1 **Dynamic surveillance of mosquitoes and their viromes in Wuhan during 2020**

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18

19 ## Abstract

20 Mosquitoes are medically important arthropod vectors and harbor a great variety of

21 viruses. The population density, species and virome of mosquitoes varies according to

22 geography and climate. To investigate the dynamic changes in the species

23 composition and diversity of mosquitoes and their viromes in Wuhan, China, a total

24 of 2,345 adult mosquitoes collected from different habitats including an urban

25 residential area, two hospitals, a scenic area, and a pig farm in a rural region from

26 April to October 2020 were subjected to morphological identification, RT-qPCR and

27 metagenomic sequencing. The results indicated that the dominant presence of *Culex*

28 mosquitoes was observed in both urban regions (90.32%, 1538/1703) and the pig farm

29 (54.98%, 353/642). Viromes of *Culex* showed dynamic changes during the collection

30 time. Several viruses, such as Culex flavivirus, Alphamesonivirus 1, Hubei mosquito

31 virus 2 and Hubei mosquito virus 4, had seasonal changes and unimodal increases or

32 declines. Other viruses, such as Wuhan mosquito virus 6, Hubei virga-like virus 2 and

33 Zhejiang mosquito virus 3, were stable in all collected *Culex* and should be potential

34 members of "core viromes". This study improves the understanding of the dynamic

35 composition of mosquitoes and the viromes they carry and provides useful

36 information for informing mosquito control and mosquito-borne disease prevention

37 strategies.

38

39 **Keywords:** Wuhan, mosquito, virome, surveillance

40

Introduction

42 Mosquitoes, as blood-sucking arthropods, are widely distributed worldwide. Many of

43 them cause severe threats to public health. Mosquitoes are vital competent vectors for

44 several mosquito-borne viruses (MBVs), such as dengue virus and chikungunya virus,

45 from which millions of people suffer every year [1][2]. In addition to MBVs that can

46 infect both vertebrates and invertebrates, various mosquito-specific viruses (MSVs)

47 have been identified, with possible applications as biological control agents against

48 mosquito-borne diseases, diagnostic therapies and novel vaccine platforms [3]. With

49 rapidly developing NGS (next-generation sequencing) technology, viral

50 metagenomics has been used to detect viral diversity and abundance, predict disease

51 outbreaks and identify novel viruses in uncultured mosquito samples [4]. Moreover,

52 viral metagenomic surveillance systems have been established to monitor the virome

53 derived from mosquito hosts [4].

54 Hubei is a province located in central China with a suitable environment for mosquito

55 breeding. During the last decade, hundreds of Japanese encephalitis cases have been

56 reported in Hubei [5]. In August 2019, the first local outbreak with a total of 50 cases

57 of dengue fever was reported in Huangzhou, Hubei Province [6]. In addition, two

58 genotypes of Banna virus (BAV) have been isolated from mosquitoes captured in

59 Hubei; this virus is a member of the *Seadornavirus* genus and may be pathogenic to

60 humans or animals [7]. Through metagenomic virome analysis, it was found that the

61 viromes of mosquitoes from Hubei Province are mainly composed of MBVs,

62 compared to the viromes of mosquitoes from Yunnan Province consisting of MSVs,

63 suggesting that the composition of the virome is highly variable by geographic region

64 [8]. For MSVs, Quang Binh virus, Culex flavivirus and Yichang virus (YCV), which

65 have the ability to reduce the replication of DENV-2 when coinfected in mosquitoes,

66 were also reported to be present in the mosquitoes from Hubei Province [9,10].

67 Wuhan is the capital city of Hubei Province, with an annual mean temperature of

68 15.8°C to 17.5°C and annual precipitation of 1,150 to 1,450 mm of rain, which makes

69 this area suitable for mosquito breeding. During the COVID-19 epidemic, in early

70 2020, a large-scale disinfection procedure was applied within the environment of the

71 whole city [11]. Therefore, compared to previous years, changes in the species or

72 density of mosquito populations may occur in Wuhan. In addition, there is still limited

73 information about the viral diversity and abundance of mosquitoes in Wuhan. In this

74 study, mosquitoes were collected monthly from the field in Wuhan from April to

75 October 2020. Dynamic surveillance of the mosquitoes and their virome and the

76 detection of BAV and Japanese encephalitis virus (JEV) in mosquitoes were

77 conducted.

78

Methods and Materials

79

Sampling sites

80

81 The sampling sites were selected in five representative regions of Wuhan, and the

82 sampling times were from late April to late October 2020 (SFig. 1) as described

83 previously [12]. The sites contain two hospitals, First People's Hospital of Jiangxia

84 District (30° 22′ 23″ N, 114° 18′ 56″ E) and Huoshenshan Hospital (30° 31′ 42″ N,

85 114° 4′ 50″ E); one urban residential area, Huanan seafood market (30° 37′ 7″ N, 114°

86 15′ 25″ E); one scenic area, East Lake (30° 37′ 6″ N, 114° 15′ 27″ E); and one pig

87 farm in a rural region, Huangpi Pig Farm (30° 52′ 52″ N, 114° 22′ 30″ E).

88

Mosquito collection and sample preparation

89

90 Mosquitoes were collected using light traps with an attractant (Maxttrac, China) set

91 on the shore ponds or in the bushes. Mosquitos were collected monthly from April to

92 October, except in the pig farm, which was only trapped in May. The traps were set

93 from 19:00 to 22:00 pm for 3-7 days every month. Then, the mosquitoes were

94 separated, identified, and stored at -80°C.

95   The mosquitoes were assigned to different pools based on their respective collection

96   site, month, species and sex. Each mosquito pool (20 mosquitoes/pool) was triturated

97   by the cryogenic grinding method using a High-Speed Low-Temperature Tissue

98   Grinding Machine (Servicebio, China) running two 30-second cycles at 50 Hz. After

99   sufficient grinding, 600 μL of Roswell Park Memorial Institute (RPMI) medium was

100 added for homogenization [13]. Mosquito macerates were clarified by centrifugation

101 at $10,000 \times g$ (4°C for 30 min) to remove cell debris and bacteria. Supernatants were

102 stored at -80°C until further use.

103

104 **RNA extraction and RT-qPCR**

105 RNA was extracted from 200 μL of homogenized supernatant sample using Direct-zol

106 RNA MiniPrep (Zymo Research, USA) following the manufacturer's instructions.

107 The quantitative real-time reverse transcription PCR (RT-qPCR) mixtures for the

108 detection of viral RNA were made by a Luna® Universal Probe One-Step RT-qPCR

109 Kit (New England Biolabs, USA) in accordance with the manufacturer's instructions

110 and then placed in a thermocycler (BIO-RAD CFX96™ Real-Time System, USA).

111 Specific RT-qPCR for BAV and JEV was performed using the reported primers and

112 probes (STable 1)[7][14][15]. All oligoprimer DNAs were synthesized by TSINGKE

113 (Wuhan Branch, China). Twenty-microliter reaction mixtures containing 5 μL of viral

114 RNA and 0.8 μL of each primer were incubated at 55°C for 10 min and 95°C for 1

115 min followed by 40 cycles of 95°C for 10 s and 55°C for 30 s.

116

117 **Metagenomic sequencing**

118 The dominant mosquito genera in urban districts and pig farms were selected for

119 sequencing. Briefly, the RNA extracted from each mosquito pool was remixed to

120 form new pools via different strategies. For urban pools, 10 μL of RNA extracted

121 from pools from all the sampling areas in the same month was mixed into monthly

122 pools. For the Huangpi pig farm pools, 10 μL of RNA extracted from pools from the

123 same mosquito genus was mixed into *Anopheles* or *Culex* pools. The purity and

124 integrity of the RNA pools were checked by the NanoPhotometer® spectrophotometer

125 (IMPLEN, CA, USA) and the RNA Nano 6000 Assay Kit and Bioanalyzer2100

126 system (Agilent Technologies, CA, USA). One microgram of RNA from each pool

127 was used for library preparation with the NEBNext® UltraTMRNA Library Prep Kit

128 for Illumina® (NEB, USA) according to the manufacturer's instructions. Then, the

129 libraries were clustered on a cBot Cluster Generation System using a TruSeq PE

130 Cluster Kit v3-cBot-HS (Illumina) and sequenced using an Illumina NovaSeq 6000

131 System.

132

133 **Downstream bioinformatics analysis**

134 The paired-end reads from NGS were processed by Trim-galore [16] to trim adapters

135 and low-quality bases, and the reads of the host genome (*Anopheles* or *Culex*) were

136 discarded using bowtie2 [17] and bedtools [18]. The remaining reads were assembled

137 into contigs by Trinityrnaseq [19]. The contigs longer than 500 bp were filtered and

138 dereplicated (nucleotide identity > 95% and coverage rate > 80%) by CD-HIT [20]

139 and aligned against the nonredundant protein database from NCBI (updated in March

140 2021) for taxonomic classification using Diamond blastX [21]. The BLASTX result

141 was processed by the LCA algorithm (weighted LCA percent = 75%, e-value = 1 ×

142 10^{-5}, min Support = 1) with MEGAN [22]. Taxonomic classification was mainly

143 conducted at the family level. The trimmed reads were aligned to the contigs of each

144 sample by checkm [23] to calculate the abundance of eukaryotic viral contigs. Data

145 visualization was conducted by ComplexHeatmap [24] and the ggplot2 [25] package

146 in R [26]. A viral species that contained more than 500 reads was regarded to be

147 present in a pool.

148

149 **Result**

150 **Mosquito composition**

151 The temperature and precipitation from April to October in Wuhan during 2020 (Fig.

152 1A) were recorded, and it was found that the rainfall increased in June and July,

153 above the 10-year average data, but the average temperature was lower than that in

154 previous years [27].

155 From April to October, a total of 2,345 adult mosquitoes were collected, with 1,703

156 from urban areas and 642 from the rural pig farm. The seasonal fluctuation of

157 mosquito density in urban areas revealed a bimodal pattern, with the first peak

158 occurring in May or June and the second significantly lower peak occurring in

159 September or October (Fig. 1B and E), which was similar to previous data [28].

160 Four genera of mosquitoes were classified: *Culex*, *Aedes*, *Anopheles* and *Armigeres*

161 (Fig. 1C-D). In these four genera, the predominant species in both urban and pig

162 farms was *Culex*, which was consistent with existing reports [28]. In urban areas,

163 *Culex* mosquitoes occupied a large proportion (90.32%, 1538/1703), followed by

164 *Aedes* (9.21%, 157/1703). Both *Anopheles* (0.18%, 3/1703) and *Armigeres* (0.18%,

165 3/1703) were caught in small amounts. However, on pig farms, the main mosquito

166 species were *Culex* (54.98%, 353/642) and *Anopheles* (43.77%, 281/1703). *Armigeres*

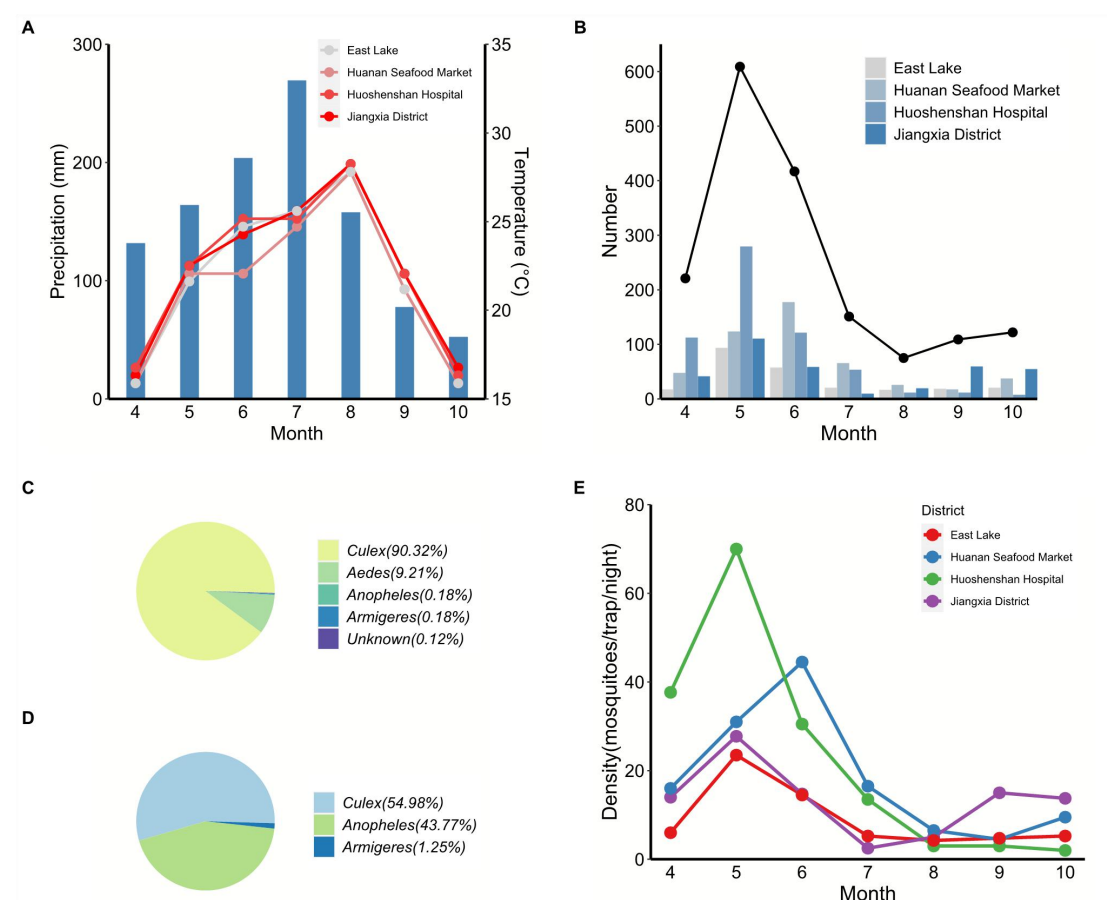167 (1.25%, 8/1703) was much less collected, while no *Aedes* was caught.

168

169

170 **Fig. 1 The species and amounts of mosquitoes collected in urban districts and Huangpi**

171 **pig farm in Wuhan from April to October during 2020.** (A) The histograms and lines show

172 the precipitation and average temperature of each month, respectively. (B) The mosquitoes

173 collected in urban districts per month. The line shows the total mosquitoes per month. (C) The

174 proportion of mosquito genera in urban districts. (D) The proportion of mosquito genera in

175 the Huangpi pig farm. (E) The abundance of mosquitoes at different collection times.

176

177 **Virus detection by RT-qPCR**

178 The 2,345 collected mosquitoes were separated into 123 pools, with 90 from urban

179 areas and 33 from the pig farm. From 90 urban pools, BAV was detected in 18, with

180 Ct values ranging from 32 to 38, and the sequencing results confirmed that 14 out of

181 18 samples were BAV positive. The BAV-positive samples were mainly distributed in

182 residential areas from May to July. For the pig farm, only one sample collected in

183 May was BAV positive (Ct = 32.71) and confirmed by sequencing. However, none of

184 the mosquito pools were JEV positive.

185

186 **Virome profile of mosquitoes collected in Wuhan**

187 The seven *Culex* monthly pools (whct-C_04 to whct-C_10) from urban areas and one

188 *Anopheles* (hpzc-A_05) and one *Culex* pool (hpzc-C_05) from the Huangpi pig farm

189 were processed for metagenomic sequencing. All nine pools contained 53,983,154

190 host-genome-removed reads in total (Table 1). A total of 2,663,238 de novo

191 assembled contigs were added. The contigs were clustered and dereplicated (longer

192 than 500 bp, nucleotide identity > 95% and coverage > 80%) into 413,645

193 nonredundant contigs. BLASTX results showed that 1,189 and 412,456 contigs were

194 aligned to eukaryotic viruses and other organisms, respectively.

195

196 **Table 1. Dominant mosquitoes in Wuhan based on metagenomic sequencing.**

Sampling site	Sample name	Mosquito genus	Month of collection	Number of mosquitoes for sequencing	Host-genome-removed reads (Viral proportion)	Number of nonredundant contigs aligned to eukaryotic viruses

	whct-C_04	*Culex*	April	220	4,765,707 (5.90%)	108
	whct-C_05	*Culex*	May	600	5,115,257 (19.26%)	176
	whct-C_06	*Culex*	June	339	8,763,700 (11.46%)	211
Urban district	whct-C_07	*Culex*	July	109	4,180,592 (12.96%)	84
	whct-C_08	*Culex*	August	31	11,091,085 (3.50%)	45
	whct-C_09	*Culex*	September	97	3,614,845 (10.60%)	63
	whct-C_10	*Culex*	October	111	2,988,426 (6.02%)	84
Huangpi pig farm	hpzc-A_05	*Anopheles*	-	281	1,677,474 (20.14%)	137
	hpzc-C_05	*Culex*		353	11,786,068 (3.37%)	281

197

198   The viromes of the collected mosquitoes were classified into 20 viral families and a

199   group of unclassified viruses (Fig. 2A). Invertebrate viruses were dominant in the

200   viromes, and the hosts of 11 viruses remain unknown (Fig. 2B). Only Hubei chryso-

201   like virus 1 (HCLV1) has a vertebrate host.

202   The abundance of each viral species in which at least one pool contained more than

203   500 reads is shown in the heatmap (Fig. 3). The viromes contained 52 viral species in

204   total. Thirty of them could be classified into 20 viral families, and the other 22 viruses

205   lacked accurate taxonomic classification. Unclassified viruses composed a large

206   proportion of the viromes in all pools.

207   Eighteen viral families were present in viromes of *Culex* mosquito pools from urban

208   districts. Viral diversity in urban districts was altered by month and showed two peaks.

209   The highest viral diversity occurred in the pool whct-C_06 (Fig. 3A). Several viruses,

210   such as Culex flavivirus and Zhejiang mosquito virus 3, had significant changes in

211   relative abundance in these months. The family *Orthomyxoviridae* had the most reads

212   in the pools from urban districts and contained four members (Wuhan mosquito virus

213   3, Wuhan mosquito virus 4, Wuhan mosquito virus 5 and Wuhan mosquito virus 6)

214   that were recently identified in Hubei Province (Fig. 3). Culex flavivirus and Quang

215   Binh virus, both MSVs, were present in the pools. The *Culex* pool of the Huangpi pig

216   farm, which contained 13 viral families and 4 unique viruses (Atrato Sobemo-like

217   virus 4, Yongsan sobemo-like virus 1, Primus virus and Ochlerotatus-associated

218   narna-like virus), had viromes similar to those of urban districts. In all *Culex* pools,

219   Wuhan Mosquito virus 6, Hubei virga-like virus 2 and Zhejiang mosquito virus 3

220   could be found. The differences in viral composition between the urban and rural

221   areas was mainly found for viral families such as *Parvoviridae* and *Circoviridae.*

222   The viromes in *Anopheles* mosquitoes were significantly lower than those in *Culex*

223   mosquitoes. The *Anopheles* pool had six unique viruses, and the most abundant virus

224   was Xincheng anphevirus. Nine viral species could be found in the two mosquito

225   species from the Huangpi pig farm, four of which (Wuhan mosquito virus 5, Atrato

226   virus, Hubei Beny-like virus 1 and *Aedes alboannulatus* orthomyxo-like virus) were

227   absent in *Culex* mosquitoes in urban districts.
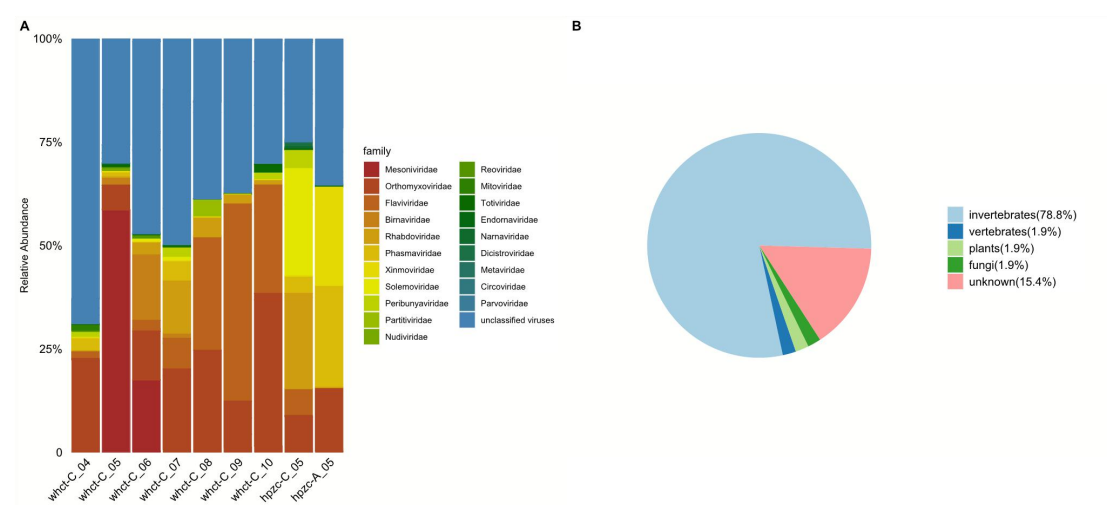
228

229

230   **Fig. 2 Families and hosts of viruses in the viromes of mosquitoes in different pools.** (A) The
231   map shows the relative abundance of viral families in each pool. (B) The composition of the hosts
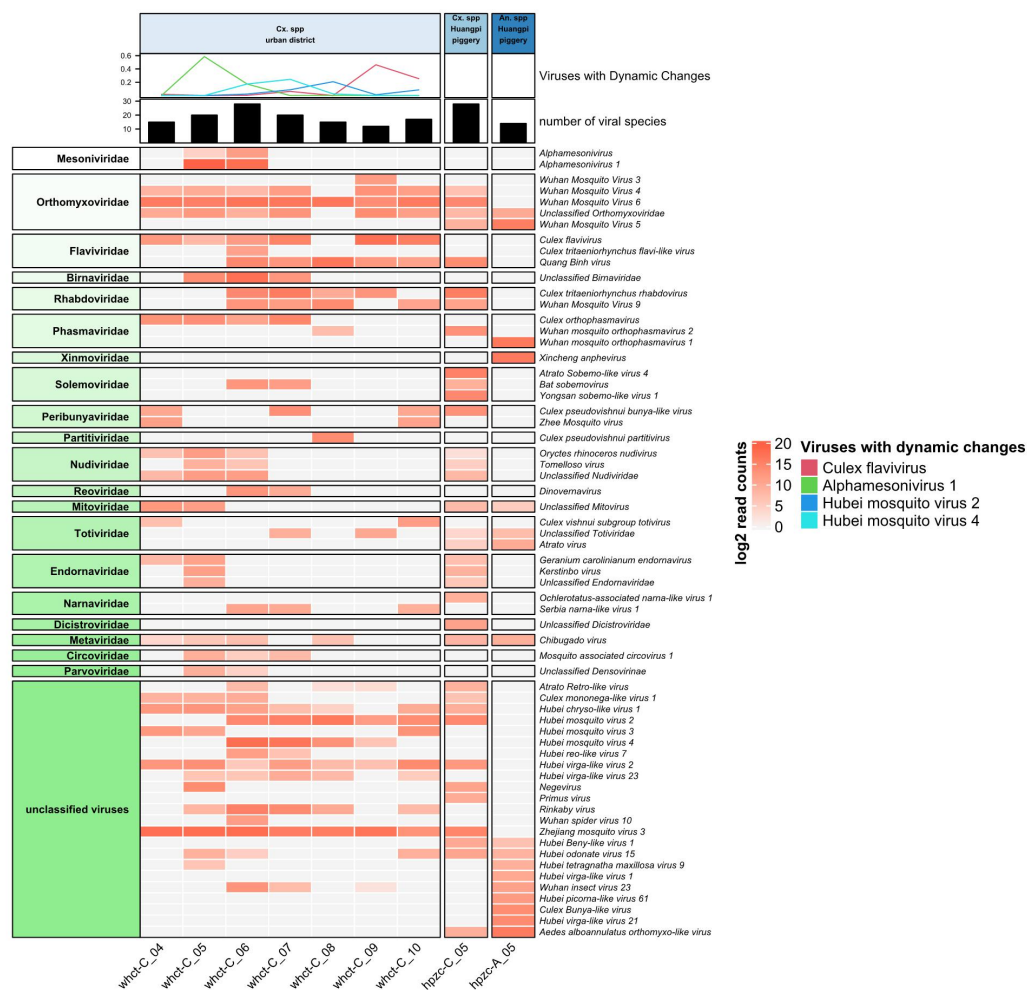232   of all 52 viral species.

233

Fig. 3 The abundance of viruses in each pool. Viral species contained more than 500 reads in at least one pool. The bar plot shows the number of viral species in each sample. The line plot at the top shows four viruses that had dynamic changes at different collection times.

Discussion

MBVs are pervasive public health problems on a global scale, and effective surveillance programs for both vectors and pathogens are needed. In this study, we completed 7 months of mosquito monitoring in Wuhan during 2020 to determine the dynamic changes in mosquito populations and their viromes.

Between-site habitat differences, climates and longer seasonal fluctuations can influence the number of mosquitoes [29]. Although efforts were made to sample throughout the whole year, the heavy rainfall from June to September did reduce the sampling quantity of mosquitos in Wuhan. The highest abundances of the mosquito catch in urban areas were in May, but that number plummeted in August (Fig. 1).

249   Apart from the highest precipitation in July and the highest temperature in August, the

250   low abundances of mosquito catches may also be due to the disinfection strategies

251   carried out by the government in July for students returning to school and public

252   entertainment reopening.

253   Compared to the trapped mosquitoes from 2017 to 2019 in Wuhan, which can be

254   classified into 8 species under 5 genera and 1 family [28], the diversity of mosquito

255   species was lower in this study (Fig. 1). This result could be attributed to the chemical

256   blend of the attractant not resulting in trap catches as high as those elicited by human

257   odor [30]. Furthermore, the morphological quality of mosquitoes deteriorated in large

258   and soft nets used in this study, resulting in mosquitoes that could only be classified to

259   the genus level here. Further investigations into diversity were prevented, which could

260   be the other reason for the lower diversity of mosquito genera.

261   BAV belongs to the family of *Reoviridae*, RT-qPCR results showed that BAV existed

262   in mosquitoes from both urban areas and rural pig farms in Wuhan. Although BAV

263   contigs was not detected in the mosquito pools for NGS sequencing, there were many

264   unclassified reads in *Reoviridae* in whct-C_06 and whct-C_07, which corresponded

265   with qPCR detection for BAV, probably due to the different pooling strategies in the

266   two methods.

267   Viral metagenomics has been applied in the analysis of viromes from mosquitoes in

268   many studies [8,31,32]. By NGS, 3.37% - 20.14% reads from each pool collected

269   from urban districts and a pig farm in Wuhan were aligned to viruses (Table 1).

270   Additionally, *Orthomyxoviridae* and *Flaviviridae* were prevalent in *Culex* viromes, in

271   which invertebrate viruses were dominant. Previous studies performed in Hubei

272   Province showed that the prevalent viral families in *Culex* mosquitoes are

273   *Herpesviridae* and *Adenoviridae*, whose hosts are vertebrates [8].

274   The dynamic changes in the viromes of *Culex* from urban districts in Wuhan were

275   monitored (Fig. 3). The viral diversity in the monthly pools followed a bimodal

276   distribution, and the two peaks occurred in June and October. A previous study

277   performed in the USA and Trinidad showed that several insect-specific viruses have

278   evident seasonal activity [33]. The viral diversity in different months in Wuhan may

279   also have seasonal changes. In our present study, a large proportion of viral families

280   were absent in several pools, probably due to their low abundance in all pools.

281   *Orthomyxoviridae* and *Flaviviridae* were the dominant viral families and stably

282   existed in the monthly pools (Fig. 3). The relative abundance of *Orthomyxoviridae*

283   had no obvious trend during all months but increased from May to August. Notably,

284   four virus species (Culex flavivirus, Alphamesonivirus 1, Hubei mosquito virus 2 and

285   Hubei mosquito virus 4) had significant changes in these months. Culex flavivirus

286   showed a low level of abundance in April to July and had higher abundance in

287   September to October. Alphamesonivirus 1, Hubei mosquito virus 2 and Hubei

288   mosquito virus 4 show unimodal curves, and their peaks occur in May, August and

289   June, respectively. Several of these viruses were highly abundant in a few months but

290   disappeared in the other months, suggesting that they may have a strong correlation

291   with the alternation of seasons and possibly related to the activity of the *Culex*

292   population. Unfortunately, environmental factors were not sufficiently recorded,

293   which limited further exploration of the correlation between mosquito viromes and

294   many other factors, such as climate and host composition.

295   In the heatmap (Fig. 3), Wuhan mosquito virus 3, Wuhan mosquito virus 4, Wuhan

296   mosquito virus 5 and Wuhan mosquito virus 6 belong to the genus *Quaranjavirus*,

297   family *Orthomyxoviridae* [34]. Several members of this genus, such as Quaranfil

298   virus and Johnston Atoll virus, can infect both vertebrate and invertebrate hosts and

299   result in the death of newborn mice in the laboratory [35]. The other members of

300   *Quaranjavirus* have not been properly characterized, and it should be confirmed

301   whether they are potential pathogenic to humans and livestock. Quang Binh virus,

302   Culex tritaeniorhynchus flavi-like virus (CtFLV) and Culex flavivirus, which belong

303   to the family *Flaviviridae*, have variable distributions in many regions [36][37].

304   Quang Binh virus was first isolated in Vietnam and then chronologically reported in

305   Yunnan Province and Hubei Province in China [8][38]. Recently, it was reported to

306   be detected in *Culex* from northwestern China, suggesting that the virus may have an

307   expanded geographical distribution [31]. Several arboviruses, such as CtFLV,

308   identified from the local *Culex* in Japan, were closely related to those identified in

309 Hubei Province [37]. In consideration of the frequent exchanges between China and

310 Japan, mosquitoes can be transferred through human activities. It is worth noting

311 whether viromes of mosquitoes interact between the two locations.

312 Viromes of mosquitoes in Wuhan reflected geographic and species differences. *Culex*

313 species from urban districts and the pig farm had similar viral family compositions.

314 Rhabdoviriuses were most prevalent in the pig farm, and several unclassified viruses

315 only existed in one of the two locations, which was probably due to diverse mosquito

316 hosts and environments in urban and rural areas [39]. Wuhan mosquito

317 orthophasmavirus 1 (Family *Phasmaviridae*) and Xincheng anphevirus (Family

318 *Xinmoviridae*) dominated in *Anopheles*; both are insect-specific viruses. *Anopheles*

319 and *Culex* had significantly distinct viromes, which is similar to previous studies in

320 Guadeloupe and Japan [32][37]. Wuhan mosquito virus 6, Hubei virga-like virus 2

321 and Zhejiang mosquito virus 3 were present in all the *Culex* pools (Fig. 3). Several

322 studies have reported these viruses in mosquitoes from multiple geographical regions,

323 suggesting that they are potential members of the "core viromes" of mosquitoes

324 [32][40]. Widely distributed MSVs may play a vital role in the biocontrol of

325 mosquitoes. In the unclassified virus group, HCLV1 has a vertebrate host, but the

326 evidence is insufficient because HCLV1 was isolated from the feces of *Cairina*

327 *moschata* (also known as Muscovy duck) in Australia [41]. The transmission of this

328 virus is still unknown, and many more studies are needed to explore whether birds act

329 as hosts since Muscovy ducks are widely bred in Hubei.

Acknowledgements

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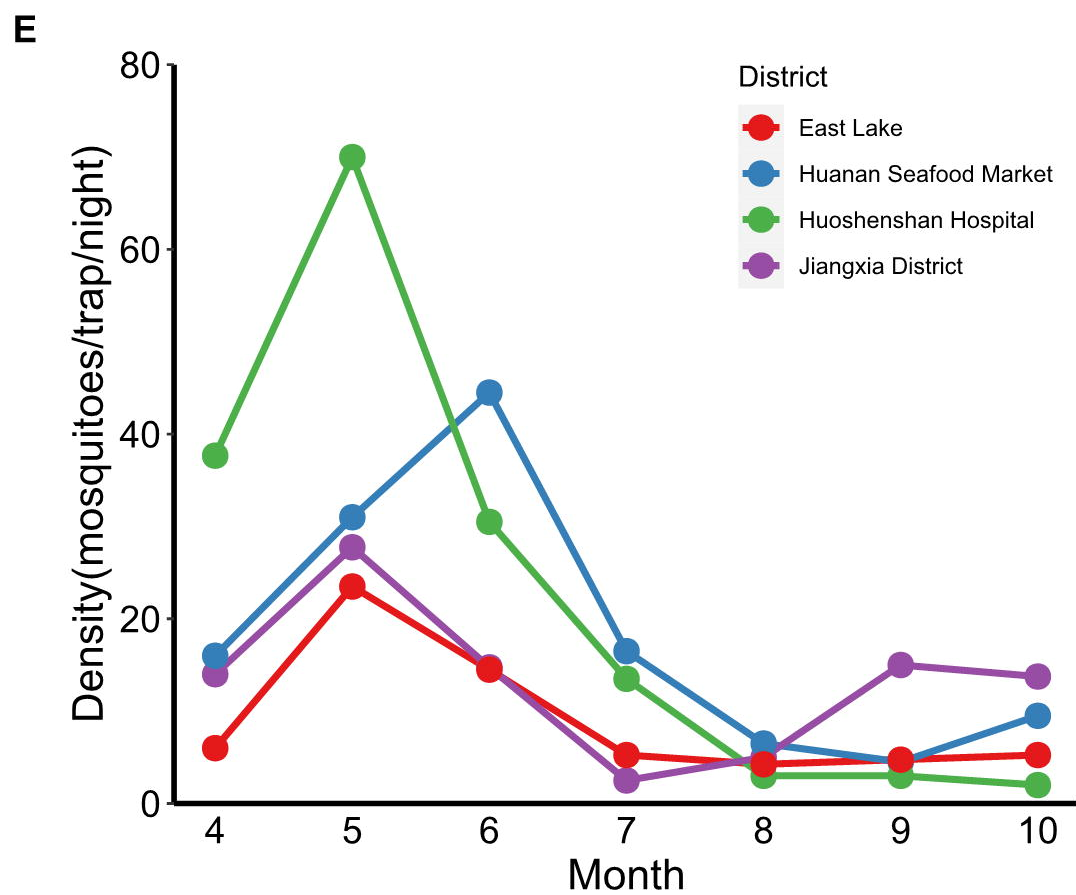415   Trinidad. Virology. 2009; 386:154–159.
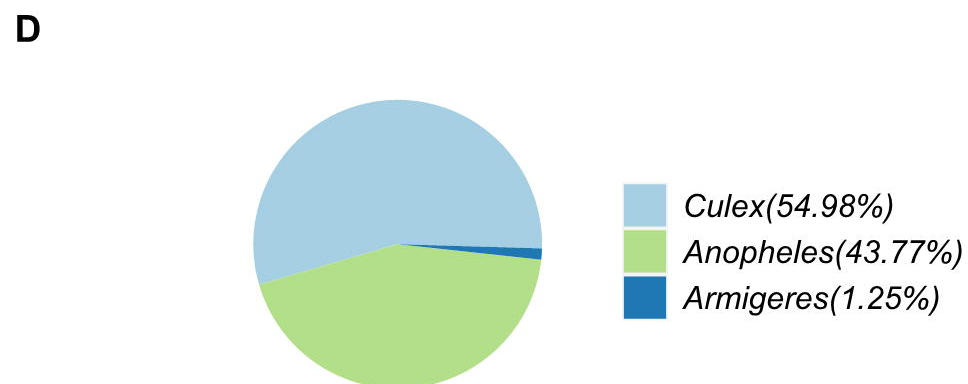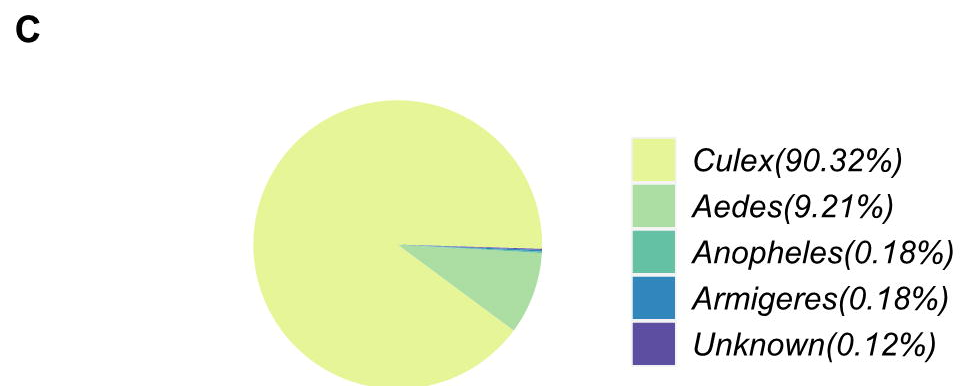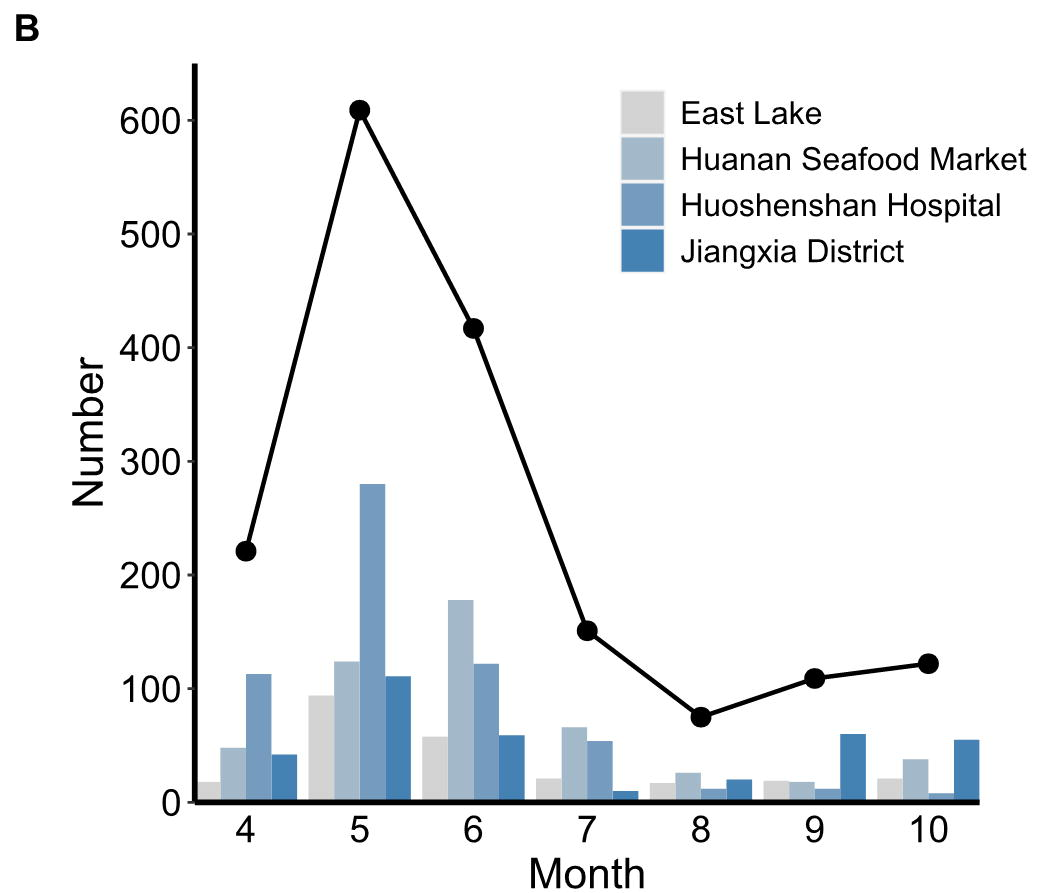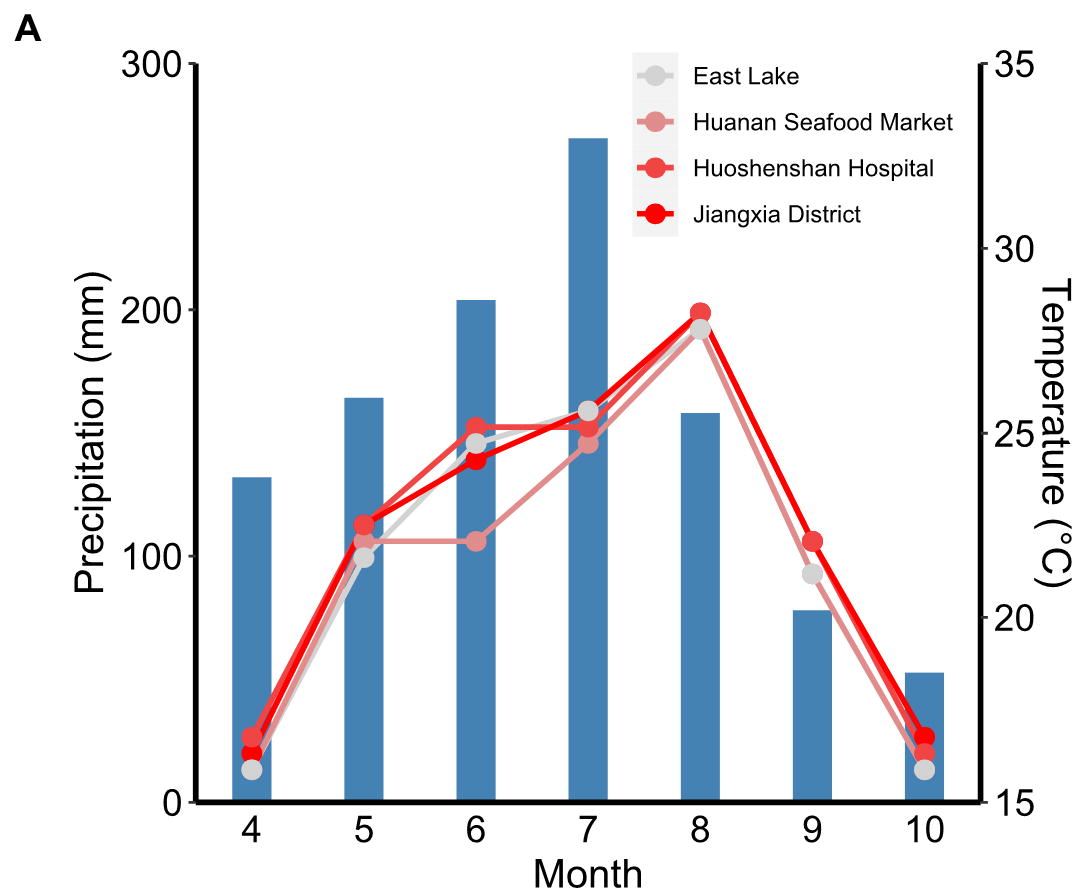
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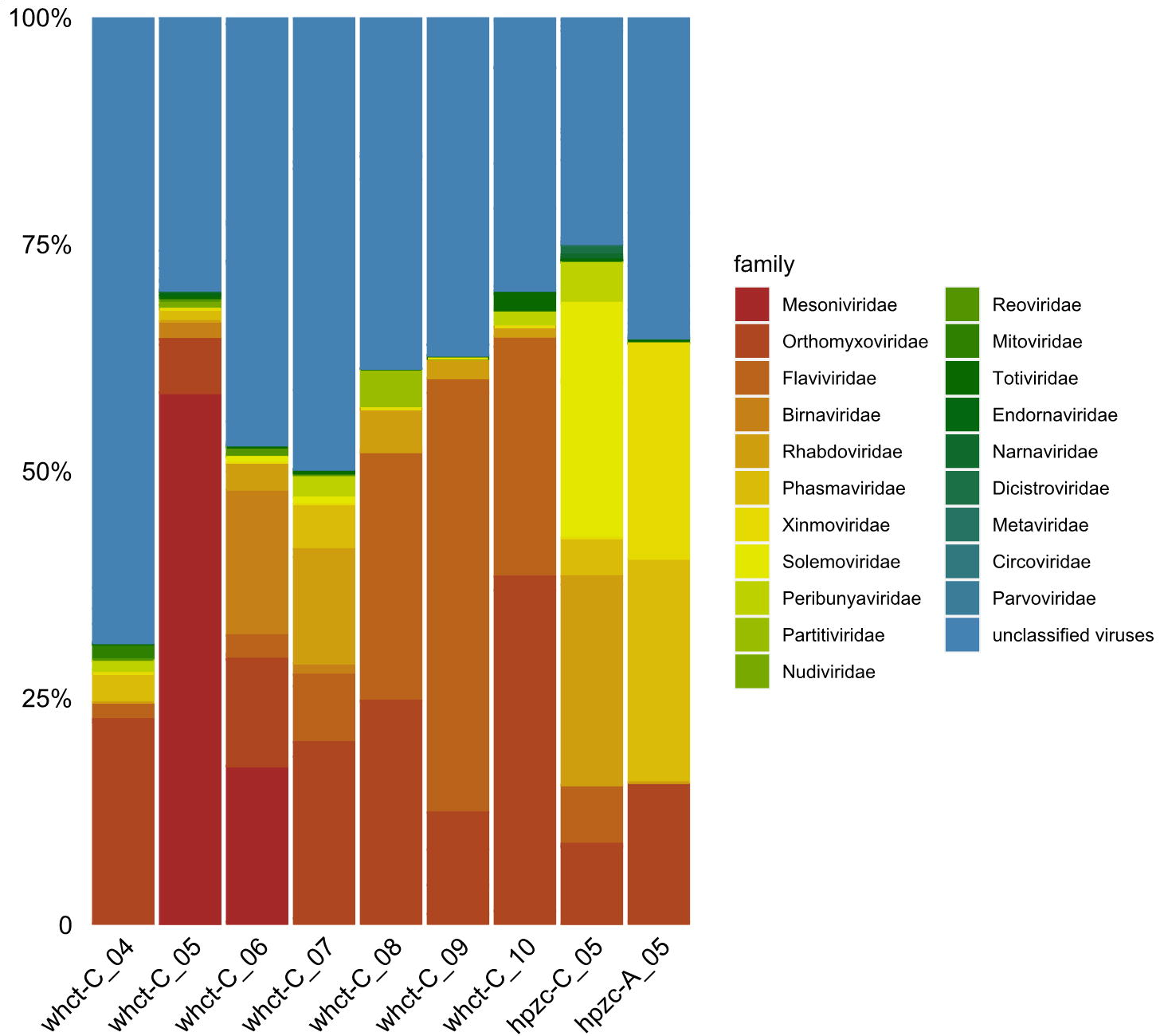418   2015; 2015:.

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