1 First report of *kdr* L1014F and *ace-1* G119S insecticide resistance in 2 Belgian *Culex* mosquitoes

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

4 Lanjiao Wang^{1#}, Alina Soto¹, Laure Remue¹, Ana Lucia Rosales Rosas¹, Lander De Coninck², Sam
5 Verwimp¹, Johanna Bouckaert¹, Mathias Vanwinkel¹, Jelle Matthijssens², 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.

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

Address correspondence to Prof. Leen Delang, leen.delang@kuleuven.be and Dr. Lanjiao Wang, wang.lanjiao@kuleuven.be

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

detoxification,⁸ and reduced cuticular penetration of insecticides. Voltage-gated sodium channels allow the passage of sodium ions across the plasma membrane of axons to initiate and propagate action potentials (electrical impulses) which are necessary for controlling insect movement and reaction to stimuli.⁹ Normally when mosquitoes get in contact with pyrethroids and DDT, these molecules bind to sodium channels and prolong their opening, thereby disrupting nerve function, inducing paralysis, knockdown and eventually death. *Knockdown resistance*, or *kdr*, is caused by certain amino acid substitutions in the sodium channel which reduce the sensitivity of these channels to pyrethroids and DDT, thus preventing paralysis and knockdown.¹⁰ Substitution L1014F (due to a single point mutation at position 1014) in the domain II S6 of the sodium channel is most commonly associated with *kdr* in *Culex* mosquitoes.¹¹ On the other hand, the target site for organophosphates and carbamates is the serine residue of the acetylcholinesterase (AChE). In the insect central nervous system, AChE catalyses the hydrolysis of the neurotransmitter acetylcholine to terminate nerve impulses.⁸ 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* mosquitoes¹².

The widespread distribution of long-lasting insecticidal bed nets for malaria control has contributed to the rapid rise and spread of highly insecticide-resistant *Anopheles* mosquitoes in Africa.¹³ 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 effectiveness of long-lasting insecticidal nets. The rapid rise in resistance in *Anopheles* mosquitoes highlighted the importance of insecticide resistance monitoring in mosquito vectors. The need for increased monitoring and surveillance of European mosquitoes was demonstrated recently in 2016 when pyrethroid resistance was detected in *Aedes albopictus* from Italy, threatening the effectiveness of outbreak response interventions.¹⁴ Another justification for monitoring insecticide resistance in mosquitoes is the potential impact of resistance mutations on the efficiency of pathogen transmission (i.e. vector competence). In *Culex quinquefasciatus* mosquitoes it was shown that the presence of one homozygous resistance mutation significantly increased WNV dissemination in the mosquito body, resulting in a higher transmission efficiency than the susceptible controls.¹⁵ This phenomenon is poorly understood, and the impact of insecticide adaptations on the vector competence for arboviruses has so far only been investigated for three arboviruses including WNV, Zika virus and dengue virus in distinct mosquito species.^{15–17}

Native mosquito species in Belgium include members of the *Culex pipiens* species complex (*Culex pipiens* biotype *pipiens* and *Culex pipiens* biotype *molestus*), *Culex torrentium*, and *Culex modestus*.^{18,19} 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

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.

2. MATERIALS AND METHODS

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 the University of Leuven.

2.2 Mosquito collection

Collections were performed from August to the beginning of October in 2019, 2020 and 2021, when the weather was adequate, without strong wind or heavy rain. Adult mosquitoes were collected using BG-Sentinel traps (Biogents® AG, Regensburg, Germany) baited with dry ice for CO₂ production and BG-lure (Biogents® AG, Regensburg, Germany) to simulate attractive host scent. The traps were emptied and repositioned between sunrise and sunset of the next day. All insects falling in the traps were transported 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 stored individually at -80°C until further processing. Ten traps were rotated between four collection sites: **Botanic Garden of Leuven** (N 50°52'41, E 4°41'21, urban habitat), **Arenberg Park of KU Leuven** (N 50°51'46, E 4°41'01, peri-urban habitat), **Bertem** (N 50°51'57, E 4°37'53, agricultural habitat), three locations in Leuven or its surrounding area, and **Mechelen** (N 51°02'34, E 4°29'08, peri-urban habitat) another Belgian city approximately 30 km from Leuven.

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 10s with a pause of 20s. The homogenate was lysed at 100°C for 10min, followed by centrifugation at 10,000 rpm for 1min 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.

Mosquito identification: *Culex pipiens* biotype *pipiens* and *Culex pipiens* biotype *molestus* were distinguished with a multiplex qRT-PCR using primers and probes described previously.²⁰ The biotyping qRT-PCR was confirmed on a subset of samples using single sequencing of the 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 manufacturer's

instructions. The cycle conditions were as follows: initial denaturation at 95°C for 10min, 40 cycles of denaturation at 94°C for 40s, elongation at 48°C for 1min, and extension at 72°C for 1min, and a final hold stage at 72°C for 2min. *Culex modestus* and *Culex torrentium* mosquitoes were identified morphologically using the key of Becker²¹ and then by COX1 Sanger sequencing.²²

2.4 PCR & gel electrophoresis

Detection of *kdr* L1014F (mutation from TTA to TTT): this protocol was adapted from Martinez-Torres et al.²³ Briefly, two PCR reactions were run in parallel using 4 primers: Cgd1 (5'-GTGGAAGTTCACCGACTTC-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 susceptible allele, and the second reaction consisted of Cgd1, Cgd2 and Cgd4 to identify the presence of the F1014 resistant allele. Taken together, the results of both reactions were used to determine the 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 2 min, 40 cycles of denaturation 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 72°C for 10min. Amplicons were separated by electrophoresis on 1.5% agarose gel and were visualised by Midori green staining under UV light with FAS-V Imaging System.

Detection of *ace-1* G119S (mutation from GGC to AGC) : This analysis was performed following the protocol described by Weill et al.¹² with minor modifications. The DNA block was amplified using the primers CxEx3dir (5'-CGACTCGGACCCACTGGT-3') and CxEx3rev (5'-GTTCTGATCAAACAGCCCCGC-3') and 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 cycle conditions were as follows: initial denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 30s, elongation at 54°C for 30s, and extension at 72°C for 1 min, with a final hold stage at 72°C for 10min. The amplicons were digested with Alul restriction enzyme (Jena Science, Sapphire, USA) following the manufacturer's instructions and separated on a 2% agarose gel. The products were visualised by Midori green staining under UV light with FAS-V Imaging System. The results were used to determine the genotype for each individual as susceptible homozygote (G/G: SS), resistant heterozygote (G/S: RS), or resistant homozygote (S/S: RR).

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 (HWE) of the allelic frequencies was evaluated using the following equation on the data from *Culex pipiens* harvested from 2019 to 2021. The χ^2 and p -values were calculated with GraphPad Prism (V.9.3). A p -value of <0.05 was considered statistically significant.

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

$$S \text{ allelic frequency} = 1 - R \text{ allelic frequency}$$

3. RESULTS

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

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 alleles for all three species was the susceptible L/L, which appeared at a frequency of 56-87% in *Culex pipiens*, 57% in *Culex modestus*, and 100% in *Culex torrentium* mosquitoes. The frequency of resistant heterozygosity (L/F) was 9.1-34% in *Culex pipiens* and 43% in *Culex modestus*. Resistant homozygosity (F/F) was the least frequent allele combination with a range of 0-12.5% in *Culex pipiens*. No homozygous resistance genotype was detected in *Culex modestus* or *Culex torrentium*. One hybrid female *Culex pipiens* (hybrid of *pipiens* and *modestus*) and one female *Culex pipiens modestus* were identified in Arenberg Park and Mechelen in 2021.

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

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). 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).

3.2 Stable presence of acetylcholinesterase insensitivity mutation 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*, the rate of heterozygous resistance often surpassed the rate of full susceptibility which was within a range of 25-57%. Heterozygosity was generally consistent over time, with a range of 25-37.5% in the urban habitat at the botanical garden and 53-57% in Bertem. At the peri-urban site (Arenberg Park), the rate of heterozygosity fell from 50% to 27% in 2019 and 2020 but returned to 50% in 2021. Homozygous resistance in *Culex pipiens* was only detected in Mechelen in 2021 (3%). Only a small number of *Culex modestus* had any resistance alleles (3.9% heterozygous, Arenberg Park, 2019), and all captured *Culex torrentium* had the full susceptibility genotype (100%).

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.

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

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

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

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

When combining the two mutations in *Culex pipiens* samples, there are seven possible genotypes that can be 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), F/F-G/G (RRSS) and F/F-G/S (RRRS) (Figure 2). The two genotypes SSRR and RRRR were not identified among the sampled Belgian mosquitoes. The L/L-G/G (SSSS) genotype was the most prevalent (> 40%) in the collections from Botanic Garden, Arenberg Park, and Mechelen. However, in Bertem, the resistant genotypes were likely more established, with the L/L-G/S (SSRS) genotype surpassing the L/L-G/G (SSSS) genotype in frequency, but not significant compared to other locations (Figure 2). In *Culex modestus*, resistant homozygotes were not observed. Only four genotypes were found in *Culex 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): 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.

4. DISCUSSION

Field mosquitoes were collected in Leuven and surrounding areas in urban (Botanic Garden), peri-urban (Arenberg Park) and agricultural (Bertem) habitats during the summer months from 2019 to 2021. *Culex pipiens* was the dominant species across all collection sites, a finding consistent with previous research conducted in this area¹⁹. The majority of *Culex pipiens* were biotype *pipiens*. However, one hybrid female *Culex pipiens* (hybrid of *pipiens* and *molestus*) and one female *Culex pipiens molestus* were identified in Arenberg Park and Mechelen in 2021, respectively. *Culex pipiens* biotype *pipiens* and biotype *molestus* can present genetic, behavioural and physiological differences. More importantly, the vector competence can vary significant between two biotypes; therefore, the identification of the subspecies level is important to assess virus transmission by field *Culex pipiens* mosquitoes.²⁴

The mutations resulting in *kdr* L1014F and *ace-1* G119S resistance were previously found in *Culex pipiens* in Morocco,²⁵ Greece,²⁶ Turkey,²⁷ California (USA)²⁸ and China²⁹. *Culex pipiens* sampled from three diverse locations in Greece had a L1014F mutation resistant frequency of 97% (40% heterozygous L/F and 57% homozygous resistant F/F), indicating intense levels of pyrethroid resistance in the country.³⁰ In contrast, the same study observed frequencies of G119S and F290V *ace-1* amino acid changes in 14% and <1% of the mosquitoes, respectively, demonstrating very low resistance levels to organophosphates and carbamates. Here, mutation frequency (heterozygous and homozygous resistant) in *Culex pipiens* mosquitoes from Belgium, we report around 13 - 44% for L1014F and 25 - 50% for G119S, respectively. It is evident that allele frequencies are location-dependent and can arise rapidly in mosquito populations given the right selection pressures. In areas with competent vectors and known/suspected arbovirus circulation, it is important to distinguish the types and degrees of insecticide resistance to inform evidence-based decision-making regarding the use of vector control. In most European countries however, the insecticide resistance profile of *Culex* mosquitoes is an important knowledge gap and potential barrier to effective vector control.

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

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

similar to the findings from Greece²⁶. We hypothesize that the high frequency of the *ace-1* G119S heterozygous resistant genotype in *Culex pipiens* is due to the high fitness costs associated when the mutation is present in the homozygote form, which is also suggested by the observation that across different studies, mosquitoes with the S/S homozygote genotype were rarely reported.³¹ Also under lab conditions a significantly higher mortality was observed in *Anopheles gambiae* pupae with the S/S (homozygote) mutation compared to their susceptible counterparts (G/G).³² As this cost is alleviated when present in the heterozygote form, repeated selection may lead to a permanent state of heterozygosity in *Culex* populations.³³ Another mutation leading to the F290V amino acid substitution was reported to be associated to AChE insensitivity as well, warranting further investigation.³⁴

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

Importantly, the co-occurrence of *kdr* L1014F and *ace-1* G119S indicated 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 transcriptomic³⁸ or proteomic methods is warranted to better understand the extent of mosquito insecticide resistance in Belgium.³⁹

In the Flanders region of Belgium, a total of 2.4 tons of pesticides were reported to be used in 2020.⁴⁰ The majority of pesticide use (98%) consisted of herbicides for plant control, of which glyphosate was the most common active ingredient (67%). This was followed by 2,4-dichlorophenoxyacetic acid (2,4-D; 17%), triclopyr (7%), 2-methyl-4-chlorophenoxyacetic acid (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 shown that at field-realistic doses, the presence of glyphosate can impair the learning ability of *Aedes aegypti* larvae⁴¹ as well as modify

the life history traits and increase insecticide resistance in *Anopheles arabiensis*.⁴² In addition, it was shown that 2,4-D and MCPA can affect AChE sensitivity in humans;⁴³ therefore, the development of *ace-1* resistance in Belgian *Culex* mosquitoes may also be due to the selection pressures caused by 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: a highway on 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.^{44,45} A common botanical pesticide is pyrethrum which, although derived from natural sources, shares a similar structure and mode of action with synthetic pyrethroid insecticides.⁴⁶ Other potential environmental contributors to resistance include natural xenobiotics (allelochemicals), industry pollutants, and domestic insecticides. However, the extent to which 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.⁶ 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 2020.⁴⁰ 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.⁴² 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.

Conclusion

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

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338 Conflict of Interest Statement

339 No conflicts of interest to declare.

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Tables

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

Year	Site	N	<i>Culex pipiens</i>			N	<i>Culex modestus</i>			N	<i>Culex torrentium</i>		
			L/L% (n)	L/F% (n)	F/F% (n)		L/L% (n)	L/F% (n)	F/F% (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)	-	-	-	-	-	-	-	-

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

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

Year	N observed			N expected			χ^2 , <i>p</i> -value
	L/L	L/F	F/F	L/L	L/F	F/F	
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

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

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

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

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

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

Year	N observed			N expected			χ^2 , <i>p</i> -value
	G/G	G/S	S/S	G/G	G/S	S/S	
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*

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

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. 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.