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Assessment of pyrethroid resistance and *Wolbachia* prevalence in pathogen-related mosquito species from southwest Germany

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Abstract

The increasing incidence of arboviral diseases in Europe, driven by the expansion of mosquito vectors due to globalisation and global warming, poses a growing threat to public health. Notably, the invasive tiger mosquito *Aedes albopictus*, a primary vector of dengue, has been rapidly expanding its range, with outbreaks becoming more frequent in various parts of the world. Insecticides targeting adult mosquitoes are commonly employed as response and protective measures for vector control, but the effectiveness of such interventions may be undermined by rising insecticide resistance, a phenomenon increasingly reported worldwide. Another promising avenue for vector control is the use of *Wolbachia*, an endosymbiotic bacterium capable of reproductive manipulation in mosquitoes, offering potential for population suppression. We evaluated permethrin (a pyrethroid insecticide) resistance in key mosquito species, including *Aedes* and *Culex*, collected from Germany through generation of LC_{50} curves utilising topical exposure assays. Additionally, the prevalence of *Wolbachia* in these populations was determined via PCR amplification of the 16S rRNA gene, followed by sequencing of selected samples. All *Aedes* populations tested exhibited susceptibility to permethrin, whilst a potential trend toward resistance was observed in the *Culex pipiens* complex, a vector of West Nile virus. However, low numbers of field-caught *Ae. albopictus* and *Ae. japonicus* preclude firm conclusions about resistance in these species. Furthermore, *Wolbachia* was detected across all tested mosquito populations, marking the first recorded presence of *Wolbachia* in *Ae. japonicus*. These findings highlight the continued efficacy of pyrethroids against *Aedes* populations in Germany and underscore the need for ongoing surveillance of insecticide resistance, particularly in *Culex* species. Additionally, the detection of *Wolbachia* in native and invasive mosquito populations opens new avenues for the exploration of biological vector control strategies in Europe. This study provides crucial preliminary data supporting the strategic use of pyrethroids and *Wolbachia* for arboviral outbreak prevention in Germany.

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Keywords

Aedes – *Culex* – vector control – insecticide resistance – *Wolbachia*

1 Introduction

Over the past two decades, Europe has witnessed the increasing emergence and spread of arboviruses, along with their mosquito vectors, some of which were previously restricted to tropical and subtropical regions (Gould and Higgs, 2009). This trend is largely attributed to the establishment of invasive mosquito species, particularly *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1894), which have successfully adapted to temperate climates (Paupy *et al.*, 2009). *Aedes albopictus* is capable of transmitting over 26 different arboviruses and has been linked to several outbreaks across southern Europe (Gossner *et al.*, 2018). A lesser-known invasive *Aedes* species, *Ae.* (*Hulecoeteomyia*) *japonicus* (Theobald, 1901), is also a capable vector (Outammassine *et al.*, 2022; Sardelis and Turell, 2001; Schaffner *et al.*, 2011). Key drivers behind this phenomenon include ongoing globalisation, climate change characterised by rising temperatures and altered precipitation patterns, as well as rapid urbanisation (Gould *et al.*, 2009). Together, these factors have created more favourable habitats for mosquitoes, enabling them to expand their geographical range across the continent. In addition to invasive mosquitoes, native mosquitoes such as *Culex* (*Culex*) *pipiens sensu lato* Linnaeus, 1758 are capable of carrying several viruses including West Nile virus (WNV) (Ciota and Krammer, 2013), and Usutu virus (USUV) (Holicki *et al.*, 2020), whilst *Ae.* (*Aedimorphus*) *vexans* (Meigen, 1830) is a potential vector of WNV and Rift Valley Fever virus (Ndiaye *et al.*, 2016). Consequently, Europe is now facing a heightened threat of vector-borne diseases including dengue (DENV), WNV, Zika virus (ZIKV), and chikungunya virus (CHIKV) (Barrett, 2018; Gossner *et al.*, 2018). This growing risk underscores the urgent need for enhanced vector surveillance, public health preparedness, and the development of effective control strategies to mitigate the spread of these diseases.

In Germany, the *Ae. albopictus* populations have been expanding rapidly in recent years (Becker, 2024). The spread is driven, at least in part, by globalisation, as *Ae. albopictus* is frequently transported to new regions via increased trade and human travel. These factors increase the risk of autochthonous disease pathogen transmission (Becker *et al.*, 2013). Climate change also plays a critical role, with hotter summer months and extreme rainfall accelerating the mosquito's development, shortening generation times, and boosting population size (Becker, 2024). Efforts to prevent the spread of mosquitoes, particularly *Ae. albopictus*, have been implemented in various regions, including the Upper Rhine in southwestern Germany. Here, two key control strategies are employed: *Bacillus thuringiensis* subsp. *israelensis* (Bti), which targets mosquito larvae, and the Sterile Insect Technique (SIT), which reduces mosquito reproduction (Becker *et al.*, 2022). Whilst both methods are effective in curbing mosquito populations, they are not sufficient to control acute arboviral outbreaks (Becker *et al.*, 2022). In Germany, although thus far no autochthonous dengue cases have been reported to date (ECDC, 2024), WNV has been detected (Fynmore *et al.*, 2021; Ziegler *et al.*, 2019) and an outbreak of USUV caused significant mortality among local bird populations (Becker *et al.*, 2017).

Another common approach for mosquito control is the use of pyrethroids, a class of fast-acting insecticides widely deployed for vector control, particularly in efforts to combat malaria (WHO, 2023) and arboviruses in south and central America and Asia (Van den Berg, 2021; WHO, 2024). In several European countries, pyrethroids have been employed to manage mosquito populations (Bonds, 2012; Flacio *et al.*, 2015; Scholte *et al.*, 2010). However, their use for vector control in Germany is currently prohibited, with exceptions potentially allowed during acute arboviral outbreaks (Nationale Expertkommission für Stechmücken, 2022). Despite this restriction, biocidal products containing pyrethroids, are available for public use in the form of insecticide sprays (ECHA, 2024).

The extensive use of pyrethroid insecticides worldwide, including in public health and in agricultural use has led to the emergence of resistance in mosquito populations, a phenomenon now observed globally (Scott *et al.*, 2015; Smith *et al.*, 2016) and increasingly reported in Europe (Bengoa *et al.*, 2017; Pichler *et al.*, 2018; Pichler *et al.*, 2022; Vereecken *et al.*, 2022). Pyrethroid resistance has been documented in *Ae. albopictus* populations in Italy with potential resistance detected in field populations from Greece (Pichler *et al.*, 2018). Additionally, *Cx. pipiens* populations in Italy and Belgium have shown resistance to pyrethroids (Bengoa *et al.*, 2017; Pichler *et al.*, 2018, 2022; Vereecken *et al.*, 2022) However, the status of permethrin resistance in mosquito species relevant to Germany remains largely unknown, warranting further investigation.

In addition to insecticide use, an innovative strategy for vector control is the use of the endosymbiotic bacterium *Wolbachia*, which naturally infects up to 40% of arthropod species, including many mosquito vectors (Zug and Hammerstein, 2012). *Wolbachia* manipulates mosquito reproduction through a process known as cytoplasmic incompatibility (Wang *et al.*, 2022) and has also been shown to affect the transmission of arboviruses (Bian *et al.*, 2010; Bourtzis *et al.*, 2014; Van den Hurk *et al.*, 2012). *Aedes albopictus* is commonly infected with two *Wolbachia* strains, *w*AlbA and *w*AlbB (Zhou *et al.*, 1998), whilst the *Cx. pipiens* complex carries the *w*Pip strain (Atyame *et al.*, 2011). *Wolbachia* has also been detected in *Ae. vexans* (Wiwatanaratanabutr, 2013), but so far, no *Wolbachia* infection has been found in *Ae. japonicus* (Huber *et al.*, 2014). The use of *Wolbachia* for population suppression and to reduce virus transmission presents a promising avenue for vector control in Europe, based on successful use in Australia, Brazil and the USA (Hoffmann *et al.*, 2014; Mains *et al.*, 2016; Pinto *et al.*, 2021).

In this study we determine the resistance status of local mosquito vectors including *Ae. albopictus, Ae. vexans, Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus* compared to well characterised insecticide susceptible and resistant mosquitoes from the African *An.* (*Anopheles*) *gambiae* Giles, 1902 complex. We further explore the *Wolbachia* prevalence and sequence diversity in these local mosquitoes.

2 Materials and methods

Collection sites of field mosquitoes

Adult and immature stages of the field mosquitoes were collected in the summer of 2023 in Baden-Württemberg, Germany (Figure 1). For the field population of *Ae. albopictus* and *Ae. japonicus*, eggs were collected in September 2023 using ovitraps (black plastic cups, half covered with water and a wooden stick for oviposition) in the

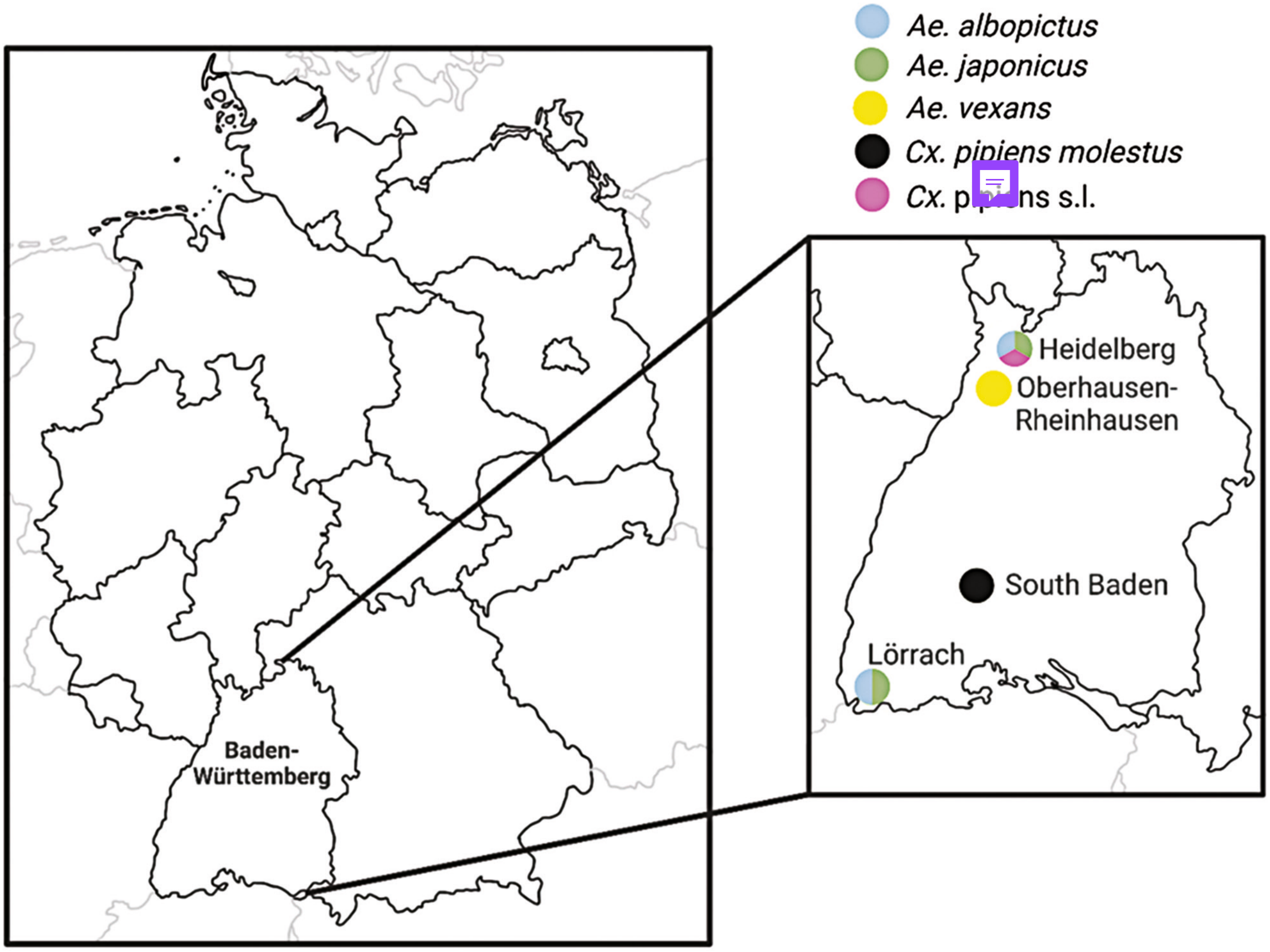

FIGURE 1 Location of mosquito collection sites within Baden-Württemberg, 2023. Blue: *Aedes albopictus*; Green: *Ae. japonicus*; yellow: *Ae. vexans*; black: *Culex pipiens* biotype *molestus*; pink: *Cx. pipiens* biotype *pipiens*. Figure created using BioRender.com.

upper Rhine region (Heidelberg and Lörrach). Larvae of the *Cx. pipiens* biotype *molestus* complex were collected from an old decommissioned septic tank in South Baden in October 2023. *Cx. pipiens* biotype *pipiens* larvae were collected from natural and artificial water containers in Heidelberg (October 2023). Adult *Ae. vexans* mosquitos were collected by CO_2 traps after a flood of the Rhine River in Oberhausen-Rheinhausen (September 2023). In each case, the species was distinguished based on morphological identification keys (Becker *et al.*, 2020). Polymerase chain reaction (PCR) confirmed the identification between the two forms of *Cx. pipiens*, as previously described (Bahnck and Fonseca, 2006).

Colony maintenance of laboratory strains

Two *An. gambiae* complex laboratory strains were used as reference strains for permethrin in this study: an *An. gambiae* susceptible (SUS) strain originally from Kisumu/Kenya; and *An. gambiae s.l.* pyrethroid resistant (RES) strain originally from Tiassalé/Côte d'Ivoire (Williams *et al.*, 2019). The Tiassalé strain was maintained under consistent selection pressure as previously described (Williams *et al*, 2019). Adult *An. gambiae* mosquitoes were sustained through routine blood meals provided by a Hemotek feeder (Hemotek Ltd, UK) with reconstituted human blood. Two laboratory strains of *Ae. albopictus* were also used. The first, originally from Emilia-Romagna, Italy, was maintained on defibrinated swine blood, while the second Heidelberg/Lörrach was maintained on human blood.

Mosquito rearing

The *Cx. pipiens* complex, *Ae. japonicus* and *Ae. albopictus* were reared at Heidelberg University Hospital under ambient conditions and natural light cycles from the beginning of summer to mid-autumn. *Anopheles gambiae* strains were reared at Heidelberg University Hospital in standard insectary conditions (27 °C, 80% humidity, 12:12 light:dark cycle, with one hour dawn and dusk). All mosquitoes were reared in large trays and fed on TetraMin fish food (Tetra, Melle, Germany). Adults were fed on a 10% sucrose solution from emergence. All mosquitoes used in this study are presumed mated.

Insecticide exposures

Topical exposure assays were performed as previously described (Lees *et al.*, 2019) to generate a dose-response curve to estimate the lethal concentration of 50% of the mosquitoes dying (LC_{50}) to permethrin of each mosquito populations (Table 1). For the field populations, the F_0 generations were tested, except for *Cx. pipiens* biotype *molestus* where the F_1 generation was additionally used. Laboratory strains of *Ae. albopictus* ITALY, (F_{24}) and *Ae. albopictus* GERMANY (F_{38}) were tested to determine their resistance status.

For topical application a range from 3–25 female mosquitoes were used for one permethrin concentration; the low numbers in some instances are due to limited availability of the field mosquitoes. For both reference strains and *Ae. albopictus* GERMANY, 20–25 mosquitoes were tested each concentration. The age of the mosquitoes was between two–five days old, except for *Ae. vexans,* which were undetermined as adult field mosquitoes were collected. Concentrations were prepared from a 10% stock solution of permethrin (PESTANAL® analytical standard, Sigma-Aldrich, St. Louis, MO, USA) by serial dilutions with acetone (Thermo Fischer Scientific) and acetone was used for the control. All mosquito populations were anesthetised by CO_2, except for the reference strains which were knocked down by cold at 4 °C. 0.5 µl

TABLE 1 Tested mosquito species and their respective populations and collection site.

Mosquito species	Strain
Ae. albopictus (n= 726)	ITALY, laboratory strain from Emilia-Romagna
Ae. albopictus (n=487)	GERMANY, laboratory strain from Heidelberg
Ae. albopictus (n=37), September 2023	Field population from Heidelberg/Lörrach
Ae. japonicus (n=35), September 2023	Field population from Heidelberg/Lörrach
Ae. vexans (n=597), September 2023	Field population from Oberhausen-Rheinhausen
Cx. pipiens biotype *pipiens* (n=614), October 2023	Field population from Heidelberg
Cx. pipiens biotype *molestus* (n=159), October 2023	Field population from South Baden
An. gambiae s.s., (n=747)	Kisumu, laboratory strain from Kenya
An. gambiae s.l., (n=744)	Tiassalé, laboratory strain from Ivory Coast

of the respective permethrin concentrations were dispensed directly on the surface of the back of the thorax (Lees *et al.*, 2019). Mosquitoes were put back into cups and 10% sucrose solution pads were placed on the top of the net of the cups. The reference mosquito strains and the laboratory *Ae. albopictus* strains were held at standard insectary conditions (27 °C, 80% humidity). All other mosquitoes were left at ambient temperature. Mortality was recorded after 24 h, and mosquitoes were classified as either dead or alive. Mosquitoes considered dead include dead and immobile mosquitoes.

Generation of dose-response curves and calculations of LC50 values

Dose-response curves were produced by a non-linear regression analysis per mosquito population using GraphPad Prism version 10.0.2 (Boston, MA, USA). Lethal doses for 50% with 95% confidence intervals were calculated using GraphPad Prism. Dose-response curves were compared by statistical analysis using extra-sum-of-square F tests in GraphPad Prism. Resistance ratios were calculated by dividing the LC_{50} of each population with the susceptible *An. gambiae* SUS strain.

Screening of **Wolbachia** *prevalence in mosquito populations*

All mosquito populations were tested for *Wolbachia* using polymerase chain reaction, by amplification of the *Wolbachia* specific 16S rRNA gene. Briefly, individual mosquitoes stored at -20 °C were homogenised in 100 µl STE buffer (Sigma-Aldrich) with a pestle. Samples of *Ae. japonicus* were kept in ethanol and were washed with phosphate-buffered saline (PBS) (Sigma-Adrich) before homogenising. Homogenates were incubated at 95 °C for 10 min and centrifugated at 16,000×*g* for 3 min. Each supernatant containing gDNA was removed and stored in a new tube.

The 438 bp fragment of the 16S rRNA gene was amplified in a 25 µl PCR containing 1 µl gDNA, 2.5 µl of 10× DreamTaq Green buffer (1× final concentration; Thermo Fisher Scientific, Waltham, MA, USA), 0.5 µl of 10 nm dNTPs (0.2 mM final concentration; Thermo Fisher Scientific), 0.25 µl DreamTaq polymerase (1 U per reaction, Thermo Fisher Scientific), 18.75 µl UltraPure Distilled Water (Invitrogen, Waltham, MA, USA) and 1 µl each of the forward primer (CATACCTATTCGAAGGGATAG) and reverse primer (AGCTTCGAGTGAAACCAATTC) at 0.4 µM final concentration (Werren and Windsor, 2000). Instead of the 1 µl sample gDNA, 1 µl UltraPure Distilled Water was added for the negative control. For the positive control, 1 µl genomic DNA from *Drosophila melanogaster* known to be *wMel* positive was added, generously donated by Prof Steffen Lemke. PCR was performed under the following thermocycler conditions: initial denaturation at 95 °C for 5 min, followed by 2 cycles of denaturation at 95 °C for 2 min, annealing at 60 °C for 1 min and 72 °C for 1 min. After, 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and 72 °C for 1 min. A final extension step at 72 °C for 10 min.

Wolbachia *species identification*

A subset of *Wolbachia* positive PCR samples were sent for sequencing. These samples were purified using the commercially available QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), using 30µl of water for elution. The samples were sent to GENEWIZ Germany (Leipzig, Germany) for Sanger sequencing. *Wolbachia* sequences were trimmed with FinchTV (v1.5.0). Consensus sequences and the alignment was created using Geneious Prime (v2024.0.5). A phylogenetic tree of the alignment was calculated with Bayesian estimation of phylogeny using MrBayes (v3.2.6) in Phylogeny.fr (Dereeper *et al.*, 2008) and was modified with the [illegible]otool iTOL (v6.8.1) (Letunic and Bork, 2024).

3 Results

Culex *biotype identification*

PCR analysis confirmed the identity of a subset of female *Culex* mosquitoes. Larvae collected from the natural habitat below ground were identified as *Cx. pipiens* biotype *molestus* (n=15) and larvae collected in natural and artificial water containers were classified as *Cx. pipiens* biotype *pipiens* (n=30).

Permethrin dose-response bioassay

The susceptibility to permethrin was assessed for all mosquito populations and the dose-response curves are shown in Figure 2. A total of 4,146 female mosquitoes were tested (Supplementary Table S1). LC_{50} values of each population and the resistance ratios (RRs) are shown in Table 2 and statistical comparisons in Table 3.

For tested *Ae. vexans* and laboratory *Ae. albopictus* populations, no permethrin resistance was observed, with RRs < 1 and significantly lower LC_{50} values compared to the *An. gambiae* susceptible control ($P<0.0001$). In contrast, the *Cx. pipiens* populations exhibited elevated LC_{50} values of 0.018% (95% CI [0.0012–0.0024];

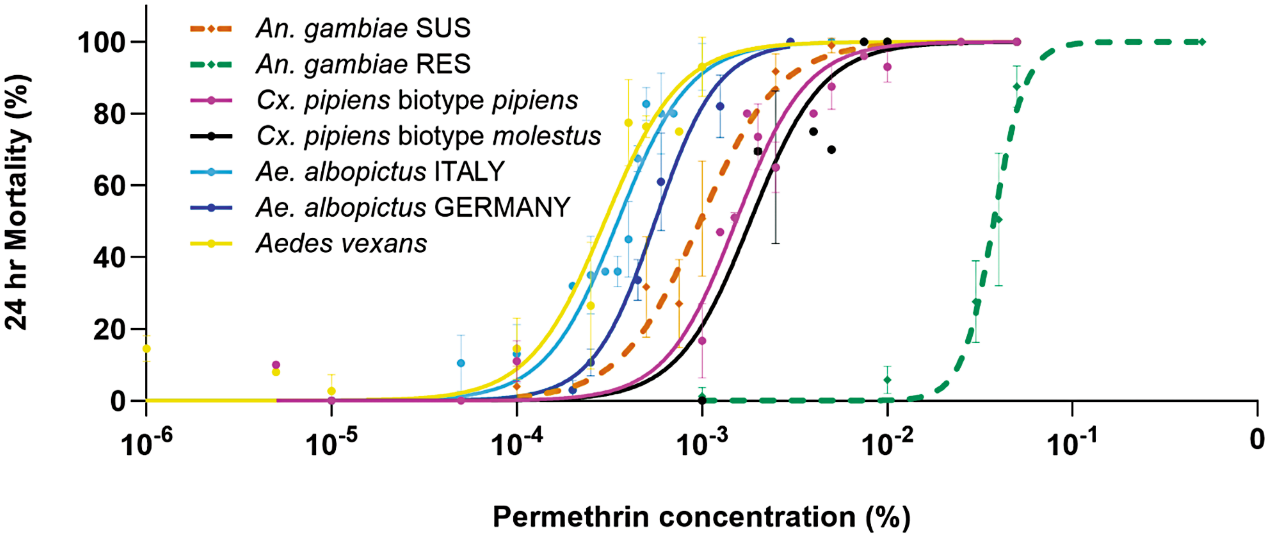

FIGURE 2 Dose-response curves with permethrin by topical exposure of mosquito populations. *Anopheles gambiae* susceptible (SUS, orange) and *An. gambiae* resistant (RES, green) are shown in bold, dashed as susceptible and resistance resistant comparator strains. *Culex pipiens* biotype *pipiens* (pink), *Cx. pipiens* biotype *molestus* (black), *Ae. albopictus* ITALY (light blue), *Ae. albopictus* GERMANY (dark blue) and *Ae. vexans* (yellow) are shown. Permethrin concentration in percentage (%) in a logarithmic scale (x axis) and 24-hour post permethrin exposure mortality rate in percentage (%) (y-axis). Error bars show 95% confidence interval, data is constrained at 0 and 100%.

TABLE 2 LC_{50} mortality values, 95% confidence interval (CI) and resistance ratios (RRs) of tested populations after 24 h topical exposure.[1]

Mosquito population	LC50 (%)	95% CI (%)	Resistance ratio (RR)
An. gambiae SUS	0.00097	0.00085–0.0011	
An. gambiae RES	0.038	0.035–0.040	38.5
Cx. pipiens biotype *molestus*	0.0018	0.0012–0.0024	1.9
Cx. pipiens biotype *pipiens*	0.0015	0.0014–0.0017	1.6
Ae. vexans	0.00030	0.00025–0.00035	0.31
Ae. albopictus ITALY	0.00035	0.00031–0.00039	0.36
Ae. albopictus GERMANY	0.00056	0.00051–0.00062	0.57

[1] LC_{50} were generated using dose-response curves (Figure 2). LC_{50} and 95% CI intervals were calculated with GraphPad Prism. RRs were obtained by dividing LC_{50} of each population with the susceptible *Anopheles gambiae* SUS strain.

P =0.0001) for *Cx. pipiens* biotype *molestus* and 0.015% (95% CI [0.0013–0.0017]; *P* =0.0002) for *Cx. pipiens* biotype *pipiens* (Table 3). The RR values were > 1 compared to the susceptible *An. gambiae* SUS strain with an LC_{50} of 0.00097% (95% CI [0.00085–0.0011]) suggesting some low level of pyrethroid tolerance (ECDC, 2023). However, when compared to the highly resistant *An. gambiae RES* strain, all tested *Culex* populations had significantly lower resistance (*P* <0.0001) (Table 3). Two drawbacks are evident for this analysis; firstly, a different species is being used as the baseline, there are possible discrepancies in baseline susceptibilities; and secondly, the *Ae. vexans* populations were collected as adults and thus the age could not be determined.

Due to limited numbers of *Ae. japonicus* and *Ae. albopictus* field populations, not enough data was generated to create dose-response curves. However, three different concentrations were tested for *Ae. albopictus* field population and two concentrations for *Ae. japonicus* (Supplementary Figures S1A and S1B). From the three concentrations of *Ae. albopictus* the data indicates that all mosquitoes were dead at 0.009% but not at 0.00001%. However, for each concentration only four to ten mosquitoes were tested (Supplementary Figure S1A). Similarly for *Ae. japonicus*, 100% mortality was observed at a concentration of 0.0015% permethrin, but only eight mosquitoes were exposed at this concentration (Supplementary Figure S1B).

TABLE 3 Comparison of reference strains to tested populations using extra-sum-of-square F tests.[1]

Mosquito population	*P*-value *An. gambiae* SUS	*P*-value *An. gambiae* RES	F (DFn, DFd) value *An. gambiae* SUS	F (DFn, DFd) value *An. gambiae* RES
An. gambiae SUS		< 0.0001		242 (2, 49)
Cx. pipiens biotype *molestus*	0.0012	< 0.0001	8.26 (2, 34)	129 (2, 33)
Cx. Pipiens biotype *pipiens*	0.0002	< 0.0001	10.2 (2, 48)	220 (2, 47)
Ae. vexans	< 0.0001	< 0.0001	58.9 (2, 51)	298 (2, 50)
Ae. albopictus ITALY	< 0.0001	< 0.0001	55.2 (2, 52)	262 (2, 51)
Ae. albopictus GERMANY	< 0.0001	< 0.0001	17.9 (2,44)	293 (2, 43)

[1] Each tested population was compared to the susceptible and resistance *Anopheles gambiae* strain. *P*-values and F-values were calculated with extra-sum-of-square F test and generated with GraphPad Prism. A *P*-value < 0.05 was taken as significant.

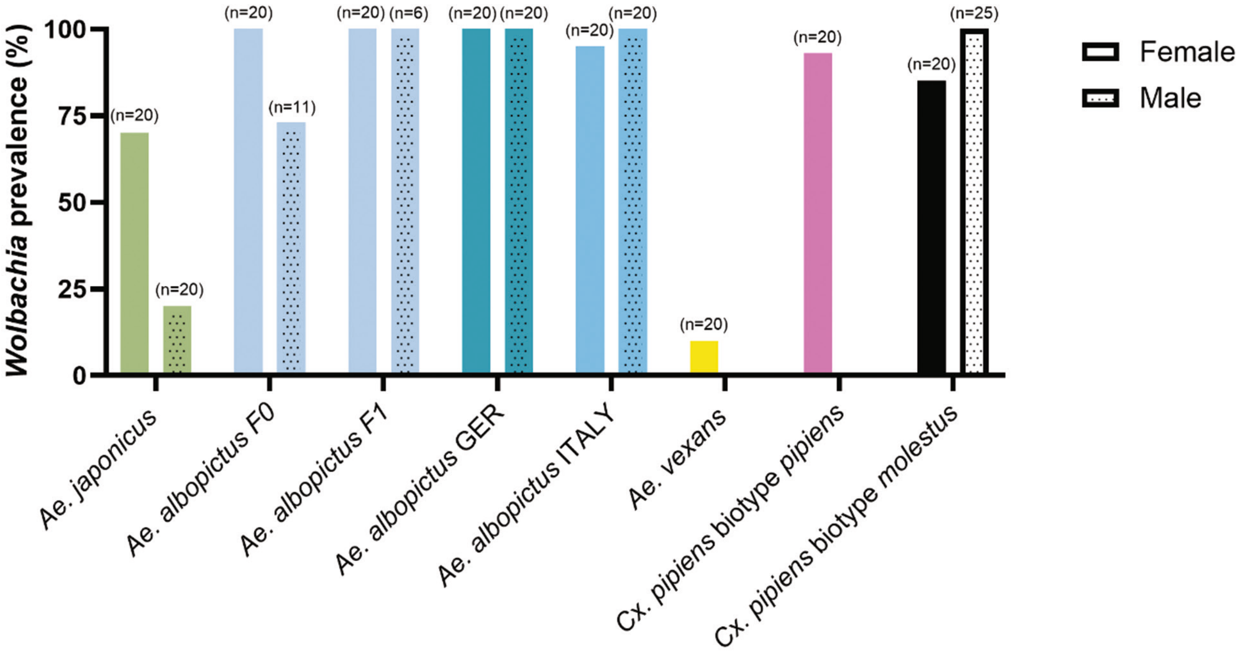

FIGURE 3 *Wolbachia* prevalence in percentage (%) in different mosquito populations. Females (block colour) and males (dotted) of each species (x-axis) were tested for *Wolbachia* prevalence (y-axis). No males for *Culex pipiens* were tested. Number of mosquitoes tested is shown above the bars.

Wolbachia *prevalence*

Detection of *Wolbachia* in all collected mosquito populations was performed with PCR by amplification of the conserved *Wolbachia* S16 rRNA gene. All populations were found to be infected with *Wolbachia* of different frequencies (Figure 3 and Supplementary Table S2). Highest rates of *Wolbachia* were found in *Ae. albopictus* and *Culex* species. All *Ae. albopictus* populations showed similar frequencies of *Wolbachia* infections, except for *Ae. albopictus* F_0 males which were found to be infected at a lower frequency. In contrast, only 10% females were found to be infected with *Wolbachia* in *Ae. vexans.* Interestingly, *Ae. japonicus* show a female bias towards infection. *Wolbachia* was detected for the first time in *Ae. japonicus* with a frequency of 20% for males and 70% for females.

Wolbachia *phylogenetic tree*

A subset of *Wolbachia* positive samples were sequenced and adequate sequences aligned to generate a phylogenetic tree with Bayesian estimation (Supplementary Table S3). For reference, sequences of *w*Mel (LC108848.1), *w*Pip (U23709.1) and *Tetranychus urticae* (MN123078.1, due to high similarity) were used (Figure 4). Overall, all sequences showed high similarities with *Ae. japonicus*, *Ae. vexans* and the *Cx. pipiens* complex grouped together

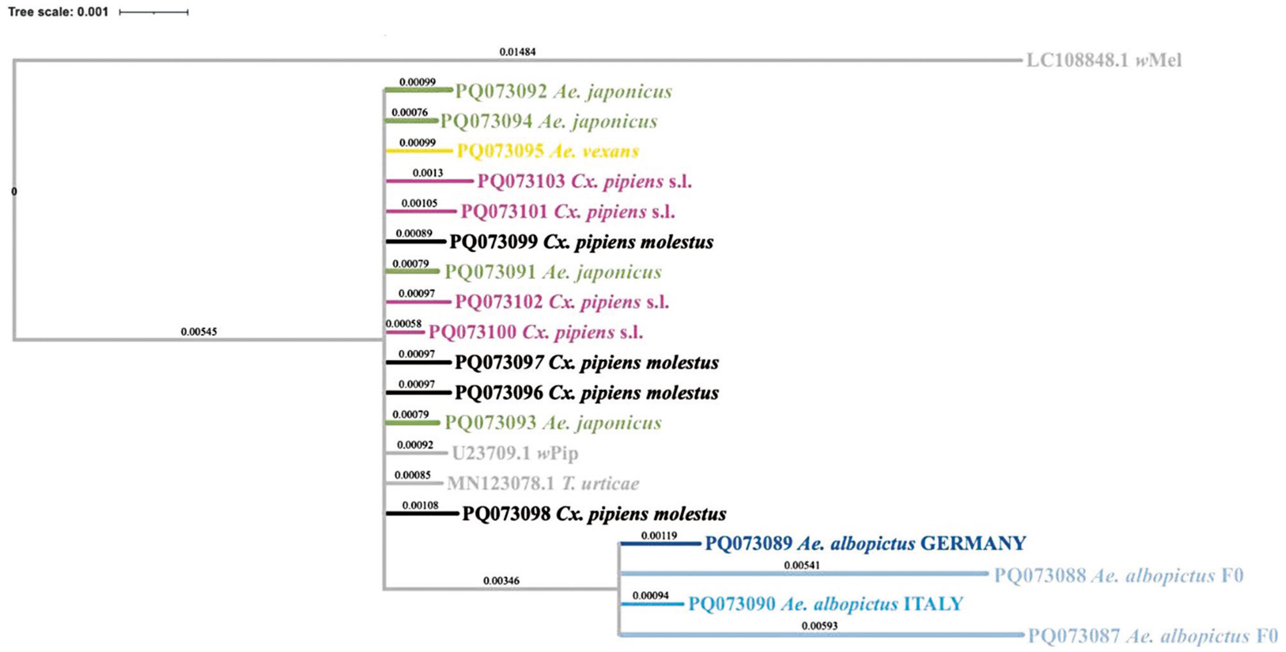

FIGURE 4 Phylogenetic tree of aligned *Wolbachia* sequences. Phylogenetic tree of *Wolbachia* sequences produced here with references of sequences of *w*Mel (LC108848.1), *w*Pip (U23709.1) and *T. urticae* (MN123078.1). Branch lengths correspond to number of substitutions per site. Phylogenetic tree of the alignment was calculated with Bayesian estimated of phylogeny using Mr Bayes in Phylogeny.fr and figure created using iTOL (v.6.8.1).

with *w*Pip and *T. urticae Wolbachia*. In contrast, *Wolbachia* sequences from all *Ae. albopictus* populations were grouped together in one clade.

4 Discussion

Little is known about the toxicity of pyrethroid insecticides to different German mosquito species. Here we show that field-caught *Cx. pipiens* populations show a tendency towards resistance whilst the *Aedes* tested here show high levels of susceptibility to pyrethroid insecticides. All mosquito species tested show infection with *Wolbachia*, highlighting its potential use for mosquito control in southwestern Germany.

Both *Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus* show some potential increased resistance to pyrethroid insecticides, in line with data showing resistance to permethrin and deltamethrin in Italy, Spain and Belgium (Bengoa *et al.*, 2017; Pichler *et al.*, 2022; Vereecken *et al.*, 2022). Nevertheless, the resistance level observed here in Germany is likely much lower than in those populations, where survival following exposure to WHO diagnostic doses of 0.05 and 0.75% deltamethrin and permethrin respectively, is high (Pichler *et al.*, 2022; Vereecken *et al.*, 2022). Although different bioassay approaches were used across studies, the lack of high survival at diagnostic doses in our populations indicates comparatively low resistance. In this study, *Cx. pipiens* biotype *molestus* appeared more resistant than *Cx. pipiens* biotype *pipiens*, which could be due to different habitats. The *Cx. pipiens* biotype *molestus* tested here were collected from an old decommissioned septic tank and are therefore, more likely to have been exposed to chemicals, potentially increasing tolerance to toxins (Nkya *et al.*, 2014; Philbert *et al.*, 2014).

The *Aedes* populations tested here showed higher susceptibility to pyrethroids when compared to a lab susceptible *An. gambiae* population; however, this data must be interpreted with caution, as these mosquitoes belong to a different genus and may exhibit distinct physiological responses to insecticides. Although data on direct comparisons between species using topical exposures are lacking, data from Centers for Disease Control and Prevention (CDC) bottle bioassays within mosquitoes from the same genus are available. For example, a susceptible strain of *Ae.* (*Stegomyia*) *aegypti* (Linnaeus, 1762) had an LC_{50} of 0.000045% when exposed to permethrin, whereas for the susceptible *Culex* line the LC_{50} was 0.0003% (Esterly *et al.*, 2020; Flores *et al.*, 2013; Maestre-Serrano *et al.*, 2020). These values provide context for interpreting susceptibility in field-collected populations, although differences in insecticide exposure methods must be considered. Species-specific factors include cuticle thickness, cuticle permeability and the expression of detoxification genes which may influence insecticide susceptibility, limiting direct comparisons between genera (Black *et al.*, 2021; Ingham *et al.*, 2023). Nevertheless, *Ae. albopictus* GERMANY and ITALY lab colonies have been kept in the laboratory with no insecticide exposure for numerous generations, and due to the associated fitness cost of pyrethroid resistance (Martins *et al.*, 2012), it is likely any resistance was lost in colony (Grossman *et al.*, 2018); therefore, the data strongly indicate that the *Ae. vexans* are susceptible, with LC_{50}s approximating these colonies. Despite managing to acquire field-caught *Ae. albopictus* and *Ae. japonicus* we were unable to ascertain resistance levels due to high levels of death during rearing.

Wolbachia screening revealed that all species tested here contained the bacteria. *Aedes albopictus* had high levels of prevalence in line with expectations. Further, both *Culex* biotypes had high prevalence of infection, an increase on a prior study showing 10–100% infection (Mahilum *et al.*, 2003). The prevalence in *Ae. japonicus* was lower than other species; however, *Wolbachia* infection of this species has never been reported before (Huber *et al.*, 2014). The final species showing the lowest prevalence is *Ae. vexans* and no data exists describing infection in Europe; however, it has been reported to be infected with *Wolbachia* in Thailand (Wiwatanaratanbutr, 2013). Interestingly, the *Wolbachia* found in native *Culex* mosquitoes and *Ae. japonicus* are very similar despite large evolutionary and ecological differences. Indeed, the first reported occurrence of *Ae. japonicus* in Germany was in 2008 (Schaffner *et al.,* 2009), indicating local acquisition of *Wolbachia*. Taken together, this data indicates that *Wolbachia*-mediated control of mosquito vectors has high potential in Germany. The results here have several limitations: 16S is not sufficient alone for species characterisation and additional markers such as *ftsZ*, wsp,or MLST should be used (Inácio da Silva, 2023); further, some sequence quality was low necessitating trimming. Additional studies are needed to confirm the close phylogenetic relationship posed here and whether *Wolbachia* infections, particularly in *Ae. japonicus,* are stable or transient.

Although pyrethroid insecticides are generally restricted in Germany, resistance monitoring data such as those presented here can support both routine surveillance and targeted responses under emergency derogation, in line with guidelines from the Nationale Expertenkommision für Stechmücken (Nationale Expertkommission für Stechmücken, 2022). Understanding insecticide susceptibility alongside *Wolbachia* prevalence could be critical to curb future arboviral outbreaks, it is important to understand the prevalence within the European context. Indeed, insecticide resistance monitoring has identified resistance for several mosquito species in a number of European countries (ECDC, 2023), specifically in *Culex,* as seen in this study. Nevertheless, resistance data within the European context is overall sparse both temporally and geographically, highlighting a critical gap in knowledge. In a similar vein, knowledge of *Wolbachia* carriage in disease vectors in Europe is limited, with a few studies showing high *Wolbachia* carriage of *Culex* in Norway and Sweden (Berman and Hesson, 2021; Lilja *et al.*, 2024) and in *Ae. albopictus* across Europe (Belo, 2024). Implementing broad European monitoring programmes to assess both the susceptibility to public health insecticides and the carriage and type of *Wolbachia* present in local mosquitoes will better equip global health authorities for informed interventions during future arboviral outbreaks.

5 Conclusions

The expanding geographic range of mosquito species with the associated risk of arboviral infection (Gould and Higgs, 2009; Paupy *et al.*, 2009) necessitates characterising resistance to difference insecticide classes which can be used in emergency scenarios. Our study demonstrates that field populations of *Aedes* mosquitoes in southwestern Germany remain largely susceptible to pyrethroids while *Cx. pipiens* biotypes show some evidence of reduced susceptibility. *Wolbachia* infection was detected in all species tested, with high prevalence in *Ae. albopictus* and *Cx. pipiens*, and novel detection in *Ae. japonicus*, suggesting potential for *Wolbachia*-based control strategies in the region. Together, these results provide region-specific data that can guide integrated vector control approaches. Given the potential for future arboviral outbreaks, especially as mosquito vectors continue to adapt to changing environmental conditions, a multifaceted approach combining chemical, biological, and genetic strategies will be crucial for effective vector control and the prevention of disease transmission across Europe.

Supplementary material

Supplementary material can be found online at https://doi.org/10.52004/2054930X-20251030 under Supplementary Materials.

Table S1. Raw data of dose-response bioassay with permethrin on different mosquito populations.

Table S2. *Wolbachia* prevalence in different mosquito populations for different mosquito populations.

Table S3. Mosquito strain with corresponding *Wolbachia* sequence location on NCBI.

Figure S1. Dose responses for field populations with low n.

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Authors' contributions

NP and TS carried out all rearing and laboratory experiments in the study. NB and AP carried out and coordinated all collections. AP provided the *Ae. albopictus* ITALY and *Ae. albopictus* GERMANY eggs and VAI and NB conceived the study. VAI provided guidance throughout. NP, TS, VAI and NB drafted the manuscript.

Conflict of interest

The authors state no conflict of interest.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files. The DNA Sequences generated in this study have been deposited in the NCBI GenBank data base under accession numbers: PQ073087, PQ073088, PQ073089, PQ073091, PQ073092, PQ073093, PQ073094, PQ073095, PQ073096, PQ073097, PQ083098, PQ073099, PQ073100, PQ073101, PQ073102, and PQ073103.

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