¹ First report of *kdr* L1014F and *ace-1* G119S insecticide resistance in

2 Belgian *Culex* (Diptera: Culicidae) mosquitoes

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4	Short running title: Insecticide res	istance in Belgian <i>Culex</i> mosquitoes
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18 Abstract

19	The emergence of West Nile virus and Usutu virus in Europe poses a significant risk to public health.
20	In the absence of efficient antiviral therapy or vaccine candidates, the only strategy to control these
21	arboviruses is to target the Culex (Diptera: Culicidae) mosquito vector. However, the selection
22	pressure caused by exposure to insecticides for vector control or agricultural pest control can lead to
23	insecticide resistance, thereby reducing the efficacy of insecticide-based vector control interventions.
24	In Culex mosquitoes, two of the most common amino acid substitutions associated with insecticide
25	resistance are the <i>kdr</i> L1014F in voltage gated sodium channels and G119S in acetylcholinesterase.
26	In this study, Culex pipiens biotype pipiens, Culex torrentium, and Culex modestus were sampled from
27	2019 to 2021 in three distinct environmental habitats (urban, peri-urban, and agricultural) in and
28	around the city of Leuven, Belgium. Individual mosquitoes were screened for two mutations resulting
29	in L1014F and G119S amino acid substitutions. Both mutations were observed in Culex pipiens and
30	Culex modestus but not in Culex torrentium mosquitoes across the four collection sites. Furthermore,
31	multi-resistance or cross-resistance in <i>Culex pipiens</i> could be a threat in these areas, as both mutations
32	were observed at low frequencies.
33	These results provide the first report of kdr L1014F and ace-1 G119S resistance in Culex pipiens and

34 *Culex modestus* mosquitoes from Belgium, highlighting the importance of mosquito surveillance to
 35 design effective arbovirus outbreak control strategies.

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37 Key words: Insecticide resistance, kdr L1014F, ace-1 G119S, multi-resistance, mosquito surveillance

38 1. INTRODUCTION

39 *Culex* mosquitoes (family: *Culicidae*), such as *Culex pipiens* and *Culex modestus*, are efficient vectors 40 of human and animal pathogens. In Europe, *Culex* mosquitoes are the primary vectors of West Nile 41 virus (WNV) and Usutu virus (USUV), two arthropod-borne viruses (arboviruses) of the *Flaviviridae* 42 family (Fros et al. 2015). Both WNV and USUV are maintained in an enzootic cycle, amplifying in birds 43 and transmitted by mosquitoes to incidental hosts such as humans and horses.

Sporadic outbreaks of WNV in humans have arisen in southern and southwestern Europe since 1963. 44 45 The most significant outbreak occurred in 2018 with 1,548 locally-acquired cases and 166 deaths 46 reported among several southern European countries ("West Nile virus infection. In: ECDC. Annual 47 epidemiological report for 2018." 2019). There is increasing evidence that the circulation of WNV is 48 spreading towards northern Europe, and this was exemplified in 2018 in Germany and in 2020 in the 49 Netherlands where the first autochthonous cases of WNV in humans and animals were reported 50 (Bakonyi and Haussig 2020). USUV, on the other hand, has established widespread endemicity in 51 Europe. The virus first emerged in 1996 in Italy and has since spread gradually across the continent 52 causing major outbreaks and mortality in birds (Vilibic-Cavlek et al. 2020). Few human cases of USUV have been reported, yet there is zoonotic potential in areas where competent vector species are 53 54 present. Furthermore, the co-circulation of WNV and USUV is poorly understood and may threaten 55 public health in the future (Nikolay 2015). A small proportion of WNV and USUV infections in humans 56 results in severe neurological complications and even death, yet there are no efficient antiviral drugs 57 or vaccine candidates available for preventing or treating these virus infections. The emergence of 58 WNV and USUV in Europe thus poses a significant increased risk to public health. However, in the 59 event of an outbreak, the only currently available tools to reduce infections with these viruses are 60 mosquito control interventions.

Adulticiding by fogging is the first-line choice for vector control against *Culex* mosquitoes during
 emergency situations. This method can rapidly reduce the infectious female mosquito population in

63 treated areas; however, adult control is not recommended for WNV outbreak prevention due to the 64 unpredictable nature of outbreaks (Bellini et al. 2014). Other interventions targeting *Culex* mosquitoes 65 include larval source reduction (such as environmental management and removal of breeding sites), 66 larviciding using chemical or biological agents, and personal protection methods such as repellents 67 and mosquito-proofing homes (Bellini et al. 2014). Pyrethroids, organophosphates, carbamates, and 68 organochlorides are the main chemical classes of insecticides available for use in public health. 69 However, because of long-term safety issues, pyrethroids are the only class allowed for mosquito 70 adulticide in Europe.

71 A potential consequence of using insecticides for vector control or agricultural pest control is the 72 development of insecticide resistance mechanisms in mosquitoes. The main mechanisms responsible 73 for insecticide resistance in insects include target gene mutations, resulting in amino acid alterations 74 leading to permanent changes in the insecticide target site, increased metabolic activity of enzymes 75 involved in insecticide detoxification, and reduced cuticular penetration of insecticides (Hemingway 76 et al. 2004). Voltage-gated sodium channels allow the passage of sodium ions across the plasma 77 membrane of axons to initiate and propagate action potentials (electrical impulses) which are 78 necessary for controlling insect movement and reaction to stimuli (Dong et al. 2014). When 79 mosquitoes come into contact with pyrethroids and DDT, these molecules bind to sodium channels 80 and prolong their opening, disrupting nerve function and inducing paralysis, knockdown and 81 eventually death. Knockdown resistance (kdr) is caused by certain amino acid substitutions in sodium 82 channels, which reduce the sensitivity of these channels to pyrethroids and DDT, thus preventing 83 paralysis and knockdown (Soderlund 2008). The substitution L1014F is a single point mutation at 84 position 1014 in the domain II S6 of sodium channels is most common kdr subsititution in Culex 85 mosquitoes (Davies et al. 2007). An alternate resistance mechanism is the serine residue of 86 acetylcholinesterase (AChE), the target site for organophosphates and carbamates. In the insect 87 central nervous system, AChE catalyses the hydrolysis of the neurotransmitter acetylcholine to 88 terminate nerve impulses (Hemingway et al. 2004). AChE insensitivity is caused by mutations in the ace-1 gene, resulting in substitutions such as G119S, which reduce the sensitivity of AChE to
 insecticides in *Culex* and *Anopheles* mosquitoes (Weill et al. 2004).

91 In Africa, the widespread distribution of long-lasting insecticidal bed nets for malaria control has 92 contributed to the rapid rise and spread of highly insecticide-resistant Anopheles mosquitoes (Glunt 93 et al. 2015). As a result, the efficacy of key malaria control interventions was significantly reduced, creating the need for novel combinations of insecticides with differing modes of action to regain the 94 95 effectiveness of long-lasting insecticidal nets. The widespread impact of insecticide resistance in 96 Anopheles mosquitoes highlights the importance of resistance monitoring in all mosquito vectors. The 97 need for increased monitoring and surveillance of European mosquitoes was demonstrated recently 98 in 2016 when pyrethroid resistance was detected in Aedes albopictus from Italy, threatening the 99 effectiveness of outbreak response interventions (Pichler et al. 2018). Furthermore, an additional 100 cause for concern is the potential impact of resistance mutations on the efficiency of pathogen 101 transmission (i.e. vector competence). In Culex quinquefasciatus mosquitoes it was shown that the 102 presence of one homozygous resistance mutation significantly increased WNV dissemination in the 103 mosquito body, resulting in a higher transmission efficiency than susceptible controls (Atyame et al. 104 2019). This phenomenon is poorly understood, and the impact of insecticide adaptations on the vector 105 competence for arboviruses has so far only been investigated for three arboviruses including WNV, 106 Zika virus and Dengue virus in distinct mosquito species (Atyame et al. 2019, Deng et al. 2021, Parker-107 Crockett et al. 2021).

In Belgium, native mosquito species include members of the *Culex pipiens* species complex (*Culex pipiens* biotype *pipiens* and *Culex pipiens* biotype *molestus*), *Culex torrentium*, and *Culex modestus* (Boukraa et al. 2015, Wang et al. 2021a). To date, there is no information regarding the presence of insecticide resistance in mosquito species from Belgium or neighbouring countries. In this study, we investigated the presence of two common insecticide resistance mechanisms, *kdr* L1014F and *ace-1* G119S, in *Culex pipiens* biotype *pipiens*, *Culex torrentium*, and *Culex modestus* mosquitoes from

Belgium. Mosquitoes were sampled in three distinct environmental habitats (urban, peri-urban, and agricultural) in and around the city of Leuven from 2019 to 2021. The insecticide resistance mutations reported here will provide insight for evidence-based vector control for the prevention and mitigation of arbovirus outbreaks in Belgium.

118 2. MATERIALS AND METHODS

119 **2.1 Ethics statement**

Permits for field collections in private habitats (Bertem and Mechelen) were obtained from the landowners. Permits for collection in the Botanic Garden were obtained from the City Green Management of Leuven. Permits for collection in Arenberg Park were obtained from the security responsible of KU Leuven.

124 2.2 Mosquito collections

125 Collections were performed from August to the beginning of October in 2019, 2020 and 2021, when 126 the weather was adequate, without strong wind or heavy rain. Adult mosquitoes were collected using 127 BG-Sentinel traps (Biogents[®] AG, Regensburg, Germany) baited with dry ice for CO₂ release and BG-128 lure (Biogents[®] AG, Regensburg, Germany) to simulate attractive host scent. The traps were emptied 129 and repositioned between sunrise and sunset of the next day. All captured insects were transported 130 to the insectary facility at the Rega Institute, KU Leuven. Mosquitoes were anesthetized and sorted on dry ice and then separated to genus level based on morphological characteristics. Mosquitoes were 131 132 stored individually at -80°C until further processing. Ten traps were rotated between three collection 133 sites in Leuven and one site in Mechelen (approximately 30 km from Leuven): Leuven urban habitat 134 (Botanic Garden of Leuven, N 50°52'41, E 4°41'21), Leuven peri-urban habitat (Arenberg Park of KU 135 Leuven, N 50°51'46, E 4°41'01), Leuven agricultural habitat (Bertem, N 50°51'57, E 4°37'53), and 136 Mechelen peri-urban habitat (N 51°02'34, E 4°29'08).

137 **2.3 DNA extraction & mosquito identification**

DNA extraction: Following morphological identification, mosquitoes were transferred to tubes with
2.8 mm ceramic beads (Precellys[®], Bertin, USA) and 500 µl phosphate-buffered saline (1X PBS) solution
and homogenised with a Precellys Evolution homogeniser at 2 cycles of 6800 rpm for 10 sec with a
pause of 20 sec. The homogenate was lysed at 100°C for 10 min, followed by centrifugation at 10,000
rpm for 1 min to spin down the tissue debris, and 150 µl of supernatant was transferred to a new tube.
DNA extraction was performed using the QIAamp DNA kit (Qiagen[®], Hilden, Germany) according to
the manufacturer's protocol.

145 Mosquito identification: Culex pipiens biotype pipiens and Culex pipiens biotype molestus were 146 distinguished with a multiplex gRT-PCR using primers and probes described previously (Rudolf et al. 147 2013). The biotyping qRT-PCR was confirmed on a subset of samples using single sequencing of the 148 mosquito cytochrome oxidase 1 (COX1) gene. A 25 µl reaction volume was prepared for each reaction using the Low ROX One-Step qRT-PCR 2X MasterMix kit (Eurogentec[®], Seraing, Belgium) following the 149 150 manufacturer's instructions. The cycle conditions were as follows: initial denaturation at 95°C for 10 151 min, 40 cycles of denaturation at 94°C for 40 sec, elongation at 48°C for 1 min, and extension at 72°C 152 for 1 min, and a final hold stage at 72°C for 2 min. *Culex modestus* and *Culex torrentium* mosquitoes 153 were identified morphologically using the key of Becker (Becker et al. 2010) followed by COX1 Sanger 154 sequencing (Wang et al. 2021a).

155 2.4 PCR & gel electrophoresis

Detection of kdr L1014F (mutation from TTA to TTT): The protocol for kdr L1014F detection was adapted from a protocol described elsewhere (Martinez-Torres et al. 1999). Briefly, two PCR reactions were run in parallel using 4 primers: Cgd1 (5'-GTGGAACTTCACCGACTTC-3'), Cgd2 (5' GCAAGGCTAAGAAAAGGTTAAG-3'), Cgd3 (5'-CCACCGTAGTGATAGGAAATTTA-3') and Cgd4 (5'-CCACCGTAGTGATAGGAAATTTT-3'). A 20 μl reaction volume was prepared for each reaction using the GoTaq[®] Green Master 2X Mix (M7122, Promega, Belgium) following the manufacturer's instructions. The first reaction contained the primers Cgd1, Cgd2 and Cgd3 to identify the presence of the L1014

163 susceptible allele, and the second reaction consisted of Cgd1, Cgd2 and Cgd4 to identify the presence 164 of the F1014 resistant allele. Taken together, the results of both reactions were used to determine the 165 genotype for each individual as susceptible homozygote (L/L: SS), resistant heterozygote (L/F: RS), or resistant homozygote (F/F: RR). The cycle conditions were as follows: initial denaturation at 94°C for 166 167 2 min, 40 cycles of denaturation at 94°C for 30 sec, elongation at 48°C for 30 sec, extension at 72°C 168 for 1 min, and a final hold stage at 72°C for 10 min. Amplicons were separated by electrophoresis on 169 1.5% agarose gel and were visualised by Midori green staining under UV light with FAS-V Imaging 170 System.

Detection of ace-1 G119S (mutation from GGC to AGC) : This analysis was performed following a 171 172 protocol described previously (Weill et al. 2004) with minor modifications. The DNA block was 173 amplified using the primers CxEx3dir (5'-CGACTCGGACCCACTGGT-3') and CxEx3rev (5'-174 GTTCTGATCAAACAGCCCCGC-3') and a 20 µl reaction volume was prepared for each reaction using the 175 GoTaq[®] Green Master 2X Mix (M7122, Promega, Belgium) following the manufacturer's instructions. 176 The cycle conditions were as follows: initial denaturation at 94°C for 2 min, 40 cycles of denaturation 177 at 94°C for 30 sec, elongation at 54°C for 30 sec, extension at 72°C for 1 min, with a final hold stage at 178 72°C for 10 min. The amplicons were digested with Alul restriction enzyme (Jena Science, Sapphire, 179 USA) following the manufacturer's instructions and separated on a 2% agarose gel. The products were 180 visualised by Midori green staining under UV light with FAS-V Imaging System. The results were used 181 to determine the genotype for each individual as susceptible homozygote (G/G: SS), resistant heterozygote (G/S: RS), or resistant homozygote (S/S: RR). 182

183 **2.5 Statistical analysis**

For the distribution of genotypic and allelic frequencies resulting in the *kdr* L1014F or *ace-1* G119S, analysis was performed among different collecting sites, years, and species by GraphPad Prism 9 (V.9.3). A Hardy–Weinberg equilibrium of the resistant (R) and susceptible (S) allelic frequencies was evaluated using the equations below on the data from *Culex pipiens* collected from 2019 to 2021. The

- 188 X^2 and *p*-values were calculated with GraphPad Prism (V.9.3). A p-value of <0.05 was considered
- 189 statistically significant.
- 190 R allelic frequency= $\frac{RS+2RR}{2(RS+RR+SS)}$;
- 191 S allelic frequency = 1- R allelic frequency

192 **3. RESULTS**

193 **3.1 Frequency and stability of** *kdr* **over 3 years**

Culex pipiens were captured from 2019 to 2021 at the urban and peri-urban sites in Leuven. The collection of *Culex pipiens* expanded to the Leuven agricultural site from 2020 to 2021 and to the Mechelen peri-urban site in 2021. *Culex modestus* were collected at the Leuven peri-urban site in 2019, and *Culex torrentium* were captured at the Leuven urban and peri-urban sites in 2020 and the Leuven urban site in 2021. One hybrid female *Culex pipiens* (hybrid of *pipiens* and *molestus*) and one female *Culex pipiens molestus* were identified at the Leuven peri-urban site and Mechelen peri-urban site in 2021, respectively.

The mutation frequencies at position 1014 of the domain II S6 of the sodium channel of *Culex pipiens*, *Culex modestus* and *Culex torrentium* mosquitoes are presented in Table 1. The most common allele combination for *Culex pipiens* and *Culex modestus* was the susceptible L/L, followed by resistant heterozygosity (L/F). Resistant homozygosity (F/F) presented at a low frequency in *Culex pipiens*, whereas no homozygous resistance genotype was detected in *Culex modestus*, and no resistant heterozygosity or homozygous resistance was detected in *Culex torrentium*.

The observed frequency of resistance genotypes in *Culex pipiens* was consistent over time in each collection site. The Hardy-Weinberg equilibrium was used to measure the stability of mutations in the population of *Culex pipiens* from all locations over time (Table 2). Ultimately, the difference between the observed and expected *kdr* L1014F genotype frequencies each year were not significantly different and are therefore expected to remain in equilibrium (constant).

212 **3.2 Frequency and stability of AChE insensitivity over 3 years**

The mutation frequencies in the *ace-1* gene resulting in the G119S amino acid change in *Culex pipiens*, *Culex modestus* and *Culex torrentium* from 2019-2021 are presented in Table 3. In *Culex pipiens*, AChE susceptibility (G/G) was detected at a higher frequency to heterozygous resistance (G/S) at the urban 216 site (2019-2021) and peri-urban site (2020) in Leuven and the peri-urban site in Mechelen (2021). An 217 equal proportion of susceptible (G/G) and heterozygous resistant (G/S) mosquitoes were observed at 218 the Leuven peri-urban site in 2019 and again in 2021. The rate of heterozygous resistance (G/S) 219 surpassed the rate of full susceptibility (G/G) in *Culex pipiens* at the agricultural site from 2020 to 2021. 220 AChE homozygous resistance (S/S) was only observed in a single mosquito captured at the Mechelen 221 peri-urban site (2021). Almost all captured *Culex modestus* had the full susceptibility (G/G) genotype, 222 yet a small minority presented with heterozygous resistant (G/S) alleles (Leuven peri-urban site, 2019). 223 All captured Culex torrentium at the urban (2020-2021) and peri-urban (2020) sites in Leuven were 224 observed with susceptible (G/G) AChE alleles.

The observed rate of heterozygosity (G/S) in *Culex pipiens* was generally consistent over time across all locations, except at the Leuven peri-urban site where the rate of heterozygosity fell from 50% to 27% from 2019 to 2020 but returned to 50% in 2021. The Hardy-Weinberg equilibrium of genotypes in *Culex pipiens* across all collection sites remained constant from 2019 to 2020 (Table 4). However, in 2021 the rates of G/G and S/S genotypes were lower and the rate of G/S heterozygosity was higher than the expected frequencies. Therefore, we may observe a higher rate of *ace-1* G119S heterozygous resistance in *Culex pipiens* in this region in the near future.

232 **3.3 Distribution of L1014F and G119S in** *Culex pipiens* collected from different habitat types

233 In this study, three habitat types including urban, peri-urban, and agricultural were chosen for 234 mosquito collections. For L1014F kdr allele detection, the L/L (susceptible homozygote) combination 235 was most dominant (>60%) in all collection sites. At the Leuven peri-urban site, L/F (resistant 236 heterozygote: 15%) was less frequently observed than at the other two collection sites in Leuven 237 (around 30%), but this difference was not significant. In addition, no F/F (resistant homozygote) was 238 observed in the agricultural habitat (Figure 1, A). For the *ace-1* G119S genotype, the proportion of G/S 239 (resistant heterozygote) alleles was lower in the urban site (25-37.5%) than in the agricultural area 240 (53-57%) and in peri-urban sites (33% -50%) (Figure 1, B), however, more samples were needed to draw a significant conclusion. The only mosquito harbouring the S/S (resistant homozygote) allele was
observed in Mechelen. Interestingly, similar proportions of both mutations were observed in the periurban area of Mechelen (Figure 1, A and B).

244 3.4 Frequency of L1014F and G119S multi-resistance

245 When combining the two resistance mechanisms in the *Culex pipiens* sampled in this study, there are 246 seven possible genotypic profiles: L/L-G/G (SSSS), L/L-G/S (SSRS), L/L-S/S (SSRR), L/F-G/G (RSSS), L/F-247 G/S (RSRS), F/F-G/G (RRSS) and F/F-G/S (RRRS) (Figure 2). The two genotypes RSRR and RRRR were not 248 identified among the sampled Belgian mosquitoes. The SSSS genotype was the most prevalent (>40%) 249 in the collections from the urban and peri-urban sites in Leuven and the peri-urban site in Mechelen. 250 However, from the agricultural site, the resistant genotypes were likely more established, with the 251 SSRS genotype (40%) surpassing the SSSS genotype (23%-36%) in frequency, but not significantly 252 when compared to other locations (Figure 2). In *Culex modestus*, resistant homozygotes were not 253 observed. Only four genotypes were found in *Culex modestus* including SSSS (53%), SSRS (2%), RSSS (43%), and RSRS (2%). In Culex torrentium, only the SSSS profile was detected. Interestingly, the Culex 254 255 pipiens, Culex modestus and Culex torrentium mosquitoes collected from the same location (Leuven 256 peri-urban site) were found to have different genotypes.

4. DISCUSSION

258 Field mosquitoes were collected in Leuven and surrounding areas in urban, peri-urban, and agricultural habitats during the summer months for three consecutive years (2019-2021). Culex 259 260 pipiens was the dominant species across all collection sites, a finding consistent with previous research 261 conducted in this area (Wang et al. 2021b). The majority of *Culex pipiens* were biotype pipiens. Only 262 one hybrid female *Culex pipiens* and one female *Culex pipiens molestus* were identified in this study. 263 *Culex pipiens* biotype *pipiens* and biotype *molestus* can present genetic, behavioural and physiological 264 differences. More importantly, vector competence can vary significantly between the two biotypes; 265 therefore, the identification of the subspecies level is important to assess the risk of arbovirus 266 transmission by field *Culex pipiens* mosquitoes (Abbo et al. 2021).

267 The kdr L1014F and ace-1 G119S amino acid substitutions were previously observed in Culex pipiens 268 from Morocco (Bkhache et al. 2016), Greece (Kioulos et al. 2014), Turkey (Guz et al. 2020), California, 269 USA (Yoshimizu et al. 2020) and China (Liu et al. 2019). Culex pipiens sampled from three diverse 270 locations in Greece had a L1014F mutation resistant frequency of 97% (40% heterozygous L/F and 57% 271 homozygous resistant F/F), demonstrating intense levels of pyrethroid resistance in the country 272 (Fotakis et al. 2017). In contrast, the same study observed frequencies of G119S and F290V ace-1 273 amino acid changes in 14% and <1% of the mosquitoes, respectively, representing very low resistance 274 to organophosphates and carbamates. In this study, we report a heterozygous and homozygous 275 resistance frequency of 13-44% for kdr and 25-50% for AChE insensitivity in Culex pipiens mosquitoes 276 from Belgium. It is evident that the rate of resistance is location-dependent and can arise rapidly in 277 mosquito populations given the right selection pressures. In areas with competent vectors and known 278 or suspected arbovirus circulation, it is important to distinguish the types and degrees of insecticide 279 resistance to inform evidence-based decision-making regarding the use of vector control. In most 280 European countries, however, the insecticide resistance profile of *Culex* mosquitoes is an important 281 knowledge gap and potential barrier to effective vector control.

No reports on insecticide resistance in *Culex modestus and Culex torrentium* mosquitoes were found in literature. Interestingly, our study observed a frequency of 43% *kdr* resistant heterozygosity (L/F) in *Culex modestus*, whereas the frequency of G119S *ace-1* was low (3.9% heterozygous). In contrast, we did not observe any resistance alleles in *Culex torrentium* mosquitoes. A larger sample size will be needed to confirm these results, although *Culex torrentium* is not an abundant species found in these locations.

288 The mutation for *ace-1* G119S was observed at a higher frequency in the heterozygous state compared 289 to the homozygous state. Only one female with the S/S resistant homozygote genotype was found, 290 similar to the findings from Greece (Kioulos et al. 2014). We assume that the high frequency of the 291 ace-1 G119S heterozygous resistant genotype in *Culex pipiens* is due to the high fitness costs 292 associated when the mutation is present in the homozygote form. This theory is also suggested by 293 other studies where mosquitoes with the S/S homozygote genotype were rarely reported (Weetman 294 et al. 2015). Moreover, under laboratory conditions, a significantly higher mortality was observed in 295 Anopheles gambiae pupae with the S/S (homozygote) substitution compared to pupae with the G/G 296 (susceptible) genotype (Djogbénou et al. 2010). As these fitness costs are alleviated in the 297 heterozygote form, repeated selection may lead to a permanent state of heterozygosity in Culex 298 populations (Berticat et al. 2002). Another mutation leading to the F290V amino acid substitution was 299 reported to be associated to AChE insensitivity as well, warranting further investigation in Belgian Culex mosquitoes (Alout et al. 2007). 300

A limitation to our study was the small sample size which did not allow us to perform insecticide bioassays such as the WHO cylinder test or CDC bottle bioassay. The detection of these two resistance genotypes does not represent all potential resistance mechanisms that may be involved, such as the changement of the cuticular penetration of insecticides, as well as the degree of phenotypic resistance in these mosquitoes (Donnelly et al. 2009). However, as there is evidence of a correlation between *kdr* resistance genotypes and phenotypic resistance to pyrethroids in *Culex pipiens pipiens*, we expect

that there must be some degree of phenotypic resistance to pyrethroids in Belgian *Culex pipiens* with *kdr* L/L or L/F alleles (Tmimi et al. 2018). In comparison with the *Culex pipiens* from Morocco (with 42% frequency of resistant alleles, showing resistance levels 33 times higher than the susceptible strain to permethrin), the *Culex pipiens* from Belgium (with approximately 20% frequency of resistant alleles) are expected to have a lower resistance level to permethrin than the mosquitoes from Morocco but higher than the susceptible mosquitoes from Belgium (Tmimi et al. 2018).

Importantly, the co-occurrence of *kdr* L1014F and *ace-1* G119S suggests that multi- or cross-resistant mosquitoes could be developing in our study area, which may cause an inefficiency of pyrethroid and organophosphate insecticides if used for mosquito control in Belgium. Further measurement of the metabolic activity of enzymes (such as cytochrome p450, esterase and acetylcholinesterase) involved in insecticide detoxification via transcriptomic (Silva Martins et al. 2019) or proteomic methods is warranted to better understand the extent of mosquito insecticide resistance in Belgium (Epelboin et al. 2021).

320 In the Flanders region of Belgium, a total of 2.4 tons of pesticides were reported to be used in 2020 321 (Vlaamse Milieumaatschappij 2020). The majority of pesticide use (98%) consisted of herbicides for 322 plant control, of which glyphosate was the most common active ingredient (67%). This was followed 323 by 2,4-dichlorphenoxyacetic acid (2,4-D; 17%), triclopyr (7%), 2-methyl-4-chlorophenoxyacetic acid 324 (MCPA; 4%), and *Bacillus thuringiensis* (2%). The strong use of glyphosate, an organophosphate, may explain the development of *ace-1* G119S resistance in the *Culex pipiens* observed in our study. It was 325 326 shown that at field-realistic doses, the presence of glyphosate can impair the learning ability of Aedes 327 aegypti larvae (Baglan et al. 2018) as well as modify the life history traits and increase insecticide 328 resistance in Anopheles arabiensis (Oliver and Brooke 2018). In addition, it was shown that 2,4-D and 329 MCPA can affect AChE sensitivity in humans (Bukowska and Hutnik 2006); therefore, the development 330 of *ace-1* resistance in Belgian *Culex* mosquitoes may also be due to the selection pressures caused by 331 2,4-D and MCPA. The use of insecticides for insect control in Belgium represented 2% of all pesticide 332 use, specifically for the control of wasps and oak processionary caterpillars. The use of insecticides 333 against mosquitoes was reported in only one location at the French border to prevent the entry of 334 Aedes mosquitoes. The most common site for the application of pesticides was open pavement (78%), 335 followed by woody vegetation (13%), and sports fields (5%). In Belgian botanical gardens, the use of 336 fungicides, acaricides and rare pesticides were also reported but not specified. Aside from chemical 337 pesticides, there is evidence that natural plant chemicals and microbes can confer insecticide 338 resistance in mosquitoes (David et al. 2006, Kikuchi et al. 2012). A common botanical pesticide is 339 pyrethrum which, although derived from natural sources, shares a similar structure and mode of 340 action with synthetic pyrethroid insecticides (Isman 2008). Other potential environmental 341 contributors to resistance include natural xenobiotics (allelochemicals), industry pollutants, and 342 domestic insecticides. However, the extent to which Belgian mosquitoes in their adult or immature 343 developmental stages may be exposed to natural pesticides is unclear.

344 There is little evidence on the efficacy and cost-effectiveness of vector control interventions in Europe 345 (Bellini et al. 2014). Over recent decades, the European Union has been gradually banning the use of 346 insecticides for agriculture, including the pyrethroid permethrin and the organophosphate malathion. 347 In Flanders, there has been a consistent decline in the overall use of pesticides. Since 2010, the 348 quantity of active ingredients has fallen from 15.7 to 2.4 tons in 2020 (Vlaamse Milieumaatschappij 349 2020). It is possible that a continuous decline of pesticide use may reduce the selection pressure on 350 mosquitoes to develop insecticide resistance. However, the limited use of a small number of active 351 ingredients and the high reliance on glyphosate may facilitate the development of insecticide 352 resistance in mosquitoes if they are being exposed regularly at low concentrations (Oliver and Brooke 353 2018). As we do not expect the frequency of insecticide resistance in these mosquito populations to 354 decline, we recommend that insecticide resistance levels of mosquitoes are monitored at a larger scale 355 in Belgium and in neighbouring countries as well.

356

357 Conclusion

- 358 In this study, we present the first report of *kdr* L1014F and *ace-1* G119S insecticide resistance in *Culex*
- 359 *pipiens, Culex modestus,* and *Culex torrentium* mosquitoes from Belgium. We highlight the importance
- 360 of mosquito surveillance for the development and implementation of effective arbovirus control
- 361 strategies in the event of an outbreak.

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- 367 Conflict of Interest Statement
- 368 No conflicts of interest to declare.

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- 498 Tables
- 499 Table 1. Total number and frequency (%) of L/L (susceptible), L/F (heterozygous resistant) or F/F
- 500 (homozygous resistant) kdr alleles in Culex mosquitoes collected in the Leuven urban (U), peri-urban
- 501 (PU), and agricultural (A) sites and the Mechelen peri-urban (MP) site from 2019 to 2021.

Culex torrentium				Culex modestus				pipiens	Culex				
[:] % F/F%	L/F%	L/L%		F/F%	L/F%	L/L%		F/F%	L/F%	L/L%			
a) (no)	(no.)	(no.)	No.	(no.)	(no.)	(no.)	No.	(no.)	(no.)	(no.)	No.	Site	Year
,,) (10.)	(110.)	(110.)		(110.)	(110.)	(110.)		(110.)	(110.)	(110.)			
-	-	-	-	0	43	57	51	12.5	12.5	75	8	PU	2019
				(0)	(22)	(29)		(1)	(1)	(6)			
-	-	-	-	-	-	-	-	8.3	25	67	12	U	
								(1)	(3)	(8)			
0	0	100	2	-	-	-	-	0.3	24	73	33	PU	2020
) (0)	(0)	(2)						(1)	(8)	(24)			
-	-	-	-	-	-	-	-	0	23	77	30	А	
								(0)	(7)	(23)			
0	0	100	4	-	-	-	-	9.4	34	56	32	U	
) (0)	(0)	(4)						(3)	(11)	(18)			
-	-	-	-	-	-	-	-	3.3	10	87	30	PU	2021
								(1)	(3)	(26)			
-	-	-	-	-	-	-	-	0	37	63	30	А	
								(0)	(11)	(19)			
0	0	100	2	-	-	-	-	7.1	29	64	28	U	
) (0)	(0)	(2)						(2)	(8)	(18)			
-	-	-	-	-	-	-	-	6.0	9.1	85	33	MP	
								(2)	(3)	(28)			
))	0 (0) - - 0 (0) -	100 (4) - - 100 (2) -	4 - - 2	-	-	-	-	 9.4 (3) 3.3 (1) 0 (0) 7.1 (2) 6.0 (2) 	34 (11) 10 (3) 37 (11) 29 (8) 9.1 (3)	56 (18) 87 (26) 63 (19) 64 (18) 85 (28)	32 30 30 28 33	U PU A U	2021

503 Table 2. Hardy-Weinberg equilibrium of kdr L/L (susceptible), L/F (heterozygous resistant) or F/F

504	homozygous resistant) alleles in <i>Culex pipiens</i> .

	No	. observ	ved	Ν	o. expecte	Y^2 avalue	
Year	L/L	L/F	F/F	L/L	L/F	F/F	, p-value
2019	14	4	2	12.80	6.40	0.80	2.813, <i>p</i> =0.245
2020	65	26	4	64.04	27.92	3.04	0.450, <i>p</i> =0.799
2021	91	25	5	88.53	29.94	2.53	3.295, <i>p</i> =0.193

505 No. observed: total mosquitoes captured (all locations grouped). No. expected: expected number of

506 mosquitoes based on the Hardy-Weinberg model. The X^2 and p-values were calculated with GraphPad

507 Prism (V.9.3). A *p*-value of <0.05 was considered statistically significant.

508 Table 3. Total number and frequency (%) of G/G (susceptible), G/S (heterozygous resistant) or S/S

509 (homozygous resistant) AChE alleles in Culex mosquitoes collected in the Leuven urban (U), peri-

510 urban (PU), and agricultural (A) sites and the Mechelen peri-urban (MP) site from 2019 to 2021.

			Culex	pipiens			Culex modestus				Culex torrentium				
			G/G%	G/S%	S/S%	No.	G/G%	G/S%	S/S%		G/G%	G/S%	S/S%		
Year	Site	No.	(no.)	(no.)	(no.)		(no.)	(no.)	(no.)	No.	(no.)	(no.)	(no.)		
2019	PU	8	50	50	0	51	96	3.9	-	-	-	-	-		
			(4)	(4)	(0)		(49)	(2)							
	U	12	75	25	0	-	-	-	-	-	-	-	-		
			(9)	(3)	(0)										
2020	PU	33	73	27	0	-	-	-	-	2	100	0	0		
			(24)	(9)	(0)						(2)	(0)	(0)		
	А	30	47	53	0	-	-	-	-	-	-	-	-		
			(14)	(16)	(0)										
	U	32	62.5	37.5	0	-	-	-	-	4	100	0	0		
			(20)	(12)	(0)						(4)	(0)	(0)		
2021	PU	30	50	50	0	-	-	-	-	-	-	-	-		
			(15)	(15)	(0)										
	А	30	43	57	0	-	-	-	-	-	-	-	-		
			(13)	(17)	(0)										
	U	28	75	25	0	-	-	-	-	2	100	0	0		
			(21)	(7)	(0)						(2)	(0)	(0)		
	MP	33	64	33	3.0	-	-	-	-	-	-	-	-		
			(21)	(11)	(1)										

512 Table 4. Hardy-Weinberg equilibrium of G/G (homozygous susceptible), G/S (heterozygous

	No. observed			No. expected			
Year	G/G	G/S	s/s	G/G	G/S	s/s	X ² , <i>p</i> -value
2019	13	7	0	13.6	5.8	0.6	0.875 <i>, p</i> =0.646
2020	58	37	0	61.6	29.8	3.6	5.550, <i>p</i> =0.062
2021	70	50	1	74.6	40.8	5.6	6.137, <i>p</i> =0.047*

513 resistant) or S/S (homozygous resistant) AChE alleles in *Culex pipiens*.

514 No. observed: total mosquitoes captured (all locations grouped). No. expected: expected number of

515 mosquitoes based on the Hardy-Weinberg model. The X^2 and p-values were calculated with GraphPad

516 Prism (V.9.3). A *p*-value of <0.05 was considered statistically significant, (* = p <0.05).

517 Figures

Figure 1. Distribution of *kdr* L1014F and *ace*-1 G119S in mosquitoes from different locations. (A)
Prevalence of mosquitoes with L/L (homozygous susceptible; S/S), L/F (heterozygous resistant; R/S) or
F/F (homozygous resistant; R/R) *kdr* genotypes. (B) Prevalence of mosquitoes with G/G (homozygous
susceptible; S/S), G/S (heterozygous resistant; R/S) or S/S (homozygous resistant; R/R) *AChE*genotypes. Urban (Leuven)= botanic garden, peri-urban =Arenberg Park, agricultural =Bertem. LE=
Leuven, ME=Mechelen.



Figure 2. Frequency of combinations of *kdr* L1014F and *ace*-1 G119S in *Culex pipiens* mosquitoes from different locations. A four-letter abbreviation was used to designate the combinations of genotypes: the first two letters refer to *kdr* L1014F and the last two refer to *ace*-1. SS susceptible genotypes, RS heterozygous genotypes, RR homozygous resistant genotypes. L/L-G/G (SSSS), L/L-G/S (SSRS), L/L-S/S (SSRR), L/F-G/G (RSSS), L/F-G/S (RSRS), F/F-G/G (RRSS) and F/F-G/S (RRRS). Urban (Leuven)= botanic garden, peri-urban =Arenberg Park, agricultural =Bertem.

