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

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

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Abstract

The emergence of West Nile virus and Usutu virus in Europe is 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* mosquito vector. However, the selection pressure caused by exposure to insecticides in vector control or agricultural pest control can lead to the development of insecticide resistance 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 the voltage gated sodium channel and G119S in the 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 in Belgium. Two mutations resulting in L1014F and G119S amino acid substitutions were screened for in individual mosquitoes. Both mutations were observed in *Culex pipiens* and *Culex modestus* mosquitoes across the four collection sites, but not in *Culex torrentium*. Furthermore, multi-resistance or cross-resistance could be a developing threat, since both mutations were observed at low frequencies (resistant allele frequency <30%) and always in heterozygosity.

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 for the development of effective arbovirus control strategies in the event of an outbreak in Belgium.

Key words: Insecticide resistance, *kdr* L1014F, *ace-1* G119S, *Culex pipiens*, *Culex modestus*, Belgium

1. INTRODUCTION

Culex mosquitoes (family: *Culicidae*), such as *Culex pipiens* and *Culex modestus*, are efficient vectors of human and animal pathogens. In Europe, *Culex* mosquitoes are the primary vectors of West Nile virus (WNV) and Usutu virus (USUV), two arthropod-borne viruses (arboviruses) of the *Flaviviridae* family.[1] Both WNV and USUV are maintained in an enzootic cycle, amplifying in birds and transmitted via mosquitoes to incidental hosts such as humans and other mammals.

Sporadic outbreaks of WNV in humans have arisen in southern and southwestern Europe since 1963. The most significant outbreak occurred in 2018 with 1,548 locally-acquired cases and 166 deaths reported among several southern European countries.[2] There is increasing evidence that the circulation of WNV is spreading towards northern Europe, and this was exemplified in 2018 in Germany and more recently in 2020 in the Netherlands where the first autochthonous cases of WNV in humans and animals were reported.[3] USUV, on the other hand, has established widespread endemicity in Europe. The virus first emerged in 1996 in Italy and has since spread gradually across the continent causing major outbreaks and mortality in birds.[4] Few human cases of USUV have been reported, although there is zoonotic potential in areas where competent vector species are present. Furthermore, the co-circulation of WNV and USUV is poorly understood and may threaten public health in the future.[5] A small proportion of WNV and USUV infections in humans results in severe neurological complications and even death, yet there are no efficient antiviral drugs or vaccine candidates available for preventing or treating these virus infections. The emergence of WNV and USUV in Europe thus poses a significant increased risk to public health. However, in the event of an outbreak, the only currently available tools to reduce infections with these viruses are mosquito control interventions.

Vector control interventions effective against *Culex* mosquitoes include larval source reduction (such as environmental management and removal of breeding sites), larviciding using chemical or biological agents, and personal protection methods such as repellents and mosquito-proofing homes.[6] Adulticiding by fogging is the first-line choice during emergency situations as this method is able to rapidly reduce the infectious female mosquito population.[6] The insecticides available for use in public health are pyrethroids, organophosphates, carbamates, and organochlorides. Because of long-term safety issues, pyrethroids remain the only chemical class allowed for mosquito adulticide in Europe.

Exposure to insecticides in vector control or agricultural pest control can result in the development of insecticide resistance in mosquitoes. The main mechanisms responsible for insecticide resistance in insects include target gene mutations, resulting in amino acid alterations leading to permanent changes in the insecticide target site,[7] increased metabolic activity of enzymes involved in insecticide

58 detoxification,[8] and reduced cuticular penetration of insecticides. Voltage-gated sodium channels
59 allow the passage of sodium ions across the plasma membrane of axons to initiate and propagate
60 action potentials (electrical impulses) which are necessary for controlling insect movement and
61 reaction to stimuli.[9] Normally when mosquitoes get in contact with pyrethroids and DDT, these
62 molecules bind to sodium channels and prolong their opening, thereby disrupting nerve function,
63 inducing paralysis, knockdown and eventually death. *Knockdown resistance*, or *kdr*, is caused by
64 certain amino acid substitutions in the sodium channel which reduce the sensitivity of these channels
65 to pyrethroids and DDT, thus preventing paralysis and knockdown.[10] Substitution L1014F (due to a
66 single point mutation at position 1014) in the domain II S6 of the sodium channel is most commonly
67 associated with *kdr* in *Culex* mosquitoes.[11] On the other hand, the target site for organophosphates
68 and carbamates is the serine residue of the acetylcholinesterase (AChE). In the insect central nervous
69 system, AChE catalyses the hydrolysis of the neurotransmitter acetylcholine to terminate nerve
70 impulses.[8] AChE insensitivity is caused by mutations in the ace-1 gene, resulting in substitutions such
71 as G119S, which reduce the sensitivity of AChE to insecticides in *Culex* and *Anopheles* mosquitoes [12].

72 The widespread distribution of long-lasting insecticidal bed nets for malaria control has contributed
73 to the rapid rise and spread of highly insecticide-resistant *Anopheles* mosquitoes in Africa.[13] As a
74 result, the efficacy of key malaria control interventions was significantly reduced, creating the need
75 for novel combinations of insecticides with differing modes of action to regain the effectiveness of
76 long-lasting insecticidal nets. The rapid rise in resistance in *Anopheles* mosquitoes highlighted the
77 importance of insecticide resistance monitoring in mosquito vectors. The need for increased
78 monitoring and surveillance of European mosquitoes was demonstrated recently in 2016 when
79 pyrethroid resistance was detected in *Aedes albopictus* from Italy, threatening the effectiveness of
80 outbreak response interventions.[14] Another justification for monitoring insecticide resistance in
81 mosquitoes is the potential impact of resistance mutations on the efficiency of pathogen transmission
82 (i.e. vector competence). In *Culex quinquefasciatus* mosquitoes it was shown that the presence of one
83 homozygous resistance mutation significantly increased WNV dissemination in the mosquito body,
84 resulting in a higher transmission efficiency than the susceptible controls.[15] This phenomenon is poorly
85 understood, and the impact of insecticide adaptations on the vector competence for arboviruses has
86 so far only been investigated for three arboviruses including WNV, Zika virus and dengue virus in
87 distinct mosquito species.[15–17]

88 Native mosquito species in Belgium include members of the *Culex pipiens* species complex (*Culex*
89 *pipiens* biotype *pipiens* and *Culex pipiens* biotype *molestus*), *Culex torrentium*, and *Culex modestus*.[18,19]
90 To date, there is no information regarding the presence of insecticide resistance in mosquito species
91 from Belgium or neighbouring countries. In this study, we investigated the presence of two common

92 insecticide resistance mechanisms, *kdr* L1014F and *ace-1* G119S, in *Culex pipiens* biotype *pipiens*, *Culex*
93 *torrentium*, and *Culex modestus* mosquitoes from Belgium. Mosquitoes were sampled in three distinct
94 environmental habitats (urban, peri-urban, and agricultural) mainly in and around the city of Leuven
95 from 2019 to 2021. The insecticide resistance mutations reported here will provide more insights in
96 proper insecticide use for vector control in Belgium.

97 ## 2. MATERIALS AND METHODS

98 **2.1 Ethics statement**

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

103 **2.2 Mosquito collection**

104 Collections were performed from August to the beginning of October in 2019, 2020 and 2021, when
105 the weather was adequate, without strong wind or heavy rain. Adult mosquitoes were collected using
106 BG-Sentinel traps (Biogents® AG, Regensburg, Germany) baited with dry ice for CO_2 production and
107 BG-lure (Biogents® AG, Regensburg, Germany) to simulate attractive host scent. The traps were
108 emptied and repositioned between sunrise and sunset of the next day. All insects falling in the traps
109 were transported to the insectary facility at the Rega Institute, KU Leuven. Mosquitoes were
110 anesthetized and sorted on dry ice and then separated to genus level based on morphological
111 characteristics. Mosquitoes were stored individually at -80°C until further processing. Ten traps were
112 rotated between four collection sites: **Botanic Garden of Leuven** (N 50°52'41, E 4°41'21, urban
113 habitat), **Arenberg Park of KU Leuven** (N 50°51'46, E 4°41'01, peri-urban habitat), **Bertem** (N 50°51'57,
114 E 4°37'53, agricultural habitat), three locations in Leuven or its surrounding area, and **Mechelen** (N
115 51°02'34, E 4°29'08, peri-urban habitat) another Belgian city approximately 30 km from Leuven.

116 **2.3 DNA extraction & mosquito identification**

117 ***DNA extraction**:* Following morphological identification, mosquitoes were transferred to tubes with
118 2.8 mm ceramic beads (Precellys®, Bertin, USA) and 500 µl phosphate-buffered saline (1X PBS) solution
119 and homogenised with a Precellys Evolution homogeniser at 2 cycles of 6800 rpm for 10s with a pause
120 of 20s. The homogenate was lysed at 100°C for 10min, followed by centrifugation at 10,000 rpm for
121 1min to spin down the tissue debris, and 150 µl of supernatant was transferred to a new tube. DNA
122 extraction was performed using the QIAamp DNA kit (Qiagen®, Hilden, Germany) according to the
123 manufacturer's protocol.

124 ***Mosquito identification**: Culex pipiens* biotype *pipiens* and *Culex pipiens* biotype *molestus* were
125 distinguished with a multiplex qRT-PCR using primers and probes described previously.[20] The biotyping
126 qRT-PCR was confirmed on a subset of samples using single sequencing of the mosquito cytochrome
127 oxidase 1 (COX1) gene. A 25 µl reaction volume was prepared for each reaction using the Low ROX
128 One-Step qRT-PCR 2X MasterMix kit (Eurogentec®, Seraing, Belgium) following the manufacturer's

129 instructions. The cycle conditions were as follows: initial denaturation at 95°C for 10min, 40 cycles of
130 denaturation at 94°C for 40s, elongation at 48°C for 1min, and extension at 72°C for 1min, and a final
131 hold stage at 72°C for 2min. *Culex modestus* and *Culex torrentium* mosquitoes were identified
132 morphologically using the key of Becker[21] and then by COX1 Sanger sequencing.[22]

133 **2.4 PCR & gel electrophoresis**

134 ***Detection of kdr L1014F (mutation from TTA to TTT)***: this protocol was adapted from Martinez-Torres
135 et al.[23] Briefly, two PCR reactions were run in parallel using 4 primers: Cgd1 (5'-
136 GTGGAACTTCACCGACTTC-3'), Cgd2 (5' GCAAGGCTAAGAAAAGGTTAAG-3'), Cgd3 (5'-
137 CCACCGTAGTGATAGGAAATTTA-3') and Cgd4 (5'-CCACCGTAGTGATAGGAAATTTT-3'). A 20 µl reaction
138 volume was prepared for each reaction using the GoTaq® Green Master 2X Mix (M7122, Promega,
139 Belgium) following the manufacturer's instructions. The first reaction contained the primers Cgd1,
140 Cgd2 and Cgd3 to identify the presence of the L1014 susceptible allele, and the second reaction
141 consisted of Cgd1, Cgd2 and Cgd4 to identify the presence of the F1014 resistant allele. Taken
142 together, the results of both reactions were used to determine the genotype for each individual as
143 susceptible homozygote (L/L: SS), resistant heterozygote (L/F: RS), or resistant homozygote (F/F: RR).
144 The cycle conditions were as follows: initial denaturation at 94°C for 2 min, 40 cycles of denaturation
145 at 94°C for 30s, elongation at 48°C for 30s, and extension at 72°C for 1 min, and a final hold stage at
146 72°C for 10min. Amplicons were separated by electrophoresis on 1.5% agarose gel and were visualised
147 by Midori green staining under UV light with FAS-V Imaging System.

148 ***Detection of ace-1 G119S (mutation from GGC to AGC)*** *:* This analysis was performed following the
149 protocol described by Weill *et al*. [12] with minor modifications. The DNA block was amplified using the
150 primers CxEx3dir (5'-CGACTCGGACCCACTGGT-3') and CxEx3rev (5'-GTTCTGATCAAACAGCCCCGC-3')
151 and a 20 µl reaction volume was prepared for each reaction using the GoTaq® Green Master 2X Mix
152 (M7122, Promega, Belgium) following the manufacturer's instructions. The cycle conditions were as
153 follows: initial denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 30s, elongation at
154 54°C for 30s, and extension at 72°C for 1 min, with a final hold stage at 72°C for 10min. The amplicons
155 were digested with AluI restriction enzyme (Jena Science, Sapphire, USA) following the manufacturer's
156 instructions and separated on a 2% agarose gel. The products were visualised by Midori green staining
157 under UV light with FAS-V Imaging System. The results were used to determine the genotype for each
158 individual as susceptible homozygote (G/G: SS), resistant heterozygote (G/S: RS), or resistant
159 homozygote (S/S: RR).

160 **2.5 Statistical analysis**

161 For the distribution of genotypic and allelic frequencies resulting in the *kdr* L1014F or *ace-1* G119S,
162 analysis was performed among different collecting sites, years, and species by GraphPad Prism 9
163 (V.9.3). A Hardy–Weinberg equilibrium (HWE) of the allelic frequencies was evaluated using the
164 following equation on the data from *Culex pipiens* harvested from 2019 to 2021. The X^2 and *p*-values
165 were calculated with GraphPad Prism (V.9.3). A *p*-value of <0.05 was considered statistically
166 significant.

167 R allelic frequency= $= \frac{\mathrm{RS+2RR}}{2(\mathrm{RS+RR+SS})}$;

168 S allelic frequency = 1- R allelic frequency

169 # 3. RESULTS

170 **3.1 Stable presence of mutation leading to the *kdr* L1014F over 3 years**

171 The mutation frequencies at position 1014 of the domain II S6 of the sodium channel of *Culex pipiens*,
172 *Culex modestus* and *Culex torrentium* mosquitoes are presented in Table 1. The most common alleles
173 for all three species was the susceptible L/L, which appeared at a frequency of 56-87% in *Culex pipiens*,
174 57% in *Culex modestus*, and 100% in *Culex torrentium* mosquitoes. The frequency of resistant
175 heterozygosity (L/F) was 9.1-34% in *Culex pipiens* and 43% in *Culex modestus*. Resistant homozygosity
176 (F/F) was the least frequent allele combination with a range of 0-12.5% in *Culex pipiens*. No
177 homozygous resistance genotype was detected in *Culex modestus* or *Culex torrentium*. One hybrid
178 female *Culex pipiens* (hybrid of *pipiens* and *molestus*) and one female *Culex pipiens molestus* were
179 identified in Arenberg Park and Mechelen in 2021.

180 The frequency of resistance genotypes in *Culex pipiens* was consistent over time. The rate of L/F
181 surpassed 30% at the urban collection site (Botanical Garden of Leuven) on a yearly basis and ranged
182 from 10-25% at all other collection sites. In 2019, the rate of L/F in *Culex modestus* was surprisingly
183 high (43.1%). Although the majority of mosquitoes captured from 2019 to 2021 were fully susceptible
184 to pyrethroids, we can expect that the use of pyrethroid insecticides in these locations will not be
185 effective against the smaller proportion of resistant mosquitoes and may lead to the further selection
186 of mutations associated with *kdr*.

187 The Hardy-Weinberg equilibrium was used to measure the stability of mutations in the population of
188 *Culex pipiens* from all locations over time (Table 2). The difference between the observed and
189 expected *kdr* L1014F genotype frequencies each year were not significantly different and are
190 therefore expected to remain in equilibrium (constant).

191 **3.2 Stable presence of acetylcholinesterase insensitivity mutation over 3 years**

192 The mutation frequencies in the *ace-1* gene resulting in the G119S amino acid change in *Culex pipiens*,
193 *Culex modestus* and *Culex torrentium* from 2019-2021 are presented in Table 3. In *Culex pipiens*, the
194 rate of heterozygous resistance often surpassed the rate of full susceptibility which was within a range
195 of 25-57%. Heterozygosity was generally consistent over time, with a range of 25-37.5% in the urban
196 habitat at the botanical garden and 53-57% in Bertem. At the peri-urban site (Arenberg Park), the rate
197 of heterozygosity fell from 50% to 27% in 2019 and 2020 but returned to 50% in 2021. Homozygous
198 resistance in *Culex pipiens* was only detected in Mechelen in 2021 (3%). Only a small number of *Culex*
199 *modestus* had any resistance alleles (3.9% heterozygous, Arenberg Park, 2019), and all captured *Culex*
200 *torrentium* had the full susceptibility genotype (100%).

201 The Hardy-Weinberg equilibrium of genotypes in *Culex pipiens* across all collection sites remained
202 constant from 2019 to 2020 (Table 4). However, in 2021 the rates of G/G and S/S genotypes were
203 lower and the rate of G/S heterozygosity was higher than the expected frequencies. Therefore, we
204 may observe a higher rate of *ace-1* G119S heterozygous resistance in *Culex pipiens* in this region in the
205 near future.

206 **3.3 Distribution of L1014F and G119S in *Culex pipiens* collected from different habitat types**

207 In this study, three habitant types including urban (Botanic Garden), peri-urban (Arenberg Park and
208 Mechelen) and agricultural (Bertem) habitats were chosen for mosquito collections. For L1014F *kdr*,
209 L/L (susceptible homozygote) was most dominant (>60%) in all collection sites. In Arenberg Park, L/F
210 (resistant heterozygote) was less frequently observed than in the other two collection sites in Leuven,
211 but this difference was not significant. In addition, no F/F (resistant homozygote) was observed in the
212 agricultural habitat (Bertem) (Figure 1, A). For the *ace-1* G119S, the proportion of G/S (resistant
213 heterozygote) was lower in the urban site (Botanic Garden: 25% - 37.5%) than in the agricultural area
214 (Bertem: 53% - 57%), possibly as the result of chemical products used during agricultural pest control
215 (Figure 1, A).

216 Interestingly, similar proportions of both mutations were observed in the peri-urban area of Mechelen
217 (Figure 1, A and B), a Belgian city approximately 30km from Leuven. Furthermore, the only mosquito
218 harbouring the S/S (resistant homozygote) allele was found in Mechelen. This suggests that insecticide
219 resistance in *Culex* mosquitoes may be widespread, not only in Leuven but in other Belgian cities as
220 well.

221 **3.4 Frequency of combination of L1014F and G119S present in different locations**

222 When combining the two mutations in *Culex pipiens* samples, there are seven possible genotypes that
223 can observed, including 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),
224 F/F-G/G (RRSS) and F/F-G/S (RRRS) (Figure 2). The two genotypes RSRR and RRRR were not identified
225 among the sampled Belgian mosquitoes. The L/L-G/G (SSSS) genotype was the most prevalent (> 40%)
226 in the collections from Botanic Garden, Arenberg Park, and Mechelen. However, in Bertem, the
227 resistant genotypes were likely more established, with the L/L-G/S (SSRS) genotype surpassing the L/L-
228 G/G (SSSS) genotype in frequency, but not significant compared to other locations (Figure 2). In *Culex*
229 *modestus,* resistant homozygotes were not observed. Only four genotypes were found in *Culex*
230 *modestus* including L/L-G/G (SSSS): 53%, L/L-G/S (SSRS): 2%, L/F-G/G (RSSS): 43%, and L/F-G/S (RSRS):
231 2%. In *Culex torrentium*, only L/L-G/G (SSSS) was detected. Interestingly, *Culex pipiens, Culex modestus*

232 and *Culex torrentium* mosquitoes collected in the same location of Arenberg Park were found to have
233 different genotypes.

234 ## 4. DISCUSSION

235 Field mosquitoes were collected in Leuven and surrounding areas in urban (Botanic Garden), peri-
236 urban (Arenberg Park) and agricultural (Bertem) habitats during the summer months from 2019 to
237 2021. *Culex pipiens* was the dominant species across all collection sites, a finding consistent with
238 previous research conducted in this area[19]. The majority of *Culex pipiens* were biotype *pipiens*.
239 However, one hybrid female *Culex pipiens* (hybrid of *pipiens* and *molestus*) and one female *Culex*
240 *pipiens molestus* were identified in Arenberg Park and Mechelen in 2021, respectively. *Culex pipiens*
241 biotype *pipiens* and biotype *molestus* can present genetic, behavioural and physiological differences.
242 More importantly, the vector competence can vary significant between two biotypes; therefore, the
243 identification of the subspecies level is important to assess virus transmission by field *Culex pipiens*
244 mosquitoes.[24]

245 The mutations resulting in *kdr* L1014F and *ace-1* G119S resistance were previously found in *Culex*
246 *pipiens* in Morocco,[25] Greece,[26] Turkey,[27] California (USA)[28] and China[29]. *Culex pipiens* sampled from
247 three diverse locations in Greece had a L1014F mutation resistant frequency of 97% (40%
248 heterozygous L/F and 57% homozygous resistant F/F), indicating intense levels of pyrethroid
249 resistance in the country.[30] In contrast, the same study observed frequencies of G119S and F290V *ace-*
250 *1* amino acid changes in 14% and <1% of the mosquitoes, respectively, demonstrating very low
251 resistance levels to organophosphates and carbamates. Here, mutation frequency (heterozygous and
252 homozygous resistant) in *Culex pipiens* mosquitoes from Belgium, we report around 13 - 44% for
253 L1014F and 25 - 50% for G119S, respectively. It is evident that allele frequencies are location-
254 dependent and can arise rapidly in mosquito populations given the right selection pressures. In areas
255 with competent vectors and known/suspected arbovirus circulation, it is important to distinguish the
256 types and degrees of insecticide resistance to inform evidence-based decision-making regarding the
257 use of vector control. In most European countries however, the insecticide resistance profile of *Culex*
258 mosquitoes is an important knowledge gap and potential barrier to effective vector control.

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

265 The mutation for *ace-1* G119S was observed at a higher frequency in the heterozygous state compared
266 to the homozygous state. Only one female with the S/S resistant homozygote genotype was found,

267 similar to the findings from Greece[26]. We hypothesize that the high frequency of the *ace-1* G119S
268 heterozygous resistant genotype in *Culex pipiens* is due to the high fitness costs associated when the
269 mutation is present in the homozygote form, which is also suggested by the observation that across
270 different studies, mosquitoes with the S/S homozygote genotype were rarely reported.[31] Also under
271 lab conditions a significantly higher mortality was observed in *Anopheles gambiae* pupae with the S/S
272 (homozygote) mutation compared to their susceptible counterparts (G/G).[32] As this cost is alleviated
273 when present in the heterozygote form, repeated selection may lead to a permanent state of
274 heterozygosity in *Culex* populations.[33] Another mutation leading to the F290V amino acid substitution
275 was reported to be associated to AChE insensitivity as well, warranting further investigation.[34]

276 A limitation to our study was the small sample size which did not allow us to perform insecticide
277 bioassays. The detection of these two resistance genotypes do not represent all potential resistance
278 mechanisms that may be involved, as well as the degree of phenotypic resistance in these
279 mosquitoes.[35] However, the previously published correlation between mutations leading to *kdr* and
280 phenotypic resistance to pyrethroids in *Culex pipiens pipiens* suggests that there is probably some
281 degree of resistance to pyrethroids in a proportion of Belgian *Culex pipiens* mosquitoes with the
282 mutation reported by this research.[36] In comparison with *Culex pipiens* from Morocco (with 42%
283 frequency of resistant alleles, showing resistance levels 33 times higher than the susceptible strain to
284 permethrin by WHO Bioassay), the *Culex pipiens* from Belgium (with approximately 20% frequency of
285 resistant alleles) is expected to have a resistance level to permethrin that is much lower than the
286 resistance level of the Moroccan mosquitoes.[37]

287 Importantly, the co-occurrence of *kdr* L1014F and *ace-1* G119S indicated that multi- or cross-resistant
288 mosquitoes could be developing in our study area, which may cause an inefficiency of pyrethroid and
289 organophosphate insecticides if used for mosquito control in Belgium. Further measurement of the
290 metabolic activity of enzymes (such as cytochrome p450, esterase and acetylcholinesterase) involved
291 in insecticide detoxification via transcriptomic[38] or proteomic methods is warranted to better
292 understand the extent of mosquito insecticide resistance in Belgium.[39]

293 In the Flanders region of Belgium, a total of 2.4 tons of pesticides were reported to be used in 2020.[40]
294 The majority of pesticide use (98%) consisted of herbicides for plant control, of which glyphosate was
295 the most common active ingredient (67%). This was followed by 2,4-dichlorphenoxyacetic acid (2,4-
296 D; 17%), triclopyr (7%), 2-methyl-4-chlorophenoxyacetic acid (MCPA; 4%), and *Bacillus thuringiensis*
297 (2%). The strong use of glyphosate, an organophosphate, may explain the development of *ace-1*
298 G119S resistance in the *Culex pipiens* observed in our study. It was shown that at field-realistic doses,
299 the presence of glyphosate can impair the learning ability of *Aedes aegypti* larvae[41] as well as modify

300 the life history traits and increase insecticide resistance in *Anopheles arabiensis*.[42] In addition, it was
301 shown that 2,4-D and MCPA can affect AChE sensitivity in humans;[43] therefore, the development of
302 *ace-1* resistance in Belgian *Culex* mosquitoes may also be due to the selection pressures caused by
303 2,4-D and MCPA. The use of insecticides for insect control in Belgium represented 2% of all pesticide
304 use, specifically for the control of wasps and oak processionary caterpillars. The use of insecticides
305 against mosquitoes was reported in only one location: a highway on the French border to prevent the
306 entry of *Aedes* mosquitoes. The most common site for the application of pesticides was open
307 pavement (78%), followed by woody vegetation (13%), and sports fields (5%). In Belgian botanical
308 gardens, the use of fungicides, acaricides and rare pesticides were also reported but not specified.

309 Aside from chemical pesticides, there is evidence that natural plant chemicals and microbes can confer
310 insecticide resistance in mosquitoes.[44,45] A common botanical pesticide is pyrethrum which, although
311 derived from natural sources, shares a similar structure and mode of action with synthetic pyrethroid
312 insecticides.[46] Other potential environmental contributors to resistance include natural xenobiotics
313 (allelochemicals), industry pollutants, and domestic insecticides. However, the extent to which
314 mosquitoes in their adult or immature developmental stages may be exposed to natural pesticides is
315 unclear.

316 There is little evidence on the efficacy and cost-effectiveness of vector control interventions in
317 Europe.[6] Over recent decades, the European Union has been gradually banning the use of insecticides
318 for agriculture, including the pyrethroid permethrin and the organophosphate malathion. In Flanders,
319 there has been a consistent decline in the overall use of pesticides. Since 2010, the quantity of active
320 ingredients has fallen from 15.7 to 2.4 tons in 2020.[40] It is possible that a continuous decline of
321 pesticide use may reduce the selection pressure on mosquitoes to develop insecticide resistance.
322 However, the limited use of a small number of active ingredients and the high reliance on glyphosate
323 may facilitate the development of insecticide resistance in mosquitoes if they are being exposed
324 regularly at low concentrations.[42] As we do not expect the frequency of insecticide resistance in these
325 mosquito populations to decline, we recommend that insecticide resistance levels of mosquitoes are
326 monitored at a larger scale in Belgium and in neighbouring countries as well.

327 **Conclusion**

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

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Conflict of Interest Statement

No conflicts of interest to declare.

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465 Tables

466 **Table 1. Frequency of *kdr* L1014F alleles in *Culex* mosquitoes collected in Leuven, Belgium from 2019**
467 **to 2021.**

			Culex pipiens				*Culex modestus*				*Culex torrentium*		
Year	Site	N	L/L% (n)	L/F% (n)	F/F% (n)	N	L/L% (n)	L/F% (n)	F/F% (n)	N	L/L% (n)	L/F% (n)	F/F% (n)
2019	AP	8	75 (6)	12.5 (1)	12.5 (1)	51	57 (29)	43 (22)	0 (0)	-	-	-	-
	BG	12	67 (8)	25 (3)	8.3 (1)	-	-	-	-	-	-	-	-
2020	AP	33	73 (24)	24 (8)	0.3 (1)	-	-	-	-	2	100 (2)	0 (0)	0 (0)
	BE	30	77 (23)	23 (7)	0 (0)	-	-	-	-	-	-	-	-
	BG	32	56 (18)	34 (11)	9.4 (3)	-	-	-	-	4	100 (4)	0 (0)	0 (0)
2021	AP	30	87 (26)	10 (3)	3.3 (1)	-	-	-	-	-	-	-	-
	BE	30	63 (19)	37 (11)	0 (0)	-	-	-	-	-	-	-	-
	BG	28	64 (18)	29 (8)	7.1 (2)	-	-	-	-	2	100 (2)	0 (0)	0 (0)
	ME	33	85 (28)	9.1 (3)	6.0 (2)	-	-	-	-	-	-	-	-

468 Percentage (%) of mosquitoes with L/L (susceptible), L/F (heterozygous resistant) or F/F (homozygous
469 resistant) *kdr* genotypes. N: total mosquitoes. AP: Arenberg Park; BG: Botanic Garden; BE: Bertem;
470 ME: Mechelen.

471 **Table 2. Hardy-Weinberg equilibrium of *kdr* L1014F alleles in *Culex pipiens*.**

	N observed			N expected			X^2, *p*-value
Year	**L/L**	**L/F**	**F/F**	**L/L**	**L/F**	**F/F**	
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

472 N observed: total mosquitoes captured (all locations grouped). N expected: expected number of
473 mosquitoes based on the Hardy-Weinberg model. Mosquitoes were detected with L/L (susceptible),
474 L/F (heterozygous resistant) or F/F (homozygous resistant) *kdr* genotypes. The X^2 and *p*-values were
475 calculated with GraphPad Prism (V.9.3). A *p*-value of <0.05 was considered statistically significant.

476 **Table 3. Frequency of G119S alleles in *Culex* mosquitoes collected in Leuven, Belgium in 2019-**
477 **2021.**

			Culex pipiens				*Culex modestus*				*Culex torrentium*		
Year	Site	N	G/G% (n)	G/S% (n)	S/S% (n)	N	G/G% (n)	G/S% (n)	S/S% (n)	N	G/G% (n)	G/S% (n)	S/S% (n)
2019	AP	8	50 (4)	50 (4)	0 (0)	51	96 (49)	3.9 (2)	-	-	-	-	-
	BG	12	75 (9)	25 (3)	0 (0)	-	-	-	-	-	-	-	-
2020	AP	33	73 (24)	27 (9)	0 (0)	-	-	-	-	2	100 (2)	0 (0)	0 (0)
	BE	30	47 (14)	53 (16)	0 (0)	-	-	-	-	-	-	-	-
	BG	32	62.5 (20)	37.5 (12)	0 (0)	-	-	-	-	4	100 (4)	0 (0)	0 (0)
2021	AP	30	50 (15)	50 (15)	0 (0)	-	-	-	-	-	-	-	-
	BE	30	43 (13)	57 (17)	0 (0)	-	-	-	-	-	-	-	-
	BG	28	75 (21)	25 (7)	0 (0)	-	-	-	-	2	100 (2)	0 (0)	0 (0)
	ME	33	64 (21)	33 (11)	3.0 (1)	-	-	-	-	-	-	-	-

478 Percentage (%) of mosquitoes with G/G (susceptible), G/S (heterozygous resistant) or S/S
479 (homozygous resistant) *AChE* genotypes. N: total mosquitoes. AP: Arenberg Park; BG: Botanic Garden;
480 BE: Bertem; ME: Mechelen.

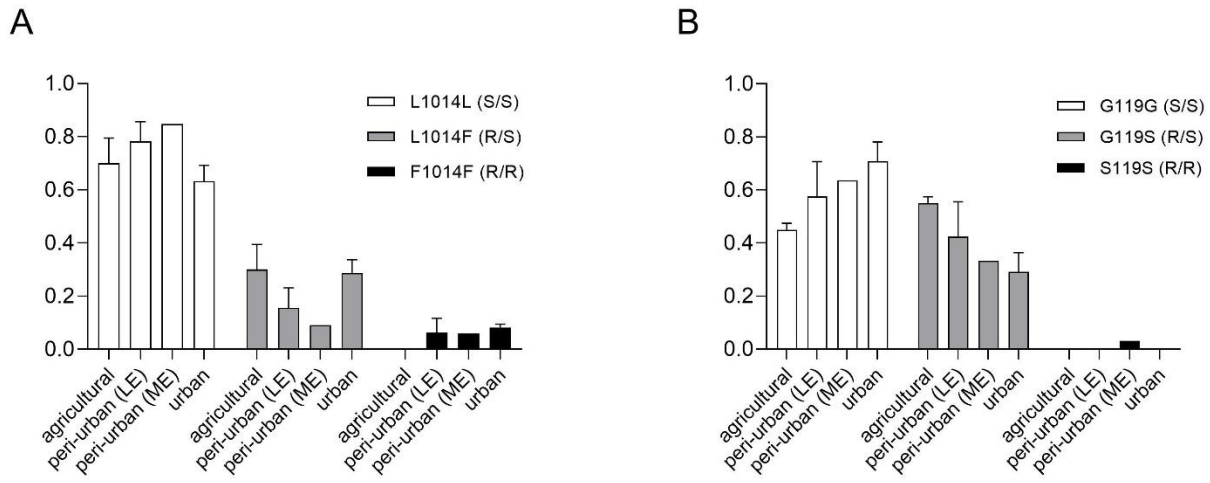
481 **Table 4. Hardy-Weinberg equilibrium of *G119S* alleles in *Culex pipiens*.**

	N observed			N 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*

482 N observed: total mosquitoes captured (all locations grouped). N expected: expected number of
483 mosquitoes based on the Hardy-Weinberg model. The X^2 and *p*-values were calculated with GraphPad
484 Prism (V.9.3). Mosquitoes were detected with G/G (homozygous susceptible), G/S (heterozygous
485 resistant) or S/S (homozygous resistant) *AChE* genotypes. The X^2 and *p*-values were calculated with
486 GraphPad Prism (V.9.3). A *p*-value of <0.05 was considered statistically significant, (* = p <0.05).

487 Figures

488 **Figure 1. Distribution of *kdr* L1014F and *ace*-1 G119S in mosquitoes from different locations.** (A)
489 Prevalence of mosquitoes with L/L (homozygous susceptible; S/S), L/F (heterozygous resistant; R/S) or
490 F/F (homozygous resistant; R/R) *kdr* genotypes. (B) Prevalence of mosquitoes with G/G (homozygous
491 susceptible; S/S), G/S (heterozygous resistant; R/S) or S/S (homozygous resistant; R/R) *AChE*
492 genotypes. LE= Leuven, ME=Mechelen.



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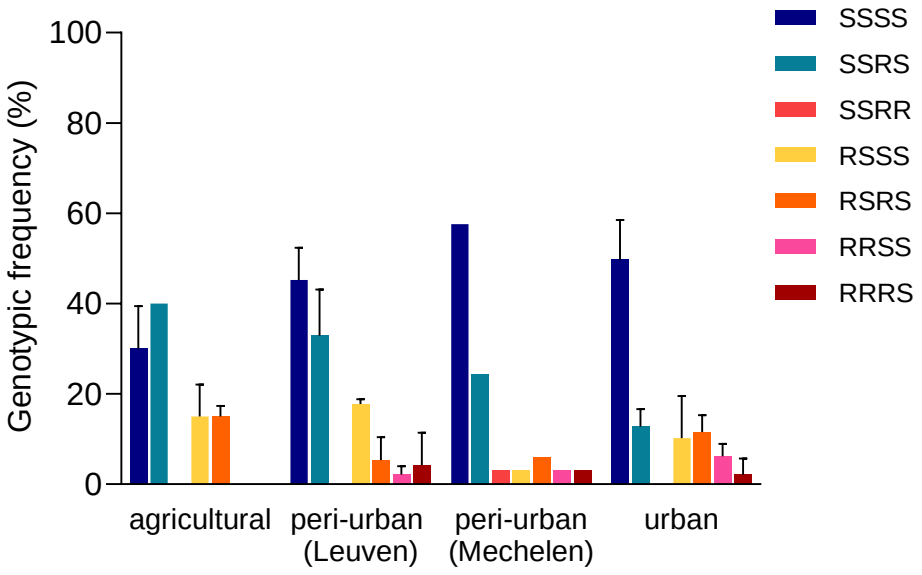
Figure 2. Frequency of combinations of *kdr* L1014F and *ace*-1 G119S in *Culex pipiens* mosquitoes from different locations. A four-letter abbreviation was used to designate the combinations of genotypes: the first two letters refer to *kdr* L1014F and the last two refer to *ace*-1. SS susceptible genotypes,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