First report of *kdr* L1014F and *ace-1* G119S insecticide resistance in Belgian *Culex* (Diptera: Culicidae) mosquitoes

Short running title: Insecticide resistance in Belgian *Culex* mosquitoes

Lanjiao Wang[1], Alina Soto[1], Laure Remue[1], Ana Lucia Rosales Rosas[1], Lander De Coninck[2], Sam Verwimp[1], Johanna Bouckaert[1], Mathias Vanwinkel[1], Jelle Matthijnssens[2], Leen Delang[1]

[1]Laboratory of Virology and Chemotherapy, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, KU Leuven, 3000, Leuven, Belgium.

[2]Laboratory of Viral Metagenomics, Rega Institute for Medical Research, KU Leuven, 3000, Leuven, Belgium.

Corresponding author:

Prof. Leen Delang,

Rega Institute for Medical Research, box 1043, Herestraat 49, Leuven 3000, Belgium

+32 16322107 / +32 16379020

leen.delang@kuleuven.be

Abstract

The emergence of West Nile virus and Usutu virus in Europe poses a significant risk to public health. In the absence of efficient antiviral therapy or vaccine candidates, the only strategy to control these arboviruses is to target the *Culex* (Diptera: Culicidae) mosquito vector. However, the selection pressure caused by exposure to insecticides for vector control or agricultural pest control can lead to insecticide resistance, thereby reducing the efficacy of insecticide-based vector control interventions. In *Culex* mosquitoes, two of the most common amino acid substitutions associated with insecticide resistance are the *kdr* L1014F in voltage gated sodium channels and G119S in acetylcholinesterase.

In this study, *Culex pipiens* biotype *pipiens*, *Culex torrentium*, and *Culex modestus* were sampled from 2019 to 2021 in three distinct environmental habitats (urban, peri-urban, and agricultural) in and around the city of Leuven, Belgium. Individual mosquitoes were screened for two mutations resulting in L1014F and G119S amino acid substitutions. Both mutations were observed in *Culex pipiens* and *Culex modestus* but not in *Culex torrentium* mosquitoes across the four collection sites. Furthermore, multi-resistance or cross-resistance in *Culex pipiens* could be a threat in these areas, as both mutations were observed at low frequencies.

These results provide the first report of *kdr* L1014F and *ace-1* G119S resistance in *Culex pipiens* and *Culex modestus* mosquitoes from Belgium, highlighting the importance of mosquito surveillance to design effective arbovirus outbreak control strategies.

Key words: Insecticide resistance, *kdr* L1014*F*, *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.

44 Sporadic outbreaks of WNV in humans have arisen in southern and southwestern Europe since 1963.
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
53 have been reported, yet there is zoonotic potential in areas where competent vector species are
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.

61 Adulticiding by fogging is the first-line choice for vector control against *Culex* mosquitoes during
62 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

89 ace-1 gene, resulting in substitutions such as G119S, which reduce the sensitivity of AChE to
90 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,
94 creating the need for novel combinations of insecticides with differing modes of action to regain the
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).

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

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

118 ## 2. MATERIALS AND METHODS

119 **2.1 Ethics statement**

120 Permits for field collections in private habitats (Bertem and Mechelen) were obtained from the
121 landowners. Permits for collection in the Botanic Garden were obtained from the City Green
122 Management of Leuven. Permits for collection in Arenberg Park were obtained from the security
123 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 CO_2 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
131 dry ice and then separated to genus level based on morphological characteristics. Mosquitoes were
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**

138 ***DNA extraction**:* Following morphological identification, mosquitoes were transferred to tubes with
139 2.8 mm ceramic beads (Precellys®, Bertin, USA) and 500 µl phosphate-buffered saline (1X PBS) solution
140 and homogenised with a Precellys Evolution homogeniser at 2 cycles of 6800 rpm for 10 sec with a
141 pause of 20 sec. The homogenate was lysed at 100°C for 10 min, followed by centrifugation at 10,000
142 rpm for 1 min to spin down the tissue debris, and 150 µl of supernatant was transferred to a new tube.
143 DNA extraction was performed using the QIAamp DNA kit (Qiagen®, Hilden, Germany) according to
144 the manufacturer's protocol.

145 ***Mosquito identification**: Culex pipiens* biotype *pipiens* and *Culex pipiens* biotype *molestus* were
146 distinguished with a multiplex qRT-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
149 using the Low ROX One-Step qRT-PCR 2X MasterMix kit (Eurogentec®, Seraing, Belgium) following the
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**

156 ***Detection of kdr L1014F (mutation from TTA to TTT)***: The protocol for *kdr* L1014F detection was
157 adapted from a protocol described elsewhere (Martinez-Torres et al. 1999). Briefly, two PCR reactions
158 were run in parallel using 4 primers: Cgd1 (5'-GTGGAACTTCACCGACTTC-3'), Cgd2 (5'
159 GCAAGGCTAAGAAAAGGTTAAG-3'), Cgd3 (5'-CCACCGTAGTGATAGGAAATTTA-3') and Cgd4 (5'-
160 CCACCGTAGTGATAGGAAATTTT-3'). A 20 µl reaction volume was prepared for each reaction using the
161 GoTaq® Green Master 2X Mix (M7122, Promega, Belgium) following the manufacturer's instructions.
162 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
166 resistant homozygote (F/F: RR). The cycle conditions were as follows: initial denaturation at 94°C for
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.

171 ***Detection of ace-1 G119S (mutation from GGC to AGC)*** *:* This analysis was performed following a
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 AluI 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
182 heterozygote (G/S: RS), or resistant homozygote (S/S: RR).

183 **2.5 Statistical analysis**

184 For the distribution of genotypic and allelic frequencies resulting in the *kdr* L1014F or *ace-1* G119S,
185 analysis was performed among different collecting sites, years, and species by GraphPad Prism 9
186 (V.9.3). A Hardy–Weinberg equilibrium of the resistant (R) and susceptible (S) allelic frequencies was
187 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= $= \frac{\mathrm{RS+2RR}}{2(\mathrm{RS+RR+SS})}$;

191 S allelic frequency = 1- R allelic frequency

192 # 3. RESULTS

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

194 *Culex pipiens* were captured from 2019 to 2021 at the urban and peri-urban sites in Leuven. The
195 collection of *Culex pipiens* expanded to the Leuven agricultural site from 2020 to 2021 and to the
196 Mechelen peri-urban site in 2021. *Culex modestus* were collected at the Leuven peri-urban site in
197 2019, and *Culex torrentium* were captured at the Leuven urban and peri-urban sites in 2020 and the
198 Leuven urban site in 2021. One hybrid female *Culex pipiens* (hybrid of *pipiens* and *molestus*) and one
199 female *Culex pipiens molestus* were identified at the Leuven peri-urban site and Mechelen peri-urban
200 site in 2021, respectively.

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

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

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

213 The mutation frequencies in the *ace-1* gene resulting in the G119S amino acid change in *Culex pipiens*,
214 *Culex modestus* and *Culex torrentium* from 2019-2021 are presented in Table 3. In *Culex pipiens*, AChE
215 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.

225 The observed rate of heterozygosity (G/S) in *Culex pipiens* was generally consistent over time across
226 all locations, except at the Leuven peri-urban site where the rate of heterozygosity fell from 50% to
227 27% from 2019 to 2020 but returned to 50% in 2021. The Hardy-Weinberg equilibrium of genotypes
228 in *Culex pipiens* across all collection sites remained constant from 2019 to 2020 (Table 4). However, in
229 2021 the rates of G/G and S/S genotypes were lower and the rate of G/S heterozygosity was higher
230 than the expected frequencies. Therefore, we may observe a higher rate of *ace-1* G119S heterozygous
231 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

241 draw a significant conclusion. The only mosquito harbouring the S/S (resistant homozygote) allele was
242 observed in Mechelen. Interestingly, similar proportions of both mutations were observed in the peri-
243 urban 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
254 (43%), and RSRS (2%). In *Culex torrentium*, only the SSSS profile was detected. Interestingly, the *Culex*
255 *pipiens, Culex modestus* and *Culex torrentium* mosquitoes collected from the same location (Leuven
256 peri-urban site) were found to have different genotypes.

257 ## 4. DISCUSSION

258 Field mosquitoes were collected in Leuven and surrounding areas in urban, peri-urban, and
259 agricultural habitats during the summer months for three consecutive years (2019-2021). *Culex*
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.

282 No reports on insecticide resistance in *Culex modestus and Culex torrentium* mosquitoes were found
283 in literature. Interestingly, our study observed a frequency of 43% *kdr* resistant heterozygosity (L/F) in
284 *Culex modestus*, whereas the frequency of G119S *ace-1* was low (3.9% heterozygous). In contrast, we
285 did not observe any resistance alleles in *Culex torrentium* mosquitoes. A larger sample size will be
286 needed to confirm these results, although *Culex torrentium* is not an abundant species found in these
287 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
300 *Culex* mosquitoes (Alout et al. 2007).

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

307 that there must be some degree of phenotypic resistance to pyrethroids in Belgian *Culex pipiens* with
308 *kdr* L/L or L/F alleles (Tmimi et al. 2018). In comparison with the *Culex pipiens* from Morocco (with
309 42% frequency of resistant alleles, showing resistance levels 33 times higher than the susceptible
310 strain to permethrin), the *Culex pipiens* from Belgium (with approximately 20% frequency of resistant
311 alleles) are expected to have a lower resistance level to permethrin than the mosquitoes from
312 Morocco but higher than the susceptible mosquitoes from Belgium (Tmimi et al. 2018).

313 Importantly, the co-occurrence of *kdr* L1014F and *ace-1* G119S suggests that multi- or cross-resistant
314 mosquitoes could be developing in our study area, which may cause an inefficiency of pyrethroid and
315 organophosphate insecticides if used for mosquito control in Belgium. Further measurement of the
316 metabolic activity of enzymes (such as cytochrome p450, esterase and acetylcholinesterase) involved
317 in insecticide detoxification via transcriptomic (Silva Martins et al. 2019) or proteomic methods is
318 warranted to better understand the extent of mosquito insecticide resistance in Belgium (Epelboin et
319 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
325 explain the development of *ace-1* G119S resistance in the *Culex pipiens* observed in our study. It was
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

use, specifically for the control of wasps and oak processionary caterpillars. The use of insecticides against mosquitoes was reported in only one location at the French border to prevent the entry of *Aedes* mosquitoes. The most common site for the application of pesticides was open pavement (78%), followed by woody vegetation (13%), and sports fields (5%). In Belgian botanical gardens, the use of fungicides, acaricides and rare pesticides were also reported but not specified. Aside from chemical pesticides, there is evidence that natural plant chemicals and microbes can confer insecticide resistance in mosquitoes (David et al. 2006, Kikuchi et al. 2012). A common botanical pesticide is pyrethrum which, although derived from natural sources, shares a similar structure and mode of action with synthetic pyrethroid insecticides (Isman 2008). Other potential environmental contributors to resistance include natural xenobiotics (allelochemicals), industry pollutants, and domestic insecticides. However, the extent to which Belgian mosquitoes in their adult or immature developmental stages may be exposed to natural pesticides is unclear.

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

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.

362 Acknowledgements

363 This project was funded by KU Leuven (C14/20/108 and starting grant STG/19/008). We would like to
364 thank Prof. Johan Neyts (Rega Institute, KU Leuven) for allowing us the use of his laboratory space and
365 equipment for our experimental work. We would like to thank the City Green Management of Leuven
366 and KU Leuven for providing the permits for the mosquito field collections from 2019 to 2021.

367 Conflict of Interest Statement

368 No conflicts of interest to declare.

369 References

370 **(West Nile virus infection. In: ECDC. Annual epidemiological report for 2018.) European Centre for**
371 **Disease Prevention and Control. 2019**. West Nile virus infection. In: ECDC. Annual
372 epidemiological report for 2018.
373 (https://www.ecdc.europa.eu/sites/default/files/documents/west-nile-fever-annual-
374 epidemiological-report-2018.pdf).

375 **Abbo, S. R., T. M. Visser, C. J. M. Koenraadt, G. P. Pijlman, and H. Wang**. **2021**. Effect of blood
376 source on vector competence of Culex pipiens biotypes for Usutu virus. Parasit. Vectors. 14:
377 194.

378 **Alout, H., A. Berthomieu, A. Hadjivassilis, and M. Weill**. **2007**. A new amino-acid substitution in
379 acetylcholinesterase 1 confers insecticide resistance to Culex pipiens mosquitoes from Cyprus.
380 Insect Biochem. Mol. Biol. 37: 41–47.

381 **Atyame, C. M., H. Alout, L. Mousson, M. Vazeille, M. Diallo, M. Weill, and A. B. Failloux**. **2019**.
382 Insecticide resistance genes affect Culex quinquefasciatus vector competence for West Nile
383 virus. Proc. R. Soc. B Biol. Sci. 286.

384 **Baglan, H., C. R. Lazzari, and F. J. Guerrieri**. **2018**. Glyphosate impairs learning in aedes aegypti
385 mosquito larvae at field-realistic doses. J. Exp. Biol. 221.

386 **Bakonyi, T., and J. M. Haussig**. **2020**. West Nile virus keeps on moving up in Europe.
387 Eurosurveillance. 25.

388 **Becker, N., D. Petrić, M. Zgomba, C. Boase, M. Madon, C. Dahl, and A. Kaiser**. **2010**. Mosquitoes
389 and their control: Second edition, Mosquitoes Their Control Second Ed. Springer-Verlag Berlin
390 Heidelberg.

391 **Bellini, R., H. Zeller, and W. Van Bortel**. **2014**. A review of the vector management methods to
392 prevent and control outbreaks of West Nile virus infection and the challenge for Europe.

393 Parasit. Vectors. 7: 323.

394 **Berticat, C., G. Boquien, M. Raymond, and C. Chevillon**. **2002**. Insecticide resistance genes induce a
395 mating competition cost in Culex pipiens mosquitoes. Genet. Res. 79: 41–47.

396 **Bkhache, M., F. Z. Tmimi, O. Charafeddine, C. Faraj, A. B. Failloux, and M. Sarih**. **2016**. First report
397 of L1014F-kdr mutation in Culex pipiens complex from Morocco. Parasit. Vectors. 9.

398 **Boukraa, S., W. Dekoninck, V. Versteirt, F. Schaffner, M. Coosemans, E. Haubruge, and F. Francis**.
399 **2015**. Updated checklist of the mosquitoes (Diptera: Culicidae) of Belgium. J. Vector Ecol. 40:
400 398–407.

401 **Bukowska, B., and K. Hutnik**. **2006**. 2,4-D and MCPA and their derivatives: Effect on the activity of
402 membrane erythrocytes acetylcholinesterase (in vitro). Pestic. Biochem. Physiol. 85: 174–180.

403 **David, J. P., S. Boyer, A. Mesneau, A. Ball, H. Ranson, and C. Dauphin-Villemant**. **2006**. Involvement
404 of cytochrome P450 monooxygenases in the response of mosquito larvae to dietary plant
405 xenobiotics. Insect Biochem. Mol. Biol. 36: 410–420.

406 **Davies, T. G. E., L. M. Field, P. N. R. Usherwood, and M. S. Williamson**. **2007**. DDT, pyrethrins,
407 pyrethroids and insect sodium channels. IUBMB Life. 59: 151–162.

408 **Deng, J., Y. Guo, X. Su, S. Liu, W. Yang, Y. Wu, K. Wu, G. Yan, and X. G. Chen**. **2021**. Impact of
409 deltamethrin-resistance in Aedes albopictus on its fitness cost and vector competence. PLoS
410 Negl. Trop. Dis. 15.

411 **Djogbénou, L., V. Noel, and P. Agnew**. **2010**. Costs of insensitive acetylcholinesterase insecticide
412 resistance for the malaria vector Anopheles gambiae homozygous for the G119S mutation.
413 Malar. J. 9: 12.

414 **Dong, K., Y. Du, F. Rinkevich, Y. Nomura, P. Xu, L. Wang, K. Silver, and B. S. Zhorov**. **2014**. Molecular
415 Biology of Insect Sodium Channels and Pyrethroid Resistance. Insect Biochem. Mol. Biol. 50: 1.

416 **Donnelly, M. J., V. Corbel, D. Weetman, C. S. Wilding, M. S. Williamson, and W. C. Black IV**. **2009**.
417 Does kdr genotype predict insecticide-resistance phenotype in mosquitoes? Trends Parasitol.
418 25: 213–219.

419 **Epelboin, Y., L. Wang, Q. G. Gianetto, V. Choumet, P. Gaborit, J. Issaly, A. Guidez, T. Douché, T.**
420 **Chaze, M. Matondo, and I. Dusfour**. **2021**. CYP450 core involvement in multiple resistance
421 strains of Aedes aegypti from French Guiana highlighted by proteomics, molecular and
422 biochemical studies. PLoS One. 16: e0243992.

423 **Fotakis, E. A., A. Chaskopoulou, L. Grigoraki, A. Tsiamantas, S. Kounadi, L. Georgiou, and J. Vontas**.
424 **2017**. Analysis of population structure and insecticide resistance in mosquitoes of the genus
425 Culex, Anopheles and Aedes from different environments of Greece with a history of mosquito
426 borne disease transmission. Acta Trop. 174: 29–37.

427 **Fros, J. J., P. Miesen, C. B. Vogels, P. Gaibani, V. Sambri, B. E. Martina, C. J. Koenraadt, R. P. van Rij,**
428 **J. M. Vlak, W. Takken, and G. P. Pijlman**. **2015**. Comparative Usutu and West Nile virus
429 transmission potential by local Culex pipiens mosquitoes in north-western Europe. One Heal. 1:
430 31–36.

431 **Glunt, K. D., A. P. Abílio, Q. Bassat, H. Bulo, A. E. Gilbert, S. Huijben, M. N. Manaca, E. Macete, P.**
432 **Alonso, and K. P. Paaijmans**. **2015**. Long-lasting insecticidal nets no longer effectively kill the
433 highly resistant Anopheles funestus of southern Mozambique. Malar. J. 14: 1–7.

434 **Guz, N., N. S. Cagatay, E. A. Fotakis, E. Durmusoglu, and J. Vontas**. **2020**. Detection of diflubenzuron
435 and pyrethroid resistance mutations in Culex pipiens from Muğla, Turkey. Acta Trop. 203:
436 105294.

437 **Hemingway, J., N. J. Hawkes, L. McCarroll, and H. Ranson**. **2004**. The molecular basis of insecticide
438 resistance in mosquitoes. Insect Biochem. Mol. Biol. 34: 653–665.

439 **Isman, M. B. 2008**. Botanical insecticides: for richer, for poorer. Pest Manag. Sci. 64: 8–11.

440 **Kikuchi, Y., M. Hayatsu, T. Hosokawa, A. Nagayama, K. Tago, and T. Fukatsu**. **2012**. Symbiont-
441 mediated insecticide resistance. Proc. Natl. Acad. Sci. U. S. A. 109: 8618–8622.

442 **Kioulos, I., A. Kampouraki, E. Morou, G. Skavdis, and J. Vontas**. **2014**. Insecticide resistance status
443 in the major West Nile virus vector Culex pipiens from Greece. Pest Manag. Sci. 70: 623–627.

444 **Liu, H., L. Xie, P. Cheng, J. Xu, X. Huang, H. Wang, X. Song, L. Liu, H. Wang, J. Kou, G. Yan, X. G.**
445 **Chen, and M. Gong**. **2019**. Trends in insecticide resistance in Culex pipiens pallens over 20
446 years in Shandong, China. Parasit. Vectors. 12: 167.

447 **Martinez-Torres, D., C. Chevillon, A. Brun-Barale, J. Bergé, N. Pasteur, and D. Pauron**. **1999**.
448 Voltage-dependent Na+ channels in pyrethroid-resistant Culex pipiens L mosquitoes. Pestic. Sci.
449 55: 1012–1020.

450 **Nikolay, B. 2015**. A review of West Nile and Usutu virus co-circulation in Europe: how much do
451 transmission cycles overlap? Trans. R. Soc. Trop. Med. Hyg. 109: 609–618.

452 **Oliver, S. V., and B. D. Brooke**. **2018**. The effect of commercial herbicide exposure on the life history
453 and insecticide resistance phenotypes of the major malaria vector Anopheles arabiensis
454 (Diptera: culicidae). Acta Trop. 188: 152–160.

455 **Parker-Crockett, C., C. R. Connelly, B. Siegfried, and B. Alto**. **2021**. Influence of Pyrethroid
456 Resistance on Vector Competency for Zika Virus by Aedes aegypti (Diptera: Culicidae). J. Med.
457 Entomol. 58: 1908–1916.

458 **Pichler, V., R. Bellini, R. Veronesi, D. Arnoldi, A. Rizzoli, R. P. Lia, D. Otranto, F. Montarsi, S. Carlin,**
459 **M. Ballardini, E. Antognini, M. Salvemini, E. Brianti, G. Gaglio, M. Manica, P. Cobre, P. Serini,**
460 **E. Velo, J. Vontas, I. Kioulos, J. Pinto, A. della Torre, and B. Caputo**. **2018**. First evidence of
461 resistance to pyrethroid insecticides in Italian Aedes albopictus populations 26 years after
462 invasion. Pest Manag. Sci. 74: 1319–1327.

463 **Rudolf, M., C. Czajka, J. Börstler, C. Melaun, H. Jöst, H. von Thien, M. Badusche, N. Becker, J.**

464 **Schmidt-Chanasit, A. Krüger, E. Tannich, and S. Becker**. **2013**. First nationwide surveillance of
465 Culex pipiens complex and Culex torrentium mosquitoes demonstrated the presence of Culex
466 pipiens biotype pipiens/molestus hybrids in Germany. PLoS One. 8: e71832.

467 **Silva Martins, W. F., C. S. Wilding, A. T. Isaacs, E. J. Rippon, K. Megy, and M. J. Donnelly**. **2019**.
468 Transcriptomic analysis of insecticide resistance in the lymphatic filariasis vector Culex
469 quinquefasciatus. Sci. Rep. 9: 11406.

470 **Soderlund, D. M. 2008**. Pyrethroids, knockdown resistance and sodium channels. Pest Manag. Sci.
471 64: 610–616.

472 **Tmimi, F. Z., C. Faraj, M. Bkhache, K. Mounaji, A. B. Failloux, and M. Sarih**. **2018**. Insecticide
473 resistance and target site mutations (G119S ace-1 and L1014F kdr) of Culex pipiens in Morocco.
474 Parasit. Vectors. 11.

475 **Vilibic-Cavlek, T., T. Petrovic, V. Savic, L. Barbic, I. Tabain, V. Stevanovic, A. Klobucar, A. Mrzljak,**
476 **M. Ilic, M. Bogdanic, I. Benvin, M. Santini, K. Capak, F. Monaco, E. Listes, and G. Savini**. **2020**.
477 Epidemiology of Usutu Virus: The European Scenario. Pathog. 2020, Vol. 9, Page 699. 9: 699.

478 **Vlaamse Milieumaatschappij**. **2020**. Duurzaam gebruik van pesticiden - 2020.

479 **Wang, L., A. L. R. Rosas, L. De Coninck, C. Shi, J. Bouckaert, J. Matthijnssens, and L. Delang**. **2021a**.
480 Establishment of Culex modestus in Belgium and a Glance into the Virome of Belgian Mosquito
481 Species. mSphere. 6.

482 **Wang, L., A. L. R. Rosas, L. De Coninck, C. Shi, J. Bouckaert, J. Matthijnssens, and L. Delang**. **2021b**.
483 Establishment of Culex modestus in Belgium and a Glance into the Virome of Belgian Mosquito
484 Species. mSphere. 6.

485 **Weetman, D., S. N. Mitchell, C. S. Wilding, D. P. Birks, A. E. Yawson, J. Essandoh, H. D. Mawejje, L.**
486 **S. Djogbenou, K. Steen, E. J. Rippon, C. S. Clarkson, S. G. Field, D. J. Rigden, and M. J.**
487 **Donnelly**. **2015**. Contemporary evolution of resistance at the major insecticide target site gene

488 Ace-1 by mutation and copy number variation in the malaria mosquito Anopheles gambiae.
489 Mol. Ecol. 24: 2656.

490 **Weill, M., C. Malcolm, F. Chandre, K. Mogensen, A. Berthomieu, M. Marquine, and M. Raymond**.
491 **2004**. The unique mutation in ace-1 giving high insecticide resistance is easily detectable in
492 mosquito vectors. Insect Mol. Biol. 13: 1–7.

493 **Yoshimizu, M. H., K. Padgett, and V. Kramer**. **2020**. Surveillance of a kdr Resistance Mutation in
494 Culex pipiens (Diptera: Culicidae) and Culex quinquefasciatus in California. J. Med. Entomol. 57:
495 645–648.

496

497

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

502

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

504 **(homozygous resistant) alleles in *Culex pipiens*.**

	No. observed			No. expected			
Year	**L/L**	**L/F**	**F/F**	**L/L**	**L/F**	**F/F**	**X^2, *p*-value**
2019	14	4	2	12.80	6.40	0.80	2.813, p=0.245
2020	65	26	4	64.04	27.92	3.04	0.450, p=0.799
2021	91	25	5	88.53	29.94	2.53	3.295, p=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*			
Year	Site	No.	G/G% (no.)	G/S% (no.)	S/S% (no.)	No.	G/G% (no.)	G/S% (no.)	S/S% (no.)	No.	G/G% (no.)	G/S% (no.)	S/S% (no.)
2019	PU	8	50 (4)	50 (4)	0 (0)	51	96 (49)	3.9 (2)	-	-	-	-	-
	U	12	75 (9)	25 (3)	0 (0)	-	-	-	-	-	-	-	-
2020	PU	33	73 (24)	27 (9)	0 (0)	-	-	-	-	2	100 (2)	0 (0)	0 (0)
	A	30	47 (14)	53 (16)	0 (0)	-	-	-	-	-	-	-	-
	U	32	62.5 (20)	37.5 (12)	0 (0)	-	-	-	-	4	100 (4)	0 (0)	0 (0)
2021	PU	30	50 (15)	50 (15)	0 (0)	-	-	-	-	-	-	-	-
	A	30	43 (13)	57 (17)	0 (0)	-	-	-	-	-	-	-	-
	U	28	75 (21)	25 (7)	0 (0)	-	-	-	-	2	100 (2)	0 (0)	0 (0)
	MP	33	64 (21)	33 (11)	**3.0** (1)	-	-	-	-	-	-	-	-

511

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

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

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

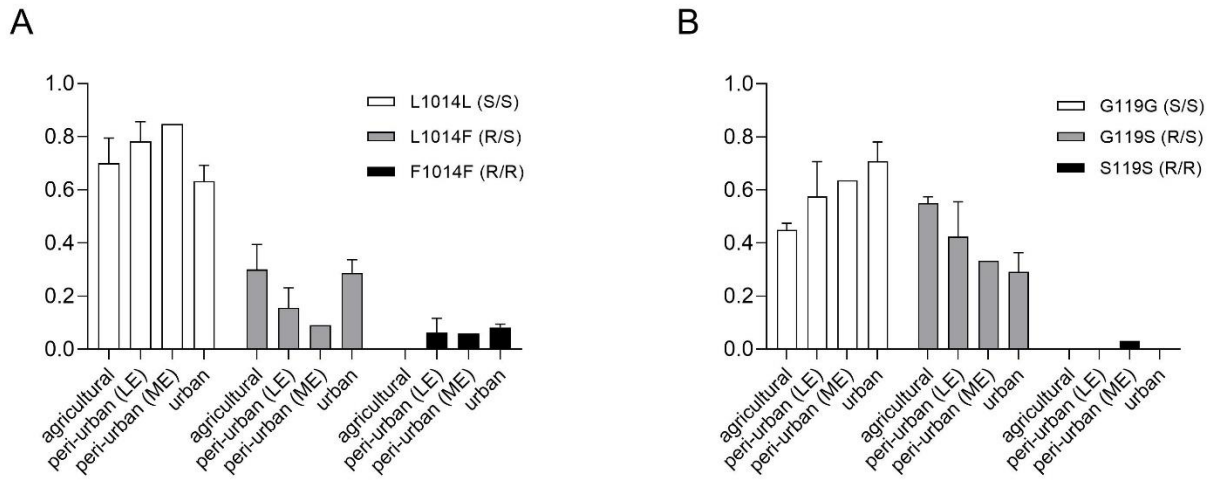
514 No. observed: total mosquitoes captured (all locations grouped). No. expected: expected number of

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

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



524

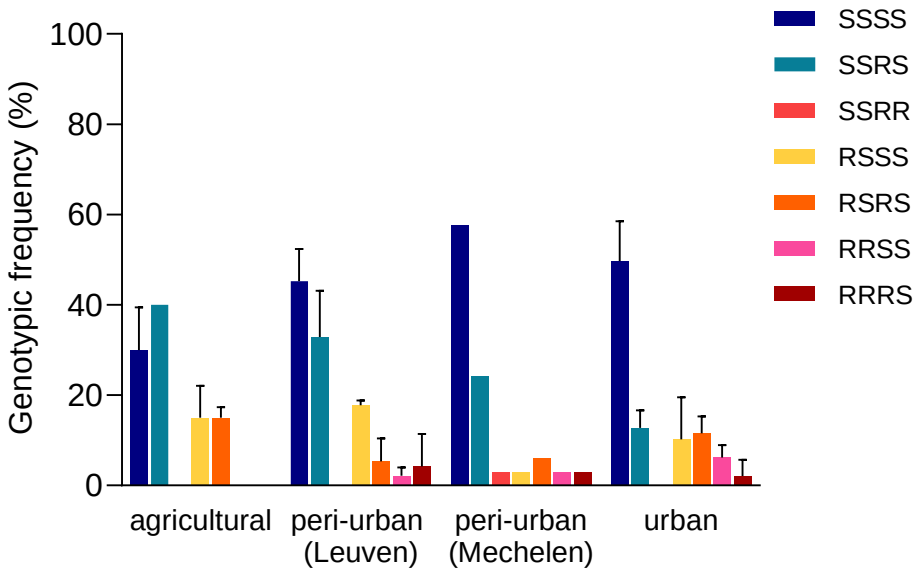



Figure 2. Frequency of combinations of *kdr* L1014F and *ace*-1 G119S in *Culex pipiens* mosquitoes from different locations. A four-letter abbreviation was used to designate the combinations of genotypes: the first two letters refer to *kdr* L1014F and the last two refer to *ace*-1. SS susceptible genotypes, 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.