1 The abundance and diversity of West Nile virus mosquito vectors in two Regional

2 Units of Greece during the onset of the 2018 transmission season

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16

17 **Abstract**

18 **Background:** West Nile virus (WNV) is a zoonotic arbovirus of great medical and veterinary

19 importance, threatening the health of humans and equines worldwide. Mosquitoes belonging

20 to the *Culex* (*Cx.*) *pipiens* complex are major vectors but numerous other mosquito species

21 have also been implicated as vectors of WNV. Due to variations in blood-feeding behaviour,

22 the different biotypes and hybrids of *Cx. pipiens* influence the transmission of WNV, from

23 enzootic cycles (between mosquitoes and birds), to spill-over transmission to humans and

24 equines.

25 **Methods**: In this study, mosquitoes were collected and analysed from two regional units

26 (RUs) of Greece with reported cases of WNV within the past 4 years; Palaio Flairo and

27 Argolida (in Attica and Peloponnese regions, respectively). Collections using different types

28   of mosquito surveillance traps were undertaken in May-June 2018 during the early period of

29   the WNV transmission season.

30   **Results**: A total of 1062 mosquitoes were collected, with Biogents Sentinel traps (BG traps)

31   collecting both a greater number of mosquitoes across all species and *Cx. pipiens* complex

32   individuals than Centres for Disease Control miniature light traps (CDC traps) or Heavy Duty

33   Encephalitis Vector Survey traps (EVS traps). Identification of collected mosquitoes (using

34   both morphological keys and molecular barcoding) confirmed the presence of additional

35   species including *Aedes (Ae.) albopictus*, *Ae. caspius* and *Culiseta (Cs.) longiareolata.* The

36   prevalence of *Cx. pipiens* biotypes in the RU of Palaio Faliro was 54.5% *pipiens* type, 20.0%

37   *molestus* type and 25.5% hybrids. In the RU of Argolida, the collection comprised 68.1%

38   *pipiens* type, 8.3% *molestus* type and 23.6% hybrids. Screening individual unfed female

39   mosquitoes for WNV (molecular xenomonitoring) resulted in detection in three females of the

40   *pipiens* type and in one hybrid; all collected from the RU of Argolida.

41   **Conclusions**: As hybrids play an important role in spill-over transmission of WNV to

42   humans and equines, these findings highlight the importance of undertaking entomological

43   surveillance programs incorporating molecular xenomonitoring at the onset of the

44   transmission season to provide an early warning system for health authorities aiming to

45   prevent WNV outbreaks in Greece.

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50   **Background**

51   West Nile virus (WNV) is an arbovirus belonging to the Japanese encephalitis group within

52   the *Flavivirus* genus (*Flaviviridae* family) and is the most widespread virus belonging to this

53   genus [1–4]. Natural transmission of WNV mainly occurs in enzootic cycles between birds

54   and competent ornithophilic mosquito vectors, with avian species being the principal

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55   maintenance and amplifying hosts of WNV as many species develop sufficient viremia for

56   onward transmission. This allows transmission to continue where competent mosquitoes are

57   present in a specific area under suitable environmental conditions [5]. Additionally, spill-over

58   transmission can occur when competent vectors feed on humans or horses. During natural

59   transmission these mammalian species are considered dead-end hosts since they cannot

60   sustain sufficient viraemia for further vector-borne transmission. However, infection in

61   humans does pose a transmission risk due to the possibility of iatrogenic transmission

62   through blood and tissue donations, in addition to the possibility of intrauterine transmission

63   or WNV being passed on through breast milk [4]. Blood and tissue donor screening is

64   essential in areas where WNV is endemic [6,7] and currently no human vaccination is

65   available, however, vaccination of horses has been shown to reduce clinical disease in this

66   species [8,9].

67   WNV was first isolated in 1937 from a woman with febrile illness in the West Nile district of

68   Uganda [10]. WNV has caused numerous recent outbreaks in North America and Europe

69   leading to major concern for human and animal health [3,11].   In North America, the

70   majority of arboviral encephalitis cases are attributable to WNV [12]. Although ~80% of

71   human WNV infections are asymptomatic, a broad clinical spectrum can result ranging from

72   a mild flu-like illness in ~20% of infected individuals (West Nile fever) to severe neurological

73   disease through infection of the central nervous system (<1% of infected individuals) that

74   can lead to death from meningitis, encephalitis, and acute flaccid paralysis [13,14]. The high

75   proportion of asymptomatic infections highlights that the number of human cases

76   demonstrating overt disease, or discovered through laboratory testing, are just the 'tip of the

77   iceberg' of the actual number of viral infections occurring within a population. Furthermore,

78   these spill-over infections in humans are likely to be far less frequent compared to the

79   amount of enzootic transmission occurring between mosquitoes and avian species. This

80   emphasises the high value of surveillance in the monitoring and prevention of major

81   outbreaks.

82   The introduction and spread of WNV in Europe is thought to have been driven by migratory

83   birds [15–18]. WNV resulted in sporadic human cases from the mid-1990s [19] with the first

84   large outbreak occurring in Romania with 393 hospitalised cases and 17 deaths [19,20].

85   From 2010, the European Center for Disease Control (ECDC) have monitored WNV cases

86   in the European Union and neighbouring countries and publishes weekly epidemiological

87   reports [21]. In Greece, WNV was first detected in the summer of 2010 in the central

88   Macedonia Region near the city of Thessaloniki, in the northern part of the country [22,23].

89   This outbreak included 262 probable and confirmed cases of WNV infection of which 197

90   were neuroinvasive cases and 35 deaths [24]. In 2011 WNV was found in both humans and

91   horses; detected from clinical and laboratory surveillance techniques [25]. In the following

92   years, cases of WNV in humans and animals were reported in central Greece and in the

93   Attica Region but there were no reported cases in 2015 or 2016. In 2017, WNV re-emerged

94   in southern Greece and in 2018 there were 311 laboratory confirmed human cases, resulting

95   in 47 deaths, showing a marked increase over 2017, with only 48 confirmed cases and 5

96   deaths [21,25]. Historical data of human cases with neurological disease in Greece from

97   2010 until present show that cases increase in August (the peak month in the transmission

98   season) and the largest case numbers were reported in August 2010 [24–26].

99   There have been over 60 species of mosquitoes in the USA implicated as potential WNV

100   vector species [4]. Seven of these species occur in Europe and have been tested for WNV

101   susceptibility including members of the *Culex (Cx.) pipiens* complex, *Ae. albopictus* and *Ae.*

102   (*Ocherlotatus*) *caspius* [27]. *Cx. pipiens* has two behaviourally different biotypes, *pipiens*

103   and *molestus*, which can form hybrids and their feeding behaviours influence their role in

104   local transmission of WNV. The *pipiens* biotype is an important species for the enzootic

105   WNV transmission cycle given its preference to feed on birds [28]. The *molestus* biotype

106   and hybrids are implicated in the spill-over transmission of WNV from avian hosts to humans

107   due to the opportunistic feeding behaviour of the *molestus* biotype [28,29]. Temperature has

108   been shown to experimentally increase WNV transmission rates of the *pipiens* and hybrid

109   biotypes but have no effect on the *molestus* biotype [30]. In order to better understand the

110   complexity of WNV transmission, entomological surveys for arboviral surveillance can be

111   undertaken to determine both the presence of potential mosquito vectors and provide

112   evidence for WNV circulation through virus detection in field-caught mosquitoes (molecular

113   xenomonitoring). Here we report the results of an entomological survey undertaken in two

114   Regional Units (RUs) of Greece (Palio Faliro in Attica region and Argolida in Peloponnese

115   region) where WNV outbreaks have previously been recorded. We determined the

116   prevalence of the *Cx. pipiens* biotypes (*pipiens*, *molestus* and hybrids) in each sampling

117   location and female mosquitoes were screened for the presence of WNV to determine

118   whether there was any evidence of virus circulation in the two RUs.

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120   **Methods**

121   **Mosquito collections**

122   The study was carried out in 2 RUs within the Attica and Peloponnese regions of Greece,

123   with three sampling locations selected from within each RU, and three trapping sites within

124   each sampling location (**Fig. 1, Table 1**). Locations for trapping in the RU of Palaio Faliro

125   were classified as urban, whereas those in the RU of Argolida were rural. In each sampling

126   location, three different traps (trapping sites) were operating for 24 hours, three times per

127   week. Trapping occurred over a six-week period (May-June 2018) during the start of the

128   WNV transmission season (based on previous historical data obtained from ECDC [31]). A

129   3x3 Latin square design [32] was applied at each site to minimize confounding factors. Traps

130   were placed more than 100m from each other and rotated every 24 hours between selected

131   positions. Three different trap types were used in each site; Biogent sentinel (BG) traps,

132   Heavy duty Encephalitis Vector Survey (EVS) traps and Centers for Disease Control

133   miniature light (CDC) traps. Dry ice was used as an attractant in all traps with approximately

134   2 kg/ trap per 24 hours. Mosquitoes were collected every 24 hours, killed on dry ice and

135   stored at -80°C. Morphological keys were used to identify individuals to species or species

136   complex level [33] and female mosquitoes were classified as unfed (no evidence of blood in

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137 their abdomen), blood fed or gravid. Individual mosquitoes were then placed in RNAlater

138 (Invitrogen) to preserve RNA for downstream molecular analysis.

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140 **Table 1**. Geographical locations with GPS co-ordinates of mosquito trapping sites within the

141 Attica and Peloponnese regions of Greece.

Region/Regional Unit	Sampling location (name)	Trapping site (street name or description)	GPS co-ordinates (decimal degrees)	
			Latitude	Longitude
Attica/Palaio Faliro	Rema Pikrodafnis	Aristeidou str	37.923972	23.710106
		Dimokritou str	37.923836	23.711511
		Sofokleous str	37.922997	23.710306
	Dimarchio	Terpsichoris str	37.928111	23.699008
		Naiadon str	37.927989	23.696631
		Athanasiadou str	37.928819	23.698006
	KAPI	Seirinon str	37.931997	23.692625
		Esperou str	37.931228	23.692983
		Atlantos str	37.931408	23.692219
Peloponnese/ Argolida	Agia Triada	Veterinary	37.636256	22.798003
		Juice factory	37.6404	22.791736
		Private house	37.638997	22.805275
	Nea Tirintha	Guard room (prisons)	37.596544	22.799989
		Sheep area (prisons)	37.594242	22.796617
		Cattle area (prisons)	37.592711	22.797669
	Dalamanara	Horse area	37.611461	22.739725
		Private house 1	37.620261	22.737842
		Private house 2	37.612106	22.738719

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DNA/RNA extraction and cDNA synthesis

145 DNA was extracted from individual male mosquitoes using QIAGEN DNeasy Blood and

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147 Tissue Kits according to manufacturer's instructions. DNA extracts were eluted in a final

148 volume of 100 μL and stored at −20°C. RNA was extracted from individual female

149 mosquitoes using Roche High Pure RNA Isolation Kits and QIAGEN RNeasy 96 kits

150 according to manufacturer's instructions. RNA extracts were eluted in a final volume of 45

151 μL and stored at −80°C. RNA was reverse transcribed into complementary DNA (cDNA)

152 using an Applied Biosystems High Capacity cDNA Reverse Transcription kit. A final volume

153 of 20 μL contained 10 μL RNA, 2 μL 10X RT buffer, 0.8 μL 25X dNTPs (100 mM), 2 μL 10X

154 random primers, 1μL reverse transcriptase and 4.2 μL nuclease-free water. Reverse

155 transcription was undertaken in a Bio-Rad T100 Thermal Cycler as follows: 25°C for 10min,

156 37°C for 120min and 85°C for 5min, with the cDNA stored at −20°C.

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Molecular identification of species

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159 Specimens morphologically identified as within the *Cx. pipiens* complex were identified to

160 species level using a combination of multiplex species-specific PCR assays [34,35].

161 Additional confirmation of species was undertaken using sequencing of conserved

162 cytochrome c oxidase 1 (*CO1*) gene fragments [36–38]. PCR products were separated and

163 visualized using 2% E-gel EX agarose gels (Invitrogen) with SYBR safe and an Invitrogen E-

164 gel iBase Real-Time Transilluminator. PCR products were submitted to Source BioScience

165 (Source BioScience Plc, Nottingham, UK) for PCR reaction clean-up, followed by Sanger

166 sequencing to generate both forward and reverse reads. Sequencing analysis was carried

167 out in MEGA7 [39] as follows. Both chromatograms (forward and reverse traces) from each

168 sample was manually checked, analyzed, and edited as required, followed by alignment by

169 ClustalW and checking to produce consensus sequences. Consensus sequences were used

170 to perform nucleotide BLAST (NCBI) database queries and sequences were compared to

171 those available from GenBank (NCBI). Representative full consensus sequences for *CO1*

172 gene fragments were submitted to GenBank and assigned accession numbers MN005042-

173 MN005056.

174

WNV screening

176 WNV detection was undertaken using a WNV-specific real-time PCR assay [40]. Reactions

177 were prepared using 5 µL of Qiagen QuantiTect SYBR® Green Master mix, a final

178 concentration of 1 µM of each primer, 1 µL of PCR grade water and 2 µL template cDNA, to

179 a final reaction volume of 10 µL. Prepared reactions were run on a Roche LightCycler® 96

180 System and PCR cycling conditions were as follows: 95°C for 10 min followed by 45 cycles

181 of 95°C for 10 sec, 60°C for 10 sec, 72°C for 20 sec. PCR products were also separated and

182 visualised using 2% E-Gel EX agarose gels (Invitrogen) with SYBR safe and an Invitrogen

183 E-Gel iBase Real-Time Transilluminator to confirm successful amplification of the 144 base

184 pair target fragment.

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WNV case mapping

187 Maps were constructed in ArcMap 10.5 (Esri, ArcGIS) using Global Administrative layers for

188 Greece (level 3), downloaded from www.gadm.org (Version 3.6) and anonymized ECDC

189 WNV case report data from "Transmission of West Nile virus, June to December 2018 –

190 Table of cases, 2018 transmission season" downloaded from www.ecdc.europa.eu. The EU

191 NUTS (Nomenclature of territorial units for statistics) level 3 regions as listed in the ECDC

192 data sheet were matched to the Global Administrative layers level 3 (municipalities) during

193 map construction, with each of the GADM level 3 municipalities matched to the

194 corresponding NUTS level 3 region and assigned the same reported case data. The data

195 from the ECDC surveillance Atlas was collected for each week of the transmission season,

196 for human and equine cases, and then combined for each region, to generate maps of

197 monthly reports.

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200 **Statistical analysis**

201 Non-parametric Mann Whitney U tests were performed in Microsoft Excel (version 16.21.1)

202 to compare the number of *Cx. pipiens* complex mosquitoes for each trap type in a given

203 sampling location.

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205 **Results**

206 **Mosquito species abundance and diversity**

207 A total of 1062 mosquitoes comprising 840 unfed females, 28 blood fed females, 9 gravid

208 females and 185 males were captured (**Table 2)**. Species belonging to the *Cx. pipiens*

209 complex were the most abundant, comprising 62.5% (n= 664) of the total collection across

210 both RUs. Additional species collected included *Cs. longiareolata* (16.1%, n= 171), *Ae.*

211 *caspius* (11.0%, n=117), *Ae. albopictus (7*.4%, n=79) and species belonging to the

212 *Anopheles (An.) maculipennis* complex (1.8%, n=19). The remaining 1.1% (n=12) of

213 mosquitoes were not possible to morphologically identify using keys due to damage during

214 trapping. Individuals of the *Cx. pipiens* complex *and Cs. longiareolata* were collected from

215 all sites within both regions. In the Attica region, *Ae. albopictus* was collected in all three

216 sites and single individuals were also collected in Agia Triada and Dalamanara within the RU

217 of Argolida. In contrast, *Ae. caspius* and *An. maculipennis* complex individuals were

218 collected in all three sites within the RU of Argolida but not from sites within the RU of Palaio

219 Faliro.

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228 **Table 2**. Mosquitoes collected from different locations in the Attica and Peloponnese regions

229 of Greece using a variety of mosquito trap types.

Region/ Regional Unit	Sampling location	Species/complex	Mosquitoes collected					% of total in location
			Females			Males	Total	
			Non blood fed	Blood fed	Gravid			
Attica/Palaio Faliro	Rema Pikrodafnis	Cx. pipiens complex	68	6	3	1	78	55.7
		Ae. albopictus	17	0	0	33	50	35.7
		Cs. longiareolata	1	1	0	7	9	6.4
		Unidentified	3	0	0	0	3	2.1
	Dimarchio	Cx. pipiens complex	47	1	0	0	48	64.9
		Ae. albopictus	8	0	0	5	13	17.6
		Cs. longiareolata	0	1	0	12	13	17.6
	KAPI	Cx. pipiens complex	106	2	2	8	118	84.3
		Ae. albopictus	4	1	0	9	14	10.0
		Cs. longiareolata	2	1	0	4	7	5.0
		Unidentified	1	0	0	0	1	0.7
Peloponnese / Argolida	Agia Triada	Cx. pipiens complex	101	2	3	9	115	54.0
		Ae. albopictus	1	0	0	0	1	0.5
		Cs. longiareolata	31	0	0	64	95	44.6
		Ae. caspius	1	0	0	0	1	0.5
		An. maculipennis complex	0	0	0	1	1	0.5
	Nea Tirintha	Cx. pipiens complex	140	3	0	4	147	49.0
		Cs. longiareolata	14	1	0	23	38	12.7
		Ae. caspius	91	2	0	1	94	31.3
		An. maculipennis complex	13	3	0	0	16	5.3
		Unidentified	5	0	0	0	5	1.7
	Dalamanara	Cx. pipiens complex	153	4	1	0	158	81.0
		Ae. albopictus	1	0	0	0	1	0.5
		Cs. longiareolata	5	0	0	4	9	4.6
		Ae. caspius	22	0	0	0	22	11.3
		An. maculipennis complex	2	0	0	0	2	1.0
		Unidentified	3	0	0	0	3	1.5
Total collected			840	28	9	185	1062	

230 Mosquitoes were morphologically identified using keys and females were classified as non-

231 blood fed (no visible blood in abdomen), blood fed or gravid.

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Species trap comparison

In both RUs, BG traps collected both more overall mosquitoes of all species, and a greater number of specimens from the *Cx. pipiens* complex than CDC traps and EVS traps (**Table 3**). As the data was not normally distributed, non-parametric Mann-Whitney tests were used to determine any significant differences in the number of *Cx. pipiens* complex mosquitoes collected using different trap types (**Table 3**). In the RU of Palaio Faliro, BG traps collected more *Cx. pipiens* complex mosquitoes (n=101) than CDC (n=46) and EVS (n=41) traps although the comparison between BG and CDC traps was not statistically significant (Mann-Whitney U=258.0, p=0.07). In the RU of Argolida BG traps collected significantly more *Cx. pipiens* complex (n=214) than CDC (n=69) and EVS (n=50) traps (Mann-Whitney U=40, p=0.02; U=32, p=0.01 respectively).

Table 3. Mann-Whitney statistical analysis comparing the number of *Cx. pipiens* complex mosquitoes collected using three traps.

Region/Regional Unit	Trap comparison	U-value	Z-score	p-value
Attica/Palaio Faliro	BG vs. CDC	258.0	1.834	0.07
	BG vs EVS	218.5	2.517	0.01
	CDC vs. EVS	342.5	0.372	0.71
Peloponnese/ Argolida	BG vs. CDC	40.0	2.256	0.02
	BG vs EVS	32.0	2.667	0.01
	CDC vs. EVS	76.5	0.385	0.70

Biogents Sentinel traps (BG traps), Centres for Disease Control miniature light traps (CDC traps) and Heavy-Duty Encephalitis Vector Survey traps (EVS traps).

Molecular identification of species

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254   Sanger sequencing of *CO1* gene fragments was undertaken to confirm morphological

255   identification of species and to also determine the species of unidentified specimens that

256   had been damaged during trapping. Representative *CO1* gene fragment sequences from

257   individuals of the *Cx. pipiens* complex from all six collection sites across both RUs were

258   obtained using a PCR assay designed for species identification for European *Cx. pipiens*

259   complex species (38) **(Table 4**).

260

261   **Table 4.** *CO1* GenBank accession numbers for representatives of species confirmed by

262   molecular identification.

Specimen code	Sampling location	Morphological identification	*CO1* gene fragment (reference)	GenBank accession number
AT1	Agia Triada	*Cx. pipiens*	[38]	MN005042
RP1	Rema Pikrodafnis	*Cx. pipiens*	[38]	MN005043
DI1	Dimarchio	*Cx. pipiens*	[38]	MN005044
DA1	Dalamanara	*Cx. pipiens*	[38]	MN005045
KA1	Kapi	*Cx. pipiens*	[38]	MN005046
NT1	Nea Tirintha	*Cx. pipiens*	[38]	MN005047
RP2	Rema Pikrodafnis	*Cs. longiareolata*	[37]	MN005048
DA2	Dalamanara	*Cs. longiareolata*	[37]	MN005049
AT2	Agia Triada	*Cs. longiareolata*	[37]	MN005050
NT2	Nea Tirintha	*Ae. caspius*	[36]	MN005051
AT3	Agia Triada	*Ae. caspius*	[36]	MN005052
DA3	Dalamanara	*Ae. caspius*	[36]	MN005053
DI2	Dimarchio	*Ae. albopictus*	[37]	MN005054
AT4	Agia Triada	*Ae. albopictus*	[37]	MN005055
RP3	Rema Pikrodafnis	*Ae. albopictus*	[37]	MN005056

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264   The location, species and *CO1* gene fragment in addition to the accession number on

265   GenBank are shown.

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267 Sequencing an additional *CO1* fragment [37] successfully confirmed the identification of *Cs.*

268 *longiareolata* (n=3) and *Ae. albopictus* (n=3). Sequencing of a third *CO1* fragment [36] was

269 required to successfully confirm *Ae. caspius* (n=3). However, Sanger sequencing of both

270 *CO1* and internal transcribed spacer – 2 (*ITS2*) fragments [41] did not produce sequences of

271 sufficient quality to successfully speciate individuals morphologically identified as within the

272 *An. maculipennis* complex. Multiplex species-specific assays [42,43] revealed the presence

273 of both biotypes of *Cx. pipiens* (*pipiens* type and *molestus* type) in addition to hybrids (**Fig.**

274 **2**). In the RU of Palaio Faliro overall 54.5% (n=79) were confirmed as the *pipiens* type,

275 20.0% (n=29) as the *molestus* type and 25.5% (n=37) as hybrids. In the RU of Argolida,

276 68.1% (n=98) were *pipiens* type, 8.3% (n=12) *molestus* type and 23.6% (n=34) hybrids.

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278 **WNV infection rates in field mosquitoes**

279 A total of 630 individual mosquitoes (229 from RU of Palaio Faliro and 401 from RU of

280 Argolida) were screened for the presence of WNV RNA and four *Cx. pipiens* complex

281 individuals were WNV positive**.** qRT PCR results were confirmed by separation and

282 visualisition of PCR products using gel electrophoresis. These positive individuals were

283 unfed females which were molecularly identified as three *pipiens* type and one hybrid, all

284 collected from the RU of Argolida at the end of May. This is interesting when compared to

285 the spatial and temporal records of human and equine cases during 2018 (**Fig. 3)** as only

286 one human case was recorded from the Peloponnese region all year, and not until August.

287 This is in contrast to the RU of Palaio Faliro where, across the whole Attica region a total of

288 159 human cases were recorded in 2018, with the first reported cases occurring in June, but

289 no WNV was detected in the mosquitoes collected from this region.

290

291 **Discussion**

292 Our mosquito trapping experiments using different adult traps show that in both regions BG

293 traps collected both a larger number of mosquitoes of all species, and a greater number of

294 individuals from the *Cx. pipiens* complex (although this was not statistically significant in the

295   RU of Palaio Faliro). Previous trap comparison studies undertaken in Europe report

296   contrasting results, ranging from BG traps in Germany collecting more *Cx. pipiens* complex

297   mosquitoes than CDC and EVS traps [44], to a study in Spain showing no statistically

298   significant differences between BG and CDC traps in collecting specimens from this

299   complex [45]. Although we measured temperature and humidity during our collection

300   periods (Additional file 1), there are a variety of additional factors that can influence the

301   collections obtained from adult mosquito traps including wind and the use of different

302   attractants. Our results highlight that using a variety of trapping types can increase the

303   species diversity of collections, however, targeting resources to just use BG traps may

304   enable a greater number of target vector species – such as individuals of the *Cx. pipiens*

305   complex – to be collected.

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307   Although different mosquito species (across multiple genera) have been demonstrated to be

308   competent vectors of WNV [46], the major vectors for WNV belong to the *Cx. pipiens*

309   complex. In this study, we collected individuals of the *Cx. pipiens* complex in addition to

310   other species including *Ae. albopictus*, *Cs. longiareolata* and *Ae. caspius* shown previously

311   to be present in Greece [47,48]. The presence of the *pipiens* type, *molestus* type and

312   hybrids in both the Attica and Peloponnese regions is consistent with previous studies in

313   Greece [22,49,50]. We found variation in the prevalence of the different types with the

314   *pipiens* type comprising 54.5% (n=79) in the RU of Palaio Faliro, 20.0% (n=20) *molestus*

315   type and 25.5% (n=37) of hybrids. These results differ from another study that had found a

316   more homogeneous *molestus* type population [49]. In RU of Argolida the biotypes of the *Cx.*

317   *pipiens* complex were 68.1% (n=98) of *pipiens* type, 8.3% (n=12) of *molestus* type and

318   23.6% (n=34) of *molestus* and *pipiens* hybrids. The high percentage of hybrids in the RU is

319   similar to a previous study conducted in the area after the 2017 outbreak that reported 37%

320   hybrids, 41% *pipiens* and 22% *molestus* types [50].

321

322   The two biotypes are morphologically indistinguishable but have genetic, biological and

323   behavioural differences. The *pipiens* type is anautogenous, so females need to consume a

324   blood meal to lay eggs [22]. Furthermore, the *pipiens* type requires a large space to swarm

325   for mating, are found above ground undergoing diapause and are primarily ornithophilic

326   (preferring to feed on birds). In contrast, the *molestus* type is autogenous and can lay eggs

327   without a blood meal. Mating can happen in confined spaces, while they live underground,

328   do not undergo diapause, and are more anthropophilic, preferentially feeding on humans.

329   Hybrid types are important in the epidemiology of WNV. In the USA, the high number of

330   WNV cases in humans was correlated to the high number of hybrids [51]. Europe is

331   considered to have more "pure" types but hybridization can result in a catholic feeding

332   behaviour (feeding both on birds and mammals) increasing the risk of mixed populations

333   acting as bridge-vectors of WNV between birds and humans/equines [49]. The feeding

334   patterns of the different mosquito species, and the different types within the species

335   complex, are important in order to identify the contribution of each vector to both the

336   enzootic maintenance of WNV in avian hosts, and the spill-over transmission to humans and

337   horses [52]. In northern Greece, the predominance of *pipiens* type could be facilitating the

338   maintenance of the enzootic cycle of the virus between mosquitos and birds in the area [49].

339   The presence of the *molestus* type and the existence of hybrids can promote an

340   opportunistic biting behaviour that could contribute to the spill-over of infection to humans

341   and equines.

342

343   In our study, we also collected several other species that have been implicated or shown to

344   be potential WNV vectors. Experimental transmission has been shown for both *Cs.*

345   *longiareolata and Ae. albopictus* [1]. Species belonging to the *An. maculipennis* group are

346   considered potential vectors of WNV [2]. Laboratory experiments have indicated that *Ae.*

347   *caspius* may be incapable of transmitting WNV [27,53], however, in some countries the high

348   densities, and detection of WNV in wild-caught specimens, have suggested this species may

349   have a potential role in transmission, particularly during an outbreak when the level of virus

350   circulation is high [54]. The presence of *Ae. albopictus*, an invasive species that has

351   expanded its range across Europe since the late 1970s, would suggest the potential for

352   transmission of additional arboviruses. *Ae. albopictus* has the ability to adapt to colder

353   temperatures and stay dormant during the winter and has previously been shown to be

354   responsible for chikungunya virus outbreaks in Italy in 2007 [55]. *Ae. albopictus* has also

355   been the principle vector responsible for dengue virus outbreaks in Hawaii in 2001-2002 and

356   Mauritius in 2009 [56,57] and is a potential vector of Zika virus [58,59]. Furthermore, it can

357   be a competent vector of WNV when experimentally tested in laboratory conditions [60] and

358   in North America natural infections have been found. The opportunistic biting behaviour of

359   *Ae. albopictus* may increase this species role as a vector of WNV. In Greece, since its first

360   reported presence in 2003 in the western part of the country, *Ae. albopictus* has now spread

361   to almost every district [48].

362

363   Detection of WNV virus RNA in four unfed *Cx. pipiens* complex specimens would indicate

364   circulation of WNV in the RU of Argolida during our collection period in May. This would be

365   supported by one human laboratory confirmed case reported in Peloponnese in 2018,

366   however, it is interesting to note the reported human case didn't occur until August,

367   suggesting WNV may have been circulating in the area for months before resulting in a case

368   of human clinical disease. The confirmation that three of the positives were *pipiens* type,

369   supports the possibility of virus circulating in an enzootic cycle, between birds and

370   mosquitoes. However, the presence of WNV in one of the hybrids also demonstrates the

371   potential for spill-over transmission to humans and equines in the area at this early time in

372   the season. In comparison, no WNV was detected in mosquitoes collected from the RU of

373   Palaio Faliro, but this area subsequently recorded a far greater number of human and

374   equine cases during 2018, highlighting the likely variations in spatial and temporal

375   transmission dynamics between these two very different localities, and the variable factors

376   that can influence risk of infection and disease during the transmission season.

377

16

Conclusions

378

379 Sampling during the onset of the 2018 WNV transmission season in the RUs of Attica and

380 Peloponnese Regions was particularly important in a year in which more than 300 human

381 cases were recorded in Greece. These results, combined with previous entomological

382 surveys conducted in Greece, show the high occurrence of hybrids between the *pipiens* and

383 *molestus* types of *Cx. pipiens.* Previous studies have demonstrated the importance of

384 hybrids as bridge vectors of WNV. Their role in spill-over transmission to humans, and the

385 presence of hybrids (and WNV infections) in RUs of Attica and Peloponnese regions of

386 Greece suggest these areas are vulnerable to outbreaks. Furthermore, 2018 was the first

387 year in Greece in which WNV human cases were recorded so early in the transmission

388 period with six human cases confirmed by late June. Future entomological surveillance

389 studies should incorporate molecular xenomonitoring to determine this potential expansion

390 of the transmission season to provide early warning systems for potential WNV outbreaks.

391 Notification of human WNV cases in Europe through the The European Surveillance System

392 (TESSy) [61] of the ECDC allows a weekly map of human cases [31]. In addition, reporting

393 of WNV encephalomyelitis in horses to the European Commission is carried out via the

394 Animal Disease Notification System (ADNS). As reported cases of WNV infection in humans

395 have been from southern and central European countries and a majority of human infections

396 are asymptomatic, it is particularly important to undertake entomological and avian

397 surveillance to determine if WNV circulation is occurring in particular area. In particular,

398 entomological surveys to determine the distribution of mosquito vectors such as *Cx. pipiens*

399 through the Pan-European VectorNet [62] will play a crucial role in an integrated approach to

400 WNV surveillance and control efforts to minimise the impact of outbreaks on veterinary and

401 public health.

402

403 **List of abbreviations**

404 **WNV:** West Nile virus

17

405 **Cx**: Culex

406 **RUs** : Regional Units

407 **BG traps**: Biogent sentinel traps

408 **CDC traps**: Centres for Disease Control miniature light traps

409 **EVS traps**: Heavy Duty Encephalitis Vector Survey traps

410 **Ae**: Aedes

411 **Cs**: Culiseta

412 **An**: Anopheles

413 **ECDC**: European Center for Disease Control

414 **CO1**: cytochrome c oxidase 1

415
416

417 **Declarations**

418 **Ethics approval and consent to participate.**

419 The study protocol was reviewed and approved by the the institutional review boards of the

420 London School of Hygiene and Tropical Medicine (#15234).

421 **Consent for publication**

422 Not applicable.

423 **Availability of data and materials**

424 All representative mosquito species sequences are available from Genbank: accession

425 numbers MN005042-MN005056. The datasets generated on Collection, extraction and PCR

426 results are available at Open Science Framework: DOI 10.17605/OSF.IO/D76QF.

427 **Competing interests**

428 The authors declare that they have no competing interests

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Authors' contributions

MB contributed to conceptualization, data curation, formal analysis, investigation, methodology and writing of the original draft. CLJ contributed to conceptualization, data curation, formal analysis, investigation, methodology, supervision, writing of the original draft and review and editing of the manuscript. IL contributed to investigation and methodology. AM contributed to conceptualization, investigation, methodology, project administration, supervision and review and editing of the manuscript. TW contributed to conceptualization, data curation, formal analysis, investigation, methodology, funding acquisition, supervision, project administration, supervision, writing of the original draft and review and editing of the manuscript. All authors read and approved the final manuscript.

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459 **References**

460 1. Hubalek Z, Halouzka J. West Nile fever--a reemerging mosquito-borne viral disease in

461 Europe. Emerg Infect Dis 1999;5(5):643–50.

462 2. Hubalek Z. Mosquito-borne viruses in Europe. Parasitol Res 2008;103:S29-43.

463 3. Brustolin M, Talavera S, Santamaría C, Rivas R, Pujol N, Aranda C, et al. Culex pipiens

464 and Stegomyia albopicta (=Aedes albopictus) populations as vectors for lineage 1 and 2

465 West Nile virus in Europe. Med Vet Entomol. 2016; 30(2):166-73.

466 4. Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL.

467 Epidemiology and transmission dynamics of West Nile virus disease. Emerging Infectious

468 Diseases. 2005;11(8):1167-73.

469 5. Vogels CBF, Hartemink N, Koenraadt CJM. Modelling West Nile virus transmission risk in

470 Europe: Effect of temperature and mosquito biotypes on the basic reproduction number. Sci

471 Rep. 2017;7(1):5022.

472 6. Custer B, Busch MP, Marfin AA, Petersen LR. The cost-effectiveness of screening the

473 U.S. blood supply for West Nile virus. Ann Intern Med. 2005;143(7):486-92.

474 7. Korves CT, Goldie SJ, Murray MB. Cost-effectiveness of alternative blood-screening

475 strategies for West Nile virus in the United States. PLoS Med. 2006;3(2):e21.

476 8. Bowen RA, Bosco-Lauth A, Syvrud K, Thomas A, Meinert TR, Ludlow DR, et al.

477 Protection of horses from West Nile virus Lineage 2 challenge following immunization with a

478 whole, inactivated WNV lineage 1 vaccine. Vaccine. 2014;32(42):5455-9.

479   9. Long MT, Gibbs EPJ, Mellencamp MW, Bowen RA, Seino KK, Zhang S, et al. Efficacy,

480   duration, and onset of immunogenicity of a West Nile virus vaccine, live Flavivirus chimera,

481   in horses with a clinical disease challenge model. Equine Vet J. 2007;39(6):491-7.

482   10. Hughes TP, Paul JH, Smithburn KC, Burke AW. A Neurotropic Virus Isolated from the

483   Blood of a Native of Uganda 1. Am J Trop Med Hyg. 1940;4:471-92.

484   11. Gubler DJ. The continuing spread of West Nile virus in the Western Hemisphere. Clin

485   Infect Dis. 2007;45(8):1039–46.

486   12. Grubaugh ND, Ebel GD. Dynamics of West Nile virus evolution in mosquito vectors.

487   Current Opinion in Virology. 2016 ;21:132-138.

488   13. Colpitts TM, Conway MJ, Montgomery RR, Fikrig E. West Nile virus: Biology,

489   transmission, and human infection. Clin Microbiol Rev. 2012;25(4):635-48.

490   14. Petersen LR, Marfin AA. West Nile virus: A primer for the clinician. Annals of Internal

491   Medicine. 2002;137(3):173-9.

492   15. Jourdain E, Schuffenecker I, Korimbocus J, Reynard S, Murri S, Kayser Y, et al. West

493   Nile Virus in Wild Resident Birds, Southern France, 2004. Vector-Borne Zoonotic Dis.

494   2007;7(3):448-52.

495   16. Linke S, Niedrig M, Kaiser A, Ellerbrok H, Müller K, Müller T, et al. Serologic evidence of

496   West Nile virus infections in wild birds captured in Germany. Am J Trop Med Hyg.

497   2007;77(2):358-64.

498   17. Figuerola J, Soriguer R, Rojo G, Tejedor CG, Jimenez-Clavero MA. Seroconversion in

499   wild birds and local circulation of West Nile virus, Spain. Emerg Infect Dis.

500   2007;13(12):1915-7.

501   18. Calzolari M, Gaibani P, Bellini R, Defilippo F, Pierro A, Albieri A, et al. Mosquito, bird and

502   human surveillance of West Nile and Usutu viruses in Emilia-Romagna Region (Italy) in

503   2010. PLoS One. 2012;7(5):e38058.

504   19. Sambri V, Capobianchi MR, Cavrini F, Charrel R, Donoso-Mantke O, Escadafal C, et al.

505   Diagnosis of west nile virus human infections: Overview and proposal of diagnostic protocols

506   considering the results of external quality assessment studies. Viruses. 2013;5(10):2329-48.

507   20. Sejvar JJ. West nile virus: an historical overview. Ochsner J. 2003;5(3):6-10.

508   21. European Centre for Disease Prevention and Control. Weekly updates: 2018 West Nile

509   fever transmission season. Available from: https://ecdc.europa.eu/en/west-nile-

510   fever/surveillance-and-disease-data/disease-data-ecdc

511   22. Papa A, Tsimitri T, Papadopoulou E, Testa T, Adamidis A, Gavana E, et al. Molecular

512   detection and isolation of West Nile virus from a human case in northern Greece, 2013. New

513   microbes new Infect. 2013;1(2):30-1.

514   23. Papa A, Danis K, Baka A, Bakas A, Dougas G, Lytras T, et al. Ongoing outbreak of west

515   nile virus infections in humans in greece, july - august 2010. Eurosurveillance. 2010;15(34).

516   pii: 19644.

517   24. Danis K, Papa A, Theocharopoulos G, Dougas G, Athanasiou M, Detsis M, et al.

518   Outbreak of West Nile virus infection in Greece, 2010. Emerg Infect Dis. 2011;17(10):1868-

519   72.

520   25. Hellenic Centre for Disease Control and Prevention (KEELPNO) - EPIET. [cited 2019

521   May 29]. Available from: https://ecdc.europa.eu/en/hellenic-centre-disease-control-and-

522   prevention-keelpno-epiet

523   26. Epidemiological update: West Nile virus transmission season in Europe, 2018. [cited

524   2019 May 29]. https://ecdc.europa.eu/en/news-events/epidemiological-update-west-nile-

525   virus-transmission-season-europe-2018

526   27. Vogels CBF, Göertz GP, Pijlman GP, Koenraadt CJM. Vector competence of European

527   mosquitoes for west Nile virus. Emerging Microbes and Infections. 2017;6(11):e96.

528   28. Fritz ML, Walker ED, Miller JR, Severson DW, Dworkin I. Divergent host preferences of

529   above- and below-ground Culex pipiens mosquitoes and their hybrid offspring. Med Vet

530   Entomol. 2015;29(2):115-23.

531   29. Osorio HC, Ze-Ze L, Alves MJ. Host-feeding patterns of Culex pipiens and other

532   potential mosquito vectors (Diptera: Culicidae) of West Nile virus (Flaviviridae) collected in

533   Portugal. J Med Entomol. 2012;49(3):717–21.

534   30. Vogels CBF, Fros JJ, Göertz GP, Pijlman GP, Koenraadt CJM. Vector competence of

535   northern European Culex pipiens biotypes and hybrids for West Nile virus is differentially

536   affected by temperature. Parasites and Vectors. 2016;9(1):393.

537   31. European Centre for Disease Prevention and Control. Weekly updates: 2018 West Nile

538   fever transmission season. https://ecdc.europa.eu/en/west-nile-fever/surveillance-and-

539   disease-data/disease-data-ecdc.

540   32. Perry JN, Wall C, Greenway AR. Latin Square designs in field experiments involving

541   insect sex attractants. Ecol Entomol. 1980; 5(4): 385-96.

542   33. Samanidou-Voyadjoglou A, Harbach RE. Keys to the adult female mosquitoes

543   (Culicidae) of Greece. Eur Mosq Bull. 2001;13(3):247-54.

544   34. Smith JL, Fonseca DM. Rapid assays for identification of members of the *Culex (Culex)*

545   *pipiens* complex, their hybrids, and other sibling species (Diptera: culicidae). Am J Trop Med

546   Hyg. 2004;70(4):339–45.

547   35. Bahnck CM, Fonseca DM. Rapid assay to identify the two genetic forms of *Culex (Culex)*

548   *pipiens* L. (Diptera: Culicidae) and hybrid populations. Am J Trop Med Hyg. 2006;75(2):251–

549   5.

550   36. Kumar NP, Rajavel AR, Natarajan R, Jambulingam P. DNA Barcodes Can Distinguish

551   Species of Indian Mosquitoes (Diptera: Culicidae). J Med Entomol. 2007;44(1):1-7.

552   37. Folmer O, Black M, Heoh W, Lutz R, Vrijenhoek R. DNA primers for amplification of

553   mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar

554   Biol Biotechnol. 1994;3(5):294-9.

555   38. Zittra C, Flechl E, Kothmayer M, Vitecek S, Rossiter H, Zechmeister T, et al. Ecological

556   characterization and molecular differentiation of Culex pipiens complex taxa and Culex

557   torrentium in eastern Austria. Parasites and Vectors. 2016;9(1);197.

558   39. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis

559   Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016;33(7):1870–4.

560   40. Linke S, Ellerbrok H, Niedrig M, Nitsche A, Pauli G. Detection of West Nile virus lineages

561   1 and 2 by real-time PCR. J Virol Methods. 2007;146(1–2):355–8.

562 41. Beebe NW, Ellis JT, Cooper RD, Saul A. DNA sequence analysis of the ribosomal DNA

563 ITS2 region for the Anopheles punctulatus group of mosquitoes. Insect Mol Biol.

564 1999;8(3):381–90.

565 42. Bahnck CM, Fonseca DM. Rapid assay to identify the two genetic forms of Culex (Culex)

566 pipiens L. (Diptera: Culicidae) and hybrid populations. Am J Trop Med Hyg. 2006;75(2):251–

567 5.

568 43. Smith JL, Fonseca DM. Rapid assays for identification of members of the Culex (Culex)

569 pipiens complex, their hybrids, and other sibling species (Diptera: culicidae). Am J Trop Med

570 Hyg. 2004;70(4):339–45.

571 44. Lühken R, Pfitzner WP, Börstler J, Garms R, Huber K, Schork N, et al. Field evaluation

572 of four widely used mosquito traps in Central Europe. Parasites and Vectors. 2014;7:268.

573 45. Roiz D, Roussel M, Munõz J, Ruiz S, Soriguer R, Figuerola J. Efficacy of mosquito traps

574 for collecting potential west nile mosquito vectors in a natural mediterranean wetland. Am J

575 Trop Med Hyg. 2012;86(4):642-8.

576 46. Ciota AT. West Nile virus and its vectors. Current Opinion in Insect Science. 2017;22:28-

577 36.

578 47. Darsie RF, Samanidou-Voyadjoglou A. Keys for the identification of the mosquitoes of

579 Greece. J Am Mosq Control Assoc. 1997;13(3):247-54.

580 48. Badieritakis, Papachristos D, Latinopoulos D, Stefopoulou, Kolimenakis, Bithas K, et al.

581 Aedes albopictus (Skuse, 1895) (Diptera: Culicidae) in Greece: 13 years of living with the

582 Asian tiger mosquito. Parasitol Res. 2017;117(2):453-460.

583 49. Gomes B, Kioulos E, Papa A, Almeida AP, Vontas J, Pinto J. Distribution and

584 hybridization of Culex pipiens forms in Greece during the West Nile virus outbreak of 2010.

585 Infect Genet Evol. 2013;16:218–25.

586 50. Mavridis K, Fotakis EA, Kioulos I, Mpellou S, Konstantas S, Varela E, et al. Detection of

587 West Nile Virus – Lineage 2 in Culex pipiens mosquitoes, associated with disease outbreak

588 in Greece, 2017. Acta Trop. 2018;182:64-68.

589 51. Ciota AT, Chin PA, Kramer LD. The effect of hybridization of Culex pipiens complex

590   mosquitoes on transmission of West Nile virus. Parasit Vectors. 2013;6(1):305.

591   52. Molaei G, Andreadis TG, Armstrong PM, Anderson JF, Vossbrinck CR. Host feeding

592   patterns of Culex mosquitoes and west nile virus transmission, northeastern United States.

593   Emerg Infect Dis. 2006;12(3):468-74.

594   53. Balenghien T, Vazeille M, Grandadam M, Schaffner F, Zeller H, Reiter P, et al. Vector

595   competence of some French Culex and Aedes mosquitoes for West Nile virus. Vector Borne

596   Zoonotic Dis. 2008;8(5):589–95.

597   54. Mancini G, Montarsi F, Calzolari M, Capelli G, Dottori M, Ravagnan S, et al. Mosquito

598   species involved in the circulation of West Nile and Usutu viruses in Italy. Vet Ital.

599   2017;53(2):97-110.

600   55. Paupy C, Delatte H, Bagny L, Corbel V, Fontenille D. Aedes albopictus, an arbovirus

601   vector: from the darkness to the light. Microbes Infect. 2009;11(14–15):1177–85.

602   56. Effler P V, Pang L, Kitsutani P, Vorndam V, Nakata M, Ayers T, et al. Dengue fever,

603   Hawaii, 2001-2002. Emerg Infect Dis. 2005;11(5):742–9.

604   57. Ramchurn SK, Moheeput K, Goorah SS. An analysis of a short-lived outbreak of dengue

605   fever in Mauritius. Euro Surveill. 2009;14(34).

606   58. Wong PS, Li MZ, Chong CS, Ng LC, Tan CH. Aedes (Stegomyia) albopictus (Skuse): a

607   potential vector of Zika virus in Singapore. PLoS Negl Trop Dis. 2013;7(8):e2348.

608   59. Grard G, Caron M, Mombo IM, Nkoghe D, Mboui Ondo S, Jiolle D, et al. Zika Virus in

609   Gabon (Central Africa) - 2007: A New Threat from Aedes albopictus? PLoS Negl Trop Dis.

610   2014;8(2):e2681.

611   60. Fortuna C, Remoli ME, Severini F, Di Luca M, Toma L, Fois F, et al. Evaluation of vector

612   competence for West Nile virus in Italian Stegomyia albopicta (=Aedes albopictus)

613   mosquitoes. Med Vet Entomol. 2015;29(4):430-3.

614   61. European Centre for Disease Prevention and Control (ECDC). The European

615   Surveillance System (TESSy). https://ecdc.europa.eu/en/publications-data/european-

616   surveillance-system-tessy.

617   62. European Centre for Disease Prevention and Control. European network for sharing

618   data on the geographic distribution of arthropod vectors, transmitting human and animal

619   disease agents (VectorNet). https://ecdc.europa.eu/en/about-us/partnerships-and-

620   networks/disease-and-laboratory-networks/vector-net.

621
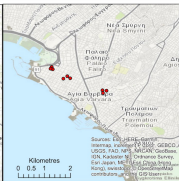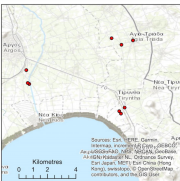622   **Figure legends**

623   **Fig. 1.** Locations of collection sites within Regional Unit of Palaio Faliro in Attica Region

624   (lower right) and Regional Unit of Argolida in Peloponnese region (lower left). Maps

625   constructed in ArcMap 10.5 (Esri, ArcGIS), using World Topographic Basemap and GPS

626   coordinates from trap locations.

627   **Fig. 2**. Prevalence rates of *Cx. pipiens* biotypes. Mosquitoes analysed using multiplex

628   species-specific PCR assays were from three sampling locations in Regional Unit of Palaio

629   Faliro in Attica Region (A) and Regional Unit of Argolida in Peloponnese region (B) of

630   Greece during May-June 2018.

631   **Fig. 3.** Reported human and equine cases of WNV in the 2018 transmission season. Maps

632   were constructed in ArcMap 10.5 (Esri, ArcGIS) using Global Administrative layers for

633   Greece (level 3), downloaded from www.gadm.org (Version 3.6) and ECDC WNV case

634   report data from "Transmission of West Nile virus, June to December 2018 – Table of cases,

635   2018 transmission season" downloaded from www.ecdc.europa.eu. The data from the

636   ECDC surveillance Atlas was collected for each week of the transmission season, for human

637   and equine cases, and then combined for each region, to generate maps of monthly reports.
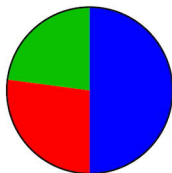
638

639   **Additonal file 1**

640   Temperature (°C) and relative humidity (%) during the collection periods in Regional Unit of

641   Palaio Faliro in Attica Region (A) and Regional Unit of Argolida in Peloponnese region (B) of

642   Greece.

Sources: Esri, HERE, Garmin, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), (c) OpenStreetMap contributors, and the GIS User Community

Sources: Esri, HERE, Garmin, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), (c) OpenStreetMap contributors, and the GIS User Community

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RU of Palaio Faliro in Attica Region

Rema Pikrodafnis
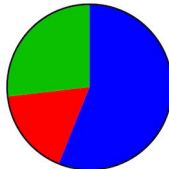
- pipiens biotype
- molestus biotype
- hybrid

Total=48

Dimarchio

- pipiens biotype
- molestus biotype
- hybrid

Total=41

KAPI
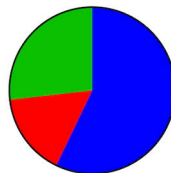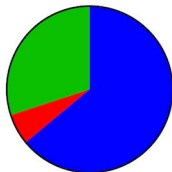
- pipiens biotype
- molestus biotype
- hybrid

Total=56

RU of Argolida in Peloponnese Region

Agia Triada

- pipiens biotype
- molestus biotype
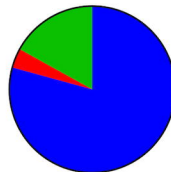- hybrid

Total=50

NeaTirintha

- pipiens biotype
- molestus biotype
- hybrid

Total=41

Dalamanara

- pipiens biotype
- molestus biotype
- hybrid

Total=53

June - Human Cases

July - Human Cases

August - Human Cases

September - Human Cases

October - Human Cases

November - Human Cases

Total Human Cases

Total Equine Cases

Legend

WNV Cases: 1 | 2-3 | 4-6 | 7-8 | 9-11 | 12-15 | 16-22 | 23-24 | 25-35 | 36-46

0 150 300 600 Kilometres