athens, and also the rural area to its east. It is the only Attica zone with significant agricultural activity, can be considered geographically isolated from the rest of the basin, and is inserted between the Penteli and Hymettus mountains. The selected RU of MS (38°59′ N 23°57′ E; total area: 222.75 km²; population: 33,423 inhabitants [64]) is located outside the Athens Basin in the northeast part of the Attica region. The MS area lies 42 km away from the center of Athens. Marathonas is an area of intense agricultural activity, while Schinias is an area of marsh and coastal forest. The National Park of Schinias constitutes the most important coastal ecosystem in Attica [66,67].

(ii) The RU of Palaio Faliro (PF): The urban area (UR) covers the south-central part of the agglomeration of Athens in the South Sector of Attica (SS) (37°54′ N 23°44′ E; total area: 69.4 km²; population: 529,826 inhabitants [64]). The selected RU of PF (37°56′ N 23°42′ E; total area: 4.574 km²; population: 64,021 inhabitants [64]) is a coastal district, situated on the east coast of the Phalerum Bay of the Saronic Gulf, 6 km southwest of the Athens city center. The seaside area of PF is an important tourist attraction with a seaside promenade, several sports venues, and a marina. The Pikrodaňí stream flows into the sea on the border of the RUs of Palaio Faliro and Alimos [68].

2.2. Mosquito Collection and Identification

The selection of the mosquito monitoring stations was performed following an on-site visit, and it was based mainly upon ecological and social characteristics, such as urban and rural sites, presence of vegetation and shading, occurrence of humans or livestock as potential hosts for adult mosquitoes, proximity to open sources of fresh or still water, nuisance complaints, and convenience of sampling.

The BG-sentinel trap (BGs) (Biogents AG, Regensburg Germany) baited with CO₂ and BG-Lure [69], which is considered an effective method for mosquito diversity and abundance characterization [2,18,32], was selected as the main monitoring tool.

The composition of mosquito fauna was investigated by the monitoring system of seven BGs traps that was established in seven selected monitoring stations. In particular, depending on the research coverage area in each RU, four traps were placed in MS area, and three in PF area, respectively (Figure 1). A summary of all sampling data concerning the two studied areas is given in Table 1.

All collected mosquito samples were transferred weekly to the Laboratory of Medical Entomology, of Public Health Policy at the University of West Attica for further analysis. Closed and chilled containers containing dry ice were used for the transportation of samples, under the scope of morphological identification of mosquitoes and molecular detection of viruses (WNV and USUV), respectively.

Mosquitoes’ identification, based on morphological characters, was performed after careful examination under a NIKON SMZ645 Stereo Microscope (Nikon Instruments Inc., Surrey, UK), using appropriate dichotomous keys [70–73].

Throughout the duration of the study, no male Culex torrentium adults were identified regarding the morphological identification of the members of Cx. pipiens s.l. complex [70,71]. Adult females were characterized morphologically as Cx. pipiens s.L., as the two species are indistinguishable morphologically [70].

Adult mosquitoes that were morphologically identified to belong to Anopheles maculipennis s.l. complex were further examined by molecular amplification methods, according to previously described protocols [74,75].

2.3. Flaviviruses Survey in Culex pipiens Pools

Screening of Culex pipiens s.l. pools was designed to evaluate the possible co-circulation of the two viruses (WNV and USUV) in the same mosquito pool. All collected mosquitoes
were maintained under cold chain conditions to preserve the virus viability, pooled according to the collection site, date, species, and sex (up to a maximum of 200 individuals per pool), and stored at $-80^\circ$C.

2.4. WNV and USUV Detection

A total of 366 *Cx. pipiens* s.l. pools were analyzed for WNV (collected under the current surveillance program). Genetic material (RNA) from mosquito pools was extracted using the Maxwell 16 Automated Nucleic Acid extraction system (Promega, Madison, WI, USA), according to the manufacturer’s instructions for Maxwell16LEV Simple RNA Tissue kit [13]. A TaqMan Real-Time PCR protocol, specific for WNV lineages 1 and 2 detection, was implemented thereafter [76].

These 366 *Cx. pipiens* s.l. pools were also analyzed for the detection of USUV, with a reverse transcription real-time PCR protocol [77] and for selected samples also with a reverse transcription conventional PCR protocol [78].

Screening of the above-mentioned pools was designed to evaluate the possible co-circulation of the two viruses (WNV and USUV) in the same pool.

2.5. Infection Rates

The minimum infection rates (MIR) and maximum likelihood estimation (MLE) were calculated using the PooledInfRate program version 4.0 (available at https://www.cdc.gov/westnile/resourcepages/mosqSurvSoft.html, accessed on 20 August 2021) [13,79]. For each region included in the study areas, the respective MIR and MLE values were calculated per 1000 mosquitoes tested. The MLE has the advantage of considering variations in pool size, while the MIR in a study area assumes the presence of a single positive mosquito in a pooled sample.

3. Molecular Methods for Identification of Anopheles Mosquitoes

Anopheles species have been incriminated as vectors for transmission of malaria worldwide [80]. Nevertheless, a common limitation in identifying morphologically related species (i.e., *An. maculipennis* s.l. complex) creates an urgent need for implementation of alternative laboratory approaches.

Unidentified by morphological characteristics, specimens of *Anopheles* spp., as well as subspecies belonging to the *An. maculipennis* complex (cryptic species), were subjected to molecular identification by PCR. The nucleotide sequence variation of the ITS2 ribosomal region (ITS2 rDNA) along with the nucleotide sequence of the mitochondrial gene region I (COI) of cytochrome oxidase, respectively, were used as the main targets of the implemented molecular protocol [81–83].

A total of 50 Anopheles adult mosquitoes, the majority of which were morphologically identified, were examined at the molecular level. Representative samples were isolated and sent for sequencing analysis [81,82].

Data Analysis

A Gaussian Generalized Estimating Equation (GEE) model was used to estimate the number of captures in the two areas, Faliro and Marathonas. GEE analysis was conducted using the package “geepack” [84,85] in R v4.0.0 (R Core Team 2013, R Foundation of Statistical Computing, Vienna, Austria).

4. Results

4.1. Mosquito Fauna Identification

A total of 46,726 (45,663 ♀♀, 1063 ♂♂) adult mosquitoes were collected in all traps from June 2017 to December 2018. The implemented entomological survey revealed the presence of 15 species, classified in six distinct genera.

According to the results in Table 2 from the GEE analysis for the *Culex* and *Aedes* species collected in both study areas, we observed the following:
Table 2. Results from the GEE analysis for the Culex and Aedes species.

|                | B (95% CI)          | Wald χ² | p   |
|----------------|---------------------|---------|-----|
| **Culex**      |                     |         |     |
| Intercept      | 117.608 (38.523–196.693) | 8.50    | 0.004 |
| Area: Falirio (ref: Marathonas) | −90.582 (−171.388, −776) | 4.83    | 0.028 |
| **Aedes**      |                     |         |     |
| Intercept      | 6.560 (1.965, 11.154) | 7830    | 0.005 |
| Area: Falirio (ref: Marathonas) | 3.038 (−4.685, 10.762) | 0.594   | 0.441 |

Culex: there were significantly less Culex captures in Falirio than in Marathonas (p = 0.028).

Aedes: the number of captures did not differ significantly between Falirio and Marathonas (p = 0.441).

A total of 37,810 (80.92%) individuals were captured in the MS area, corresponding to six genera and 15 species. Additionally, 8,916 (19.08%) individuals were captured in PF area, corresponding to three genera and four species, respectively. Three of the captured species, namely *Anopheles sacharovi*, *Culex pipiens* s.l., and *Aedes albopictus*, are of major medical importance. Furthermore, *Cx. pipiens* s.l. (88.25%) and *Ae. albopictus* (8.05%) were by far the most abundant from all the collected species (Table 3).

A total of 531 adult sampling collections (MS, n = 295; PF, n = 236) from established BGs traps were examined from both studied areas, as described in Table 1. Mosquito collection was carried out by 495 sampling collections (MS, n = 277; PF, n = 218), while 36 BGs (MS, n = 18; PF, n = 18) were problematic due to either technical failure that occurred while in operation or without catches, possibly due to the effectiveness of the local mosquito control programs conducted during the transmission period or due to unstable weather conditions, mainly in winter.

The observations regarding the adult sampling collections of the four BGs traps in the MS study area showed differences regarding species abundance, richness, and diversity. Comparing the findings from the four BGs traps in the MS area, we observed the largest numbers of mosquitoes were collected in the MS1 (33.6% of total MS catches) and MS4 (40.5%) traps, followed by MS2 (15.4%) and MS3 (10.5%), while the variety of species was enriched in the MS3 and MS4 traps, respectively (Table 3). Regarding the adult sampling collections of the three BGs traps in PF area, no differences were observed concerning the species diversity. A large number of mosquitoes collected in the PF1 (47.35% of total PF catches) trap were followed by the PF3 (38.72%) and PF2 (13.93%) traps, respectively (Table 3). Taking into consideration that the urban habitats contained more densely human-populated areas than the rural habitats, which had a higher density of livestock, we concluded that species richness and diversity recorded in the surveyed municipalities were within the expected range [32].

The results for *Cx. pipiens* s.l. and *Ae. albopictus* populations’ fluctuations per week, from June 2017 to December 2018, concerning the surveyed RUs of MS and PF are presented in Figures 2 and 3, respectively. The results for *Anopheles* spp. population fluctuations per week, for the studied period concerning the surveyed RU of MS, are presented in Figure 4.
| Mosquito Species | Marathonas-Schinias (MS) Wetland Area (WT) | Palaio Faliro (PF) Urban Area (UR) |
|------------------|-------------------------------------------|----------------------------------|
|                  | Total Number/Species (%)                  | Total Number/Species (%)         |
| *Aedes (Stegomyia) albopictus* (Skuse) | 3762 (8.05) | 991 (4.3) |
| *Aedes (Ochlerotatus) caspius* (Pallas) | 81 (0.173) | 0 |
| *Aedes (Ochlerotatus) detritus* (Haliday) | 588 (1.25) | 0 |
| *Anopheles (Anopheles) algeriensis* (Theobald) | 209 (0.45) | 0 |
| *Anopheles (Anopheles) claviger* (Meigen) | 160 (0.34) | 0 |
| *Anopheles (Anopheles) maculipennis* s.l. (Meigen) | 2 (0.0042) | 0 |
| *Anopheles (Anopheles) sacharovi* (Favre) | 93 (0.2) | 0 |
| *Culex (Culex) pipiens* (Linnaeus) | 41,236 (88.25) | 3150 (73.90) |
| *Culex (Culex) theileri* (Theobald) | 16 (0.034) | 0 |
| *Culiseta (Culiseta) annulata* (Schrank) | 11 (0.023) | 0 |
| *Culicella fumipennis* (Stephens) | 5 (0.010) | 0 |
| *Culicella longiareolata* (Macquart) | 395 (0.84) | 81 (195 (0.41) |
| *Culicella subochrea* (Edwards) | 3 (0.0064) | 0 |
| *Uranotaenia (Pseudoficalbia) unguiculata* (Edwards) | 1 (0.002) | 0 |

Table 3. Species composition and relative abundance (%) in adult mosquitoes trapped in the WT and UR areas of the Attica region that participated in the research program, 2017–2018.

Aedini denomination according to Wilkerson et al. (2015).
High numbers of *Cx. pipiens* s.l. were observed in the MS RU from June to September 2017, while in 2018, the population reached a peak in June and then remained relatively low the following months (Figure 2). In the RU of PF, the populations of *Cx. pipiens* s.l. were kept low during both years of entomological surveillance (Figure 2).

A gradual increase in *Ae. albopictus* population was recorded in the MS area since June, reaching a peak in August, followed by a gradual decline in 2017, while populations remained in low numbers in the year 2018 (Figure 3). In the PF area, relatively low numbers of *Ae. albopictus* were recorded in 2017, while there was a peak in July 2018, followed by a gradual decline in the upcoming months (Figure 3).
Of particular importance was the presence of *An. sacharovi* collected in the MS RU, showing an increase in the population in June 2017, then a gradual decrease of catches the following months, and a slight increase was observed in December 2017. In 2018, the catches of the above species were zero during the period of collection (Figure 4). There were no collections of *An. algeriensis* in 2017; this species appeared in the area from March 2018, recording high numbers in April, May, and June, and reaching the peak in May; nevertheless, the population decline from July onwards (Figure 4). *An. claviger* was first collected in July 2017, reaching peak capture rates in August, and then gradually declined in the upcoming months. In 2018, the mosquito populations were kept in low numbers, and a few catches were recorded during the summer months (Figure 4).

4.2. Flaviviruses Detection

Of the adult female *Cx. pipiens* s.l. captured, a total of 41,050 (MS, n = 34,358; PF, n = 6,692) were examined in pools for the presence of WNV and USUV. *Cx. pipiens* s.l. adults were treated as a single entity, without determining the relative composition of molestus and pipiens forms.

Out of the 366 mosquito pools tested, a total of 38 (10.4%) tested positive for WNV, including 30 positive samples in MS and 8 positive samples in PF, respectively (Table 4).

The 366 mosquito pools tested for USUV were found to be negative (Table 4). One single pool of 200 *Cx. pipiens* s.l. collected in MS in June 2018 was suspected to be possibly USUV positive (low signal upon real-time PCR assay). A reverse transcription conventional PCR protocol was performed for further analysis. The sample produced a PCR product of low intensity (faint band), which did not contribute much to resolving this issue. The PCR product was subsequently sent for sequencing analysis; however, due to the possible reduced concentration of DNA in the sample, the results were inconclusive and therefore the sample was not confirmed as a positive one. WNV screening in the same sample showed that it was positive for WNV.
Table 4. Culex pipiens pools tested for West Nile virus and Usutu virus (Nt); the number of West Nile virus- and Usutu virus-positive Culex pipiens pools (Np) per surveyed RUs; and the year and maximum likelihood estimate (MLE) of the infection rate values for the urban and wetland areas, Attica Research Program, 2017–2018.

| Surveyed RUs                                      | West Nile Virus | Usutu Virus |
|--------------------------------------------------|-----------------|-------------|
|                                                  | 2017 Np/Nt *    | 2018 Np/Nt  | 2017 Np/Nt | 2018 Np/Nt | Total No. of Positive/Tested Pools | MLE            | 2017 Np/Nt | 2018 Np/Nt | Total No. of Positive/Tested Pools |
| East Attica/Marathonas-Schinias (wetland area)   | 18/120          | 12/147      | 30/267     | 0.00010 (95% CL 0.0007–0.0014) | 0/120          | 0/147       | 0/267      |
| South Sector/Palaio (urban area)                 | 5/33            | 3/66        | 8/99       | 0.01 (95% CL 0.0006–0.0026)   | 0/33           | 0/66        | 0/99       |
| Falir (urban area)                               | 23/153          | 15/213      | 38/366     | 0/153       | 0/213       | 0/366       |

* Np/Nt = number of positive pools/number of tested pools.

Infection Rates

In the present study, MLE values both for the wetland and the urban area were almost zero (Table 4), suggesting very low circulation of the virus in the study areas, which is in accordance with the human WNF cases recorded (no cases for 2017 and three cases in the MS area in 2018) [38]. MIR was calculated by extrapolation from the real-time PCR results (the total number of positive pools in the area/total number of mosquitoes sampled in this area × 1000) and is presented in Figure 5. MIR rates indicate that the peak in WNV-infected mosquitoes coincides with high numbers of Cx. pipiens populations.

Figure 5. Minimum infection rate (MIR) of Cx. pipiens adults captured in the wetland and in the urban study area per month.

4.3. Anopheles Specimens’ Molecular Identification

The species Anopheles maculipennis s.l., Anopheles sacharovi, and Anopheles algeriensis were identified by PCR and RFLP analysis. Further analysis of the sequencing chromatograms in the ITS2 gene identified An. maculipennis s.l. and An. sacharovi species in eight of the nine tested samples, with the associated traps located in the marsh and the rural environment, respectively. Species identification based on PCR amplification of the COI gene following sequencing of the 522 bp fragment revealed the presence of An. algeriensis and An. sacharovi, with the relevant traps located in marsh, rural, and semi-arid areas, respectively.

5. Discussion and Conclusions

The epidemiology of WNV and USUV has changed dramatically over the past two decades [28,47]. Recent data showed that strains detected in humans, horses, birds, and mosquitoes mainly belong to WNV lineage 2, including the Greek WNV strains detected during 2010–2018 [30,31,39,86–89]. Various USUV lineages are co-circulating in Europe,
the majority of strains are related to European USUV lineages [49,52–54,90], although some reports indicate the presence of African USUV lineages as well [43,61,91]. USUV Europe 2 lineage is the most prevalent genetic lineage detected in birds, mosquitoes, and humans, while Europe 3 and 4 and Africa 2 and 3 lineages were detected in mosquitoes [47].

Vector competence plays an important role in vectorial capacity helping in identifying species that might be important contributors to flaviviruses transmission, implementing control measures to reduce the potential of WNV/USUV transmission [28,42], and indicating the possible role of supporting the spread of WNV/USUV during winter [20,53,88,92–95].

In this study, mosquito screening for WNV showed that the majority of positive samples for WNV were detected in the East Attica, followed by the South Sector of Attica (Table 4). In the RU of PF, WNV-positive pools in mosquitoes were detected in both years, while in the study of Bisia et al. (2020) [18], no positive pool was detected in 2018. The region of MS has a warm temperate climate with hot dry summers and mild winters and displays characteristics to sustain WNV transmission cycles [96]. Due to its ecological and geographical features, this region is considered a risk area for flavivirus transmission [67].

The higher diversity and abundance of mosquito fauna were observed in Marathonas (intense agricultural activity) and Schinias (swamp and coastal forest), confirming similar remarks from previous studies [67,97]. The spatiotemporal monitoring of land cover changes studied by Gaitanis et al. (2015) [66] in the RU of MS showed a reduction of the areas covered by semi-natural and agricultural and cover types (forests, wetlands, shrublands, and cultivated fields) and the increase of urban and mixed areas during the last 60 years. According to the results of the study and records from the resident population, the mosquito nuisance is serious from early spring onwards [97].

Regarding data on the WNV circulation in equids and birds, according to the Ministry of Rural Development and Food, no cases were detected in 2017 in the Attica region. For 2018, confirmed WNV cases in equids were recorded in West Attica and East Attica RUs, and canary birds that were positive for the virus were also detected in the Athens West Sector RU [98].

For 2018, Greece reported 317 WNV infections and 51 deaths, representing 20% of total EU cases and displaying a 6,6 higher rate than in 2017, with 48 human cases being recorded. Regarding the areas of the present study, 11 and 14 human cases were recorded in the South Sector of Athens and the East Attica RUs, respectively, with a total number of 160 cases being recorded in the Attica region for 2018 [99].

The installation and circulation of WNV in Greece is a fact, while extensive studies have been performed on its circulation since 2010 [10–12,14,18,23,30,33–35]. However, predicting periods and circulation areas of the virus are difficult due to complex interactions of multiple involved factors [23,38,100]. It is noteworthy that, although outbreaks occurred in humans every year apart from the 2015–2016 period [18,37,38,88,100], positive Cx. pipiens s.l. pools were detected in different areas of the country [9,13,14,96].

Minimal infection rates of Cx. pipiens adults for WNV in both study areas were in accordance with the mosquito population density, and the low infection rates detected are consistent with the low human case rates observed. No human cases were recorded for 2017 and three cases were detected in the wetland area in 2018 [38,99].

The molecular identification of Anopheles spp. proved to be a useful tool for supporting morphological identification. This molecular approach using two genetic markers increased taxonomic resolution helped to identify damaged specimens and to distinguish species within a complex. A deeper study on the molecular identification of the Anopheline mosquito complex is required [3], as many of the Anopheles species in the MS area are malaria vectors [96], and indigenous cases of malaria have been recorded in East Attica the years 2009, 2010, 2011, 2012, and 2015 [63]. An. maculipennis s.l. is a potential vector of malaria, and it has been considered as an important vector in the past for the spread of this disease in various regions of Greece. An. sacharovi is considered to be the principal vector of malaria, from all subspecies of the An. maculipennis s.l. complex for Mediterranean
countries and of course for Greece [67,75,83]. *An. claviger* is a potential vector of malaria, although its medical significance is not considered to be great for our country. Relatively in high numbers, *An. algeriensis* is a common and very abundant species in the area, captured in all four traps, and although it can easily be infected with malaria plasmodium, it is considered a secondary vector due to its exophily [70].

The findings of this study revealed different assemblages of mosquito species in each targeted RU. Regarding the selected RUs, there were significant differences in many of their ecological characteristics and that was the main factor for their selection. All of the species recorded in this study were collected in the MS RU, while in the PF RU, the main collected species were *Cx. pipiens* s.l., *Ae. albopictus*, and *Cs. longiareolata*, which is in accordance with the study of Bisia et al. (2020) [18]. In both surveyed RUs, *Cx. pipiens* s.l. and *Ae. albopictus* were by far the most abundant species.

This study also provides baseline information and acts as a starting point for further investigation of USUV circulation. With the continuing spread of USUV since 2001 in neighboring countries of Greece [42,47,58], it is important to monitor both viruses before the possible occurrence of an epidemic. According to the epidemiological model, Greece belongs to the areas where the USUV can be transmitted, causing a possible epidemic. It mainly indicates areas in the north of the country, for two possible reasons. First of all, in these areas, especially in the river deltas where the number of mosquitoes has increased, migratory birds appear to have stopped moving from Europe to Africa [101], and secondly, in Northern Greece, the only case so far with antibodies to the virus has been recorded [62].

In the present study, samples from Attica for the years 2017–2018 were found negative for USUV. However, despite the limitations that emerged for confirming one possibly positive sample, USUV and WNV co-circulation cannot be excluded in the future. Up to date, no cases of USUV have been reported in humans or equids in Greece, suggesting that there is no circulation of this virus or, at least, its prevalence is very low. Given the knowledge we have from other relatives of flaviviruses, such as the WNV, the risk of causing even more outbreaks in areas endemic to the USUV or in new ones that have not yet spread are quite high. It must be noted that USUV might be misdiagnosed as WNV when the diagnosis is based only on antibody detection, without testing by PCR or neutralization assays, due to cross-reactivity in serology [42,60,102].

Further investigations of both viruses could provide answers to our suspicions about both the USUV circulation in our country and the interaction with its related flavivirus. Furthermore, entomological surveillance activities should be extended to Attica, especially urban ones, as USUV appears to be equally transmitted in urban and rural areas, in contrast to WNV, where higher transmission rates are recorded in rural areas [26]. Virus surveillance within the native mosquito populations offers an opportunity to detect a virus before the emergence of disease in the susceptible host population [103].

A general comment to be made refers to the fact that *Anopheles* species identification exclusively by morphological features often presents difficulties, highlighting the necessity for implementation of a targeted molecular protocol to the species level [104]. In our case, *Anopheles* mosquitoes collected in the Marathonas-Schinias area created a need for the development of a special molecular protocol. In this manuscript, we aimed to highlight that the implemented combined research methodology proved to be a useful tool for supporting morphological identification. Furthermore, the applied molecular methodology was found to be specific and sensitive, regarding the possibility of finding positive mosquito pools for WNV.

In conclusion, the findings of this study emphasize the need for regular monitoring of the mosquito fauna in all regions of Greece, which will contribute to increasing the current knowledge about the diversity, distribution abundance, and ecology of species that are present in the regions. Related studies on mosquito fauna should be performed in all RUs of the Attica region as data on mosquito population and species distribution would be valuable, in particular on those species that are of zoonotic relevance. The implementation of integrated arbovirus surveillance programs represents a relevant and
necessary assessment of the risk of pathogen transmission in a given region, allowing for the establishment of the appropriate preventive measures.

Author Contributions: Conceptualization, N.P.; methodology, N.T. and F.S.; formal analysis, D.P.; investigation, G.B.; V.K., and N.T.; resources, A.M.; data curation, G.B.; writing—original draft preparation, S.B.; writing—review and editing, N.P. and E.P.; supervision, G.R. and C.H.; funding acquisition, A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Region of Attiki and LIFE CONOPS project. The project entitled “A systematic surveillance of vector mosquitoes for the control of mosquito-borne diseases in the Region of Attiki” financed by the Region of Attiki. The project LIFE CONOPS (LIFE12 ENV/GR/000466) funded by the European Commission in the framework of the program LIFE + Environment Policy and Governance (www.conops.gr, accessed on 30 January 2020).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors would like to thank the residents that kindly allowed us to place traps in their premises, Angeliki Stefopoulos for her kind contribution to the creation of the Figure 1, and Eleni Verykouki for her assistance in the statistical analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. European Centre for Disease Prevention and Control (ECDC). Guidelines for the Surveillance of Native Mosquitoes in Europe. Stockholm: ECDC. 2014. Available online: https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/surveillance-of%20native-mosquitoes%20-guidelines.pdf (accessed on 20 March 2020).

2. Ferraguti, M.; Martínez-de la Puente, J.; Rozí, D.; Ruiz, S.; Soriguer, R.; Figuerola, J. Effects of landscape anthropization on mosquito community composition and abundance. *Sci. Rep.* 2016, 6, 29002. [CrossRef]

3. Ruiz-Arrondo, I.; McMahon, B.J.; Hernández-Triana, L.M.; Santibañez, P.; Portillo, A.; Oteo, J.A. Surveillance of mosquitoes (Diptera, Culicidae) in a northern central region of Spain: Implications for the medical community. *Front. Vet. Sci.* 2019, 6, 86. [CrossRef]

4. Török, E.; Tomazatos, A.; Cadar, D.; Horváth, C.; Keresztes, L.; Jansen, S.; Becker, N.; Kaiser, A.; Popescu, O.; Schmidt-Chanasit, J.; et al. Pilot longitudinal mosquito surveillance study in the Danube Delta Biosphere Reserve and the first reports of Anopheles algeriensis Theobald, 1903 and Aedes hungaricus Mihalj, 1955 for Romania. *Parasit. Vectors* 2016, 9, 196. [CrossRef]

5. Barzon, L. Ongoing and emerging arbovirus threats in Europe. *J. Clin. Virol.* 2018, 107, 38–47. [CrossRef]

6. Martina, B.E.; Barzon, L.; Pilijan, G.P.; de la Fuente, J.; Rizzoli, A.; Wannmes, L.J.; Takken, W.; van Rij, R.P.; Papa, A. Human to human transmission of arthropod-borne pathogens. *Curr. Opin. Virol.* 2017, 22, 13–21. [CrossRef]

7. Weaver, S.C.; Reisen, W.K. Present and future arboviral threats. *Antivir. Res.* 2010, 85, 328–345. [CrossRef]

8. Chaskopoulou, A.; Dovas, C.I.; Chaintoutis, S.C.; Kashefi, J.; Koehler, P.; Papanastassopoulou, M. Detection and Early Warning of West Nile Virus Circulation in Central Macedonia, Greece, Using Sentinel Chickens and Mosquitoes. *Vector-Borne Zoonotic Dis.* 2013, 13, 723–732. [CrossRef]

9. Mavridis, K.; Fotakis, E.A.; Kioulos, I.; Mpellou, S.; Konstantas, S.; Varela, E.; Gewehr, S.; Diamantopoulos, V.; Vontas, J. Detection of West Nile Virus—Lineage 2 in Culex pipiens mosquitoes, associated with disease outbreak in Greece, 2017. *Acta Trop.* 2018, 182, 64–68. [CrossRef]

10. Papa, A.; Gewehr, S.; Tsioka, K.; Kalaitzopoulos, S.; Pappa, S.; Mourelatos, S. Detection of flaviviruses and alphaviruses in mosquitoes in Central Macedonia, Greece, 2018. *Acta Trop.* 2020, 202, 105278. [CrossRef]

11. Papa, A.; Tsioka, K.; Gewehr, S.; Kalaitzopoulos, S.; Pappa, S.; Mourelatos, S. West Nile virus lineage 2 in Culex mosquitoes in Thessaly, Greece, 2019. *Acta Trop.* 2020, 208, 105514. [CrossRef]

12. Papa, A.; Tsioka, K.; Gewehr, S.; Kalaitzopoulos, S.; Pervanidou, D.; Vakali, A.; Kefaloudi, C.; Pappa, S.; Louka, X.; Mourelatos, S. West Nile fever upsurge in a Greek regional unit, 2020. *Acta Trop.* 2021, 221, 106010. [CrossRef] [PubMed]

13. Patsoula, E.; Vakali, A.; Balatsos, G.; Pervanidou, D.; Beleri, S.; Tegos, N.; Baka, A.; Spanakos, G.; Georgakopoulou, T.; Tserkezou, P.; et al. West Nile Virus Circulation in Mosquitoes in Greece (2010–2013). *BioMed Res. Int.* 2016, 2016, 2450682. [CrossRef] [PubMed]

14. Patsoula, E.; Beleri, S.; Tegos, N.; Mkrtchian, R.; Vakali, A.; Pervanidou, D. Entomological Data and Detection of West Nile Virus in Mosquitoes in Greece (2014–2016), Before Disease Re-Emergence in 2017. *Vector-Borne Zoonotic Dis.* 2020, 20, 60–70. [CrossRef]

15. Cabanová, V.; Šíkutová, S.; Straková, P.; Šebesta, O.; Vichová, B.; Zubršiková, D.; Miterpáková, M.; Mendel, J.; Hurníková, Z.; Hubšek, Z.; et al. Co-Circulation of West Nile and Usutu Flaviviruses in Mosquitoes in Slovakia, 2018. *Viruses* 2019, 11, 639. [CrossRef] [PubMed]

16. Christova, I.; Papa, A.; Trifonova, I.; Panayotova, E.; Pappa, S.; Mikov, O. West Nile virus lineage 2 in humans and mosquitoes in Bulgaria, 2018–2019. *J. Clin. Virol.* 2020, 127, 104365. [CrossRef] [PubMed]
17. Fros, J.; Miesen, P.; Vogels, C.; Gaibani, P.; Sambri, V.; Martina, B.E.; Koenraadt, C.J.; van Rij, R.P.; Vlak, J.M.; Takken, W.; et al. Comparative Usutu and West Nile virus transmission potential by local Culex pipiens mosquitoes in north-western Europe. *One Health* 2015, 1, 31–36. [CrossRef]

18. Bista, M.; Jeffries, C.L.; Lytra, I.; Michaelakis, A.; Walker, T. A Comparison of Adult Mosquito Trapping Methods to Assess Potential West Nile Virus Mosquito Vectors in Greece during the Onset of the 2018 Transmission Season. *Insects* 2020, 11, 329. [CrossRef]

19. Marka, A.; Diamantidis, A.; Papa, A.; Valiakos, G.; Chaintoutis, S.C.; Doukas, D.; Tserkezou, P.; Giannakopoulou, A.; Papasypyropoulos, K.; Patsoula, E.; et al. West Nile Virus State of the Art Report of MALWEST Project. *Int. J. Environ. Res. Public Health* 2013, 10, 6534–6610. [CrossRef]

20. Calzolari, M.; Monaco, F.; Montarsi, F.; Bonilauri, P.; Ravagnan, S.; Bellini, R.; Cattoli, G.; Cordioli, P.; Cazzin, S.; Pinoni, C.; et al. New incursions of West Nile virus lineage 2 in Italy in 2013: The value of the entomological surveillance as early warning system. *Vet. Ital.* 2013, 49, 315–319.

21. Riccardo, F.; Monaco, F.; Bella, A.; Savini, G.; Russo, F.; Cagarelli, R.; Dottori, M.; Rizzo, C.; Venturi, G.; Di Luca, M.; et al. An early start of West Nile virus seasonal transmission: The added value of One Heath surveillance in detecting early circulation and triggering timely response in Italy, June to July 2018. *Eurosurveill* 2018, 23, 1800427. [CrossRef]

22. Bellini, R.; Zeller, H.; Van Bortel, W. A review of the vector management methods to prevent and control outbreaks of West Nile virus infection and the challenge for Europe. *Parasites Vectors* 2014, 7, 323. [CrossRef]

23. Gossner, C.M.; Marrama, L.; Carson, M.; Allerberger, F.; Calisti, P.; Dilaveris, D.; Lecollinet, S.; Morgan, D.; Nowotny, N.; Paty, M.C.; et al. West Nile virus surveillance in Europe: Moving towards an integrated animal-human-vector approach. *Eurosurveillance* 2017, 22, 30526. [CrossRef] [PubMed]

24. Jourdain, F.; Samy, A.M.; Hamidi, A.; Bouattour, A.; Alten, B.; Faraj, C.; Reiz, D.; Petric, D.; Perez-Ramirez, E.; Velo, E.; et al. Towards harmonisation of entomological surveillance in the Mediterranean area. *PLoS Negl. Trop. Dis.* 2019, 13, e0007314. [CrossRef] [PubMed]

25. Calisher, C.H.; Gould, E.A. Taxonomy of the virus family Flaviviridae. *Adv. Virus Res.* 2003, 59, 1–19. [PubMed]

26. Calzolari, M.; Bonilauri, P.; Bellini, R.; Albieri, A.; Defilippo, F.; Maioli, G.; Galletti, G.; Gelati, A.; Barbieri, I.; Tambia, M.; et al. Evidence of Simultaneous Circulation of West Nile and Usutu Viruses in Mosquitoes Sampled in Emilia-Romagna Region (Italy) in 2009. *PLoS ONE* 2010, 5, e14324. [CrossRef]

27. Papa, A. Emerging arboviruses of medical importance in the Mediterranean region. *J. Clin. Virol.* 2019, 115, 5–10. [CrossRef]

28. Zannoli, S.; Sambri, V. West Nile Virus and Usutu Virus Co-Circulation in Europe: Epidemiology and Implications. *Microorganisms* 2019, 7, 184. [CrossRef]

29. Hubálek, Z.; Halouzka, J. West Nile Fever—a Reemerging Mosquito-Borne Viral Disease in Europe. *Emerg. Infect. Dis.* 1999, 5, 643–650. [CrossRef]

30. Chancey, C.; Grinev, A.; Volkova, E.; Rios, M. The Global Ecology and Epidemiology of West Nile Virus. *BioMed Res. Int.* 2015, 2015, 376230. [CrossRef]

31. Haussig, J.M.; Young, J.J.; Gossner, C.M.; Mezei, E.; Bella, A.; Sirbu, A.; Pervanidou, D.; Drakulovic, M.B.; Sudre, B. Early start of the West Nile fever transmission season 2018 in Europe. *Eurosurveillance* 2018, 23, 1800428. [CrossRef]

32. Martinez-de la Puente, J.; Ferraguti, M.; Ruiz, S.; Roiz, D.; Llorente, F.; Perez-Ramirez, E.; Jimenez-Clavero, M.A.; Soriguerr, R.; Figuerola, J. Mosquito community influences West Nile virus seroprevalence in wild birds: Implications for the risk of spillover into human populations. *Sci. Rep.* 2018, 8, 2599. [CrossRef] [PubMed]

33. Chaintoutis, S.C.; Chaskopoulou, A.; Chassalevris, T.; Koehler, P.G.; Papanastassopoulou, M.; Dovas, C.I. West Nile Virus Lineage 2 Strain in Greece, 2012. *Emerg. Infect. Dis.* 2013, 19, 827–829. [CrossRef] [PubMed]

34. Danis, K.; Papa, A.; Theocharopoulos, G.; Dougas, G.; Athanasiou, M.; Detsis, M.; Baka, A.; Lytras, T.; Mellou, K.; Bonovanas, S.; et al. Outbreak of West Nile virus infection in Greece, 2010. *Emerg. Infect. Dis.* 2011, 17, 1868–1872. [CrossRef]

35. Hadjidristodoulou, C.; Pourmaras, S.; Mavrouli, M.; Marka, A.; Tserkezou, P.; Baka, A.; Billinis, C.; Katsioulis, A.; Psaroulaki, A.; Papa, A.; et al. West Nile Virus Seroprevalence in the Greek Population in 2013: A Nationwide Cross-Sectional Survey. *PLoS ONE* 2015, 10, e0143803. [CrossRef]

36. MALWEST. West Nile Virus, Epidemiology. Available online: http://www.malwest.gr/en-us/westnilevirus/informationforhealthcareprofessionals/epidemiology.aspx (accessed on 27 May 2020).

37. European Centre for Disease Prevention and Control (ECDC). Historical Data by Year—West Nile Fever Seasonal Surveillance. Available online: https://www.ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical (accessed on 20 March 2020).

38. Pervanidou, D.; Vakali, M.; Georgakopoulou, T.; Panagiotopoulos, T.; Patsoula, E.; Koliosopoulos, G.; Politis, C.; Stamoulis, K.; Gavana, E.; Pappa, S.; et al. West Nile virus in humans, Greece, 2018: The largest seasonal number of cases, 9 years after its emergence in the country. *Eurosurveillance* 2020, 25, 1900543. [CrossRef] [PubMed]

39. Papa, A.; Papadopoulou, E.; Chatzixanthioulou, C.; Glouftsiots, P.; Pappa, S.; Pervanidou, D.; Georgiou, L. Emergence of West Nile virus lineage 2 belonging to the Eastern European subclade, Greece. *Arch Virol.* 2019, 164, 1673–1675. [CrossRef]

40. Gaibani, P.; Rossini, G. An overview of Usutu virus. *Microbes Infect.* 2017, 19, 382–387. [CrossRef]

41. Nikolay, B.; Diao, M.C.; Boye, S.; Sall, A.A. Usutu virus in Africa. *Vector Borne Zoonotic Dis.* 2011, 11, 1417–1423. [CrossRef]
66. Gaitanis, A.; Kalogeropoulos, K.; Detsis, V.; Chalkias, C. Monitoring 60 Years of Land Cover Change in the Marathon Area, Greece. Land 2015, 4, 337–354. [CrossRef]
67. MALWEST 2013. Study on Presence, Seasonal Variation and Spatial Distribution of Mosquitoes and Design of an Integrated Mosquito Management Plan. Report Regarding Mosquito Species and Geographical Distribution. Available online: http://www.malwest.gr/en-us/deliverables.aspx (accessed on 27 July 2020).
68. Stefopoulou, A.; Balatsos, G.; Petraki, A.; LaDeau, S.L.; Papachristos, D.; Michaelakis, A. Reducing Aedes albopictus breeding sites through education: A study in urban area. PLoS ONE 2018, 13, e0202451. [CrossRef]
69. Biogents, A.G. The BG-Sentinel: Biogent’s Mosquito Trap for Researchers. Available online: http://www.bg-sentinel.com/ (accessed on 29 September 2020).
70. Becker, N.; Petrić, D.; Zagomba, M.; Boase, C.; Madon, M.; Dahl, C.; Kaiser, A. Mosquitoes and Their Control, 2nd ed.; Springer: Berlin/Heidelberg, Germany, 2010.
71. Darsie, R.F.J.; Samanidou-Voyadjoglou, R.E. Keys for the identification of the mosquitoes of Greece. Eur. Mosq. Bull. 2001, 10, 13–20.
72. Wilkerson, R.C.; Linton, Y.-M.; Fonseca, D.; Schultz, T.R.; Price, D.C.; Strickman, D.A. Making Mosquito Taxonomy Useful: A Stable Classification of Tribe Aedini that Balances Utility with Current Knowledge of Evolutionary Relationships. PLoS ONE 2015, 10, e0133602. [CrossRef]
73. Beleri, S.; Chatzinikolaou, S.; Nearchou, A.; Patsoula, E. Entomological study of the mosquito fauna in the regional unit of Drama, region of East Macedonia-Thrace, Greece (2015 to 2016). Vector Borne Zoonotic Dis 2017, 17, 665–671. [CrossRef] [PubMed]
74. Patsoula, E.; Samanidou-Voyadjoglou, A.; Spanakos, G.; Kremastinou, J.; Nasioulas, G.; Vakalis, N.C. Molecular characterization of the Anopheles maculipennis complex during surveillance for the 2004 Olympic Games in Athens. Med. Vet. Entomol. 2007, 21, 36–43. [CrossRef] [PubMed]
75. Tang, Y.; Hapip, A.C.; Liu, B.; Fang, C.T. Highly sensitive TaqManRT-PCR assay for detection and quantification of both lineages of West Nile virus RNA. J. Clin. Virol. 2006, 36, 177–182. [CrossRef] [PubMed]
76. Cavrini, F.; Della Pepa, M.E.; Gaibani, P.; Pierro, A.M.; Rossini, G.; Landini, M.P.; Sambri, V. A rapid and specific real-time RT-PCR assay to identify Usutu virus in human plasma, serum, and cerebrospinal fluid. J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol. 2011, 50, 221–223. [CrossRef] [PubMed]
77. Weissenböck, H.; Bakonyi, T.; Chvála, S.; Nowotny, N. Experimental Usutu virus infection of suckling mice causes neuronal and glial cell apoptosis and demyelination. Acta Neuropathol. 2004, 108, 453–460. [CrossRef]
78. Biggerstaff Brad, J. PooledInfRate, Version 4.0: A Microsoft® Office Excel© Add-In to Compute Prevalence Estimates from Pooled Samples; Centers for Disease Control and Prevention: Fort Collins, CO, USA, 2009.
79. Sinka, M.E.; Banga, M.J.; Manguin, S.; Coetzee, M.; Mbogo, C.M.; Hemingway, J.; Patil, A.P.; Temperley, W.H.; Getheing, P.W.; Kabari, C.W.; et al. The dominant Anopheles vectors of human malaria in Africa, Europe and middle East: Occurrence data, distribution maps and bionomic précis. Parasites Vectors 2010, 3, 117. [CrossRef]
80. Engler, O.; Savini, G.; Papa, A.; Figureola, J.; Groschup, M.H.; Kampen, H.; Medlock, J.; Vaux, A.; Wilson, A.J.; Werner, D.; et al. European Surveillance for West Nile Virus in Mosquito Populations. Int. J. Environ. Res. Public Health 2013, 10, 4869–4895. [CrossRef] [PubMed]
81. Kavran, M.; Zagomba, M.; Weitzel, T.; Petric, D.; Manz, C.; Becker, N. Distribution of Anopheles daciae and other Anopheles maculipennis complex species in Serbia. Parasitol. Res. 2018, 117, 3277–3287. [CrossRef] [PubMed]
82. Naddaf, S.R.; Oshaghi, M.A.; Vatandoost, H. Confirmation of Two Sibling Species among Anopheles fluviatilis Mosquitoes in Northern Iran. Parasites Vectors 2011, 4, 221–223. [CrossRef] [PubMed]
83. Stefopoulou, A.; Balatsos, G.; Petraki, A.; LaDeau, S.L.; Papachristos, D.; Michaelakis, A. Reducing Aedes albopictus breeding sites through education: A study in urban area. PLoS ONE 2018, 13, e0202451. [CrossRef]
84. Biogents, A.G. The BG-Sentinel: Biogent’s Mosquito Trap for Researchers. Available online: http://www.bg-sentinel.com/ (accessed on 29 September 2020).
85. Becker, N.; Petrić, D.; Zagomba, M.; Boase, C.; Madon, M.; Dahl, C.; Kaiser, A. Mosquitoes and Their Control, 2nd ed.; Springer: Berlin/Heidelberg, Germany, 2010.
86. Darsie, R.F.J.; Samanidou-Voyadjoglou, R.E. Keys for the identification of the mosquitoes of Greece. Eur. Mosq. Bull. 2001, 10, 13–20.
87. Wilkerson, R.C.; Linton, Y.-M.; Fonseca, D.; Schultz, T.R.; Price, D.C.; Strickman, D.A. Making Mosquito Taxonomy Useful: A Stable Classification of Tribe Aedini that Balances Utility with Current Knowledge of Evolutionary Relationships. PLoS ONE 2015, 10, e0133602. [CrossRef]
88. Beleri, S.; Chatzinikolaou, S.; Nearchou, A.; Patsoula, E. Entomological study of the mosquito fauna in the regional unit of Drama, region of East Macedonia-Thrace, Greece (2015 to 2016). Vector Borne Zoonotic Dis 2017, 17, 665–671. [CrossRef] [PubMed]
89. Patsoula, E.; Samanidou-Voyadjoglou, A.; Spanakos, G.; Kremastinou, J.; Nasioulas, G.; Vakalis, N.C. Molecular characterization of the Anopheles maculipennis complex during surveillance for the 2004 Olympic Games in Athens. Med. Vet. Entomol. 2007, 21, 36–43. [CrossRef] [PubMed]
90. Tang, Y.; Hapip, A.C.; Liu, B.; Fang, C.T. Highly sensitive TaqManRT-PCR assay for detection and quantification of both lineages of West Nile virus RNA. J. Clin. Virol. 2006, 36, 177–182. [CrossRef] [PubMed]
91. Engel, D.; Jöst, H.; Wink, M.; Börstler, J.; Bosch, S.; Garigliany, M.-M.; Jöst, A.; Czajka, C.; Lühken, R.; Ziegler, U.; et al. Reconstruction of the Evolutionary History and Dispersal of Usutu Virus, a Neglected Emerging Arbovirus in Europe and Africa. *mBio* 2016, 7, e01938-15. [CrossRef]

92. Blagrove, M.S.C.; Sherlock, K.; Chapman, G.E.; Impoinvil, D.E.; McCall, P.J.; Medlock, J.M.; Lycett, G.; Solomon, T.; Baylis, M. Evaluation of the vector competence of a native UK mosquito *Ochlerotatus detritus* (Aedes detritus) for dengue, chikungunya and West Nile viruses. *Parasites Vectors* 2016, 9, 452. [CrossRef] [PubMed]

93. Camp, J.V.; Kolodziejek, J.; Nowotny, N. Targeted surveillance reveals native and invasive mosquito species infected with Usutu virus. *Parasites Vectors* 2019, 12, 46. [CrossRef] [PubMed]

94. Mancini, G.; Montarsi, F.; Calzolari, M.; Capelli, G.; Dottori, M.; Ravagnan, S.; Lelli, D.; Chiari, M.; Santilli, A.; Quaglia, M.; et al. Mosquito species involved in the circulation of West Nile and Usutu viruses in Italy. *Vet. Ital.* 2017, 53, 97–110. [PubMed]

95. Puggioli, A.; Bonilauri, P.; Calzolari, M.; Lelli, D.; Carrieri, M.; Urbanelli, S.; Pudar, D.; Bellini, R. Does *Aedes albopictus* (Diptera: Culicidae) play any role in Usutu virus transmission in Northern Italy? Experimental oral infection and field evidences. *Acta Trop.* 2017, 172, 192–196. [CrossRef]

96. Gomes, B.; Kioulos, E.; Papa, A.; Almeida, A.P.; Vontas, J.; Pinto, J. Distribution and hybridization of *Culex pipiens* forms in Greece during the West Nile virus outbreak of 2010. *Infect. Genet. Evol.* 2013, 16, 218–225. [CrossRef]

97. Vakalis, N.; Patsoula, E.; Samanidou-Voyadjoglou, A. Mosquito surveillance and control (Chapter 11). In *Mass Gathering and Public Health. The Experience of the Athens 2004 Olympic Games*; Tsouras, A.D., Efstathiou, P.A., Eds.; WHO Europe: Geneva, Switzerland, 2007; pp. 193–208.

98. Ministry of Rural Development and Food. Available online: http://www.minagric.gr/index.php/el/for-citizen-2/nosimata-zoon/602-progrepitndeilou17 (accessed on 30 July 2021).

99. European Centre for Disease Prevention and Control (ECDC). West Nile Virus Infection, Annual Epidemiological Report for 2018. Available online: https://www.ecdc.europa.eu/en/publications-data/west-nile-virus-infection-annual-epidemiological-report-2018 (accessed on 25 May 2021).

100. Mavrouli, M.; Vrioni, G.; Kaptsimali, V.; Tsiamin, C.; Mavroulis, S.; Pervanidou, D.; Billinis, C.; Hadjichristodoulou, C.; Tsakris, A. Reemergence of West Nile virus Infections in Southern Greece, 2017. *Am. J. Trop. Med. Hyg.* 2019, 100, 420–426. [CrossRef]

101. Valiakos, G.; Touloudi, A.; Iacovakis, C.; Athanasiou, A.; Birtsas, P.; Spyrou, V.; Billinis, C. Molecular detection and phylogenetic analysis of West Nile virus lineage 2 in sedentary wild birds (Eurasian magpie), Greece, 2010. *Eurosurveill* 2011, 16, 19862. [CrossRef]

102. Llorente, F.; García-Irazábal, A.; Pérez-Ramírez, E.; Cano-Gómez, C.; Sarasa, M.; Vázquez, A.; Jiménez-Clavero, M.A. Influence of flavivirus co-circulation in serological diagnostics and surveillance: A model of study using West Nile, Usutu and Bagaza viruses. *Transbound. Emerg. Dis.* 2019, 66, 2100–2106. [CrossRef]

103. Calzolari, M.; Gaibani, P.; Bellini, R.; De Filippio, F.; Pierro, A.; Albieri, A.; Maioli, G.; Luppi, A.; Rossini, G.; Balzani, A.; et al. Mosquito, bird and human surveillance of West Nile and Usutu viruses in Emilia-Romagna Region (Italy) in 2010. *PLoS ONE* 2012, 7, e38058. [CrossRef]

104. Collins, F.H.; Kamau, L.; Ranson, H.; Vulule, J.M. Molecular entomology and prospects for malaria control. *Bull. World Health Organ.* 2003, 78, 1412–1423.