Research Article

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First evidence of resistance to pyrethroid insecticides in Italian *Aedes albopictus* populations 26 years after invasion

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Abstract

BACKGROUND: *Aedes albopictus* has spread during the last few decades all over the world. This has increased significantly the risk of exotic arbovirus transmission (e.g. chikungunya, dengue, and Zika) also in temperate areas, as demonstrated by the Chikungunya 2007 and 2017 outbreaks in northeastern and central Italy. Insecticides are an important tool for limiting the circulation of these mosquito-borne viruses. The aim of the present study was to address the gap in current knowledge of pyrethroid insecticide resistance of European *Ae. albopictus* populations, focusing on populations from Italy, Albania and Greece.

RESULTS: Bioassays for resistance to permethrin (0.75%), α-cypermethrin (0.05%) or deltamethrin (0.05%) were performed according to World Health Organization (WHO) protocols and showed reduced susceptibility (<90% mortality) of some Italian populations to permethrin and α-cypermethrin, but not to deltamethrin.

CONCLUSION: This study reports the first evidence of resistance to pyrethroids in adult Italian *Ae. albopictus* populations. Results refer to the season preceding the Chikungunya 2017 outbreak in central Italy and highlight the need to increase efforts to monitor the spread of insecticide resistance and the need to develop strategies to limit the spread of insecticide resistance, particularly in areas where extensive treatments have been carried out to contain disease outbreaks.

Supporting information may be found in the online version of this article.

Keywords: insecticide resistance; *Aedes albopictus*; pyrethroids; Chikungunya vector; vector control

1 INTRODUCTION

More than half of the human population is at risk of infection with *Aedes*-borne viruses (e.g dengue, yellow fever, chikungunya and Zika viruses), which cause thousands of deaths per year and potentially millions of debilitating and economically damaging illnesses.[1] Although these arboviral diseases are mostly endemic in the tropics, outbreaks have occurred in temperate regions, mainly mediated by *Aedes aegypti*, a major tropical vector species, which was reported repeatedly in Mediterranean countries before 1950 and only sporadically afterwards (e.g. on the northern coast of the Black Sea since 2008).[2,3]

The risk of exotic arbovirus transmission in Europe, however, has significantly increased during the last few decades as a consequence of the rapid spread of the Asian tiger mosquito *Aedes albopictus*.[4–6] This species is classified as one of the 100 most invasive species (Global Invasive Species Database; http://www

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.issg.org/database/) and, during the last 40 years, has spread from its native range in Southeast Asia all over the world, including temperate regions, thanks to the production of cold-hardy and long-lived eggs, as well as to the capacity to exploit anthropogenic water containers (e.g. tires, flowerpot saucers and water storage containers) as breeding sites.[7,8] Even though *Ae. albopictus* is a less efficient vector for most arboviruses than the more anthropophilic *Ae. aegypti*, in 2005–2006 it was responsible for large Chikungunya epidemics in Indian Ocean islands and has also been recognized as a competent vector for dengue and several other arboviruses, including Zika.[9,10] In Europe, autochthonous cases of dengue vectored by *Ae. albopictus* have been recorded in France[11] and in Croatia[12] during the last decade. Moreover, two autochthonous chikungunya outbreaks occurred in Italy (in 2007 in northeastern Italy[13,14] and in 2017 in central Italy[15]). In addition, as a consequence of its opportunistic feeding behaviour,[16,17] *Ae. albopictus* has the potential to act as a "bridge vector" of zoonotic pathogens (e.g. *Dirofilaria* spp. which cause canine dirofilariosis) to humans.[18–20]

In Europe, *Ae. albopictus* was first reported in 1979 in Albania[21] and in 1990 in Italy[22] and is now established in 12 countries. In Italy, where in the last 30 years urban and peri-urban areas have been widely colonized by *Ae. albopictus*,[22,23] national guidelines,[24] in agreement with European Centre for Disease Prevention and Control (ECDC) guidelines,[4] recommend prioritizing larval over adult control, because of the higher expected impact and the lower environmental costs of larvicidal interventions. Adulticide interventions are recommended only when infected human travellers from endemic countries are detected, in order to prevent autochthonous disease transmission, or in the case of extremely intense nuisance. Nevertheless, private citizens and some public administrations implement adulticidal control measures in order to achieve immediate and tangible, although short-term, effects on mosquito nuisance.[25–27]

Pyrethroids are the only chemicals allowed for mosquito adulticide in Europe.[28–30] The most commonly used pyrethroids are α-cypermethrin, permethrin and deltamethrin, sometimes in combination with the synergist piperonyl butoxide (PBO). These compounds are also extensively used to control the adult abundance of major tropical vector species (e.g. *Ae. aegypti*, *Culex quinquefasciatus* and *Anopheles* vectors of malaria) and are the only ones recommended by the World Health Organization (WHO) for the treatment of bed-nets. In regions where these species represent major public health problems and are thus the target of extensive control activities, the high selective pressure exerted by the pyrethroid-based interventions in public health and/or agriculture has led to increasing levels of insecticide resistance (IR) with the risk of a reduction in the efficacy of these major vector control tools.[31,32] To prevent this, WHO has drafted guidelines to monitor IR in major vector species and to avoid its insurgence and spread.[33–35]

In contrast with the extensive knowledge of IR in major tropical mosquito vector species, knowledge of IR in *Ae. albopictus* is still fragmented, as pointed out by Moyes *et al.*[32] and Vontas *et al.*.[36] Available data documenting IR is highly clustered, making comparisons difficult, as different methods were used to generate results. So far, resistance to pyrethroids has been reported in the last few years in adult populations from SouthEast Asia, the native range of *Ae. albopictus*,[37–40] as well as from the Indian subcontinent[41–43] and Africa.[44,45] In contrast, almost no reports have come from temperate areas, with the exception of those by Richards *et al.*,[46] who recorded reduced susceptibility to permethrin in the USA, and Bengoa *et al.*,[47] who revealed the first signs of resistance of Spanish *Ae. albopictus* populations to cypermethrin and possible resistance to deltamethrin and permethrin. In Italy, no resistance to pyrethroids was found in early 2000 in adult *Ae. albopictus* populations from Rome and other sites across the country.[48] Later, in 2009, also Vontas *et al.*[36] observed full susceptibility in one population from Rome (Italy) and one from Athens (Greece).

The need for a better understanding of IR in invasive mosquito species and for coordinated strategies for early detection and management of IR was recognized during the first International Workshop on 'Insecticide resistance in vectors of emerging arboviruses: Challenge and prospects for vector control' (Rio de Janeiro, Brazil, December 2016) organized by the Worldwide Insecticide Resistance Network.[32,49] Herein, we report the first evidence of resistance to permethrin and α-cypermethrin in adult *Ae. albopictus* populations primarily from Italy, which should serve as a warning for all Europe and encourage further efforts in monitoring this phenomenon.

2 MATERIALS AND METHODS

2.1 Mosquito collections and rearing

Ovitrap collections of *Ae. albopictus* eggs were carried out by local entomology teams from May to October 2016 (except for the untreated population from Rome, RM-NT, which was sampled and tested in September 2015) at 16 sites across Italy, as well as two sites from Albania and one from Greece (Supporting Information Table S1 and Figure 1). Collections at each sampling site were conducted with at least five ovitraps to avoid oversampling of siblings, and, whenever possible, at a site where adulticide treatments using pyrethroids were known to have been performed during the sampling season (labelled 'TR' in site acronyms), as well as at a second untreated site in the same area (labelled with 'NT') (Table S1). In addition to field-collected populations, a laboratory colony from Athens, Greece, selected for resistance to temephos, was included in the study to evaluate possible cross-resistance between organophosphates and pyrethroids. The carboxyl esterase amplifications found in this colony enhance resistance to temephos[50] and have been detected also in field-collected specimens from Greece, consistent with reduced susceptibility to this organophosphate detected in Greek populations in previous studies.[36] Egg samples sealed in plastic bags were sent by express courier to the Department of Public Health and Infectious Diseases (DPHID) at Sapienza University of Rome.

Larvae were reared at a larval density of 0.05 larvae ml^{-1} in the insectary of DPHID at a temperature of $+26 \pm 1\,^{\circ}$C and a relative humidity (RH) of $60 \pm 5\%$ and with a 14:10 h light:dark photoperiod, and fed with artificial dry cat-food. Pupae were

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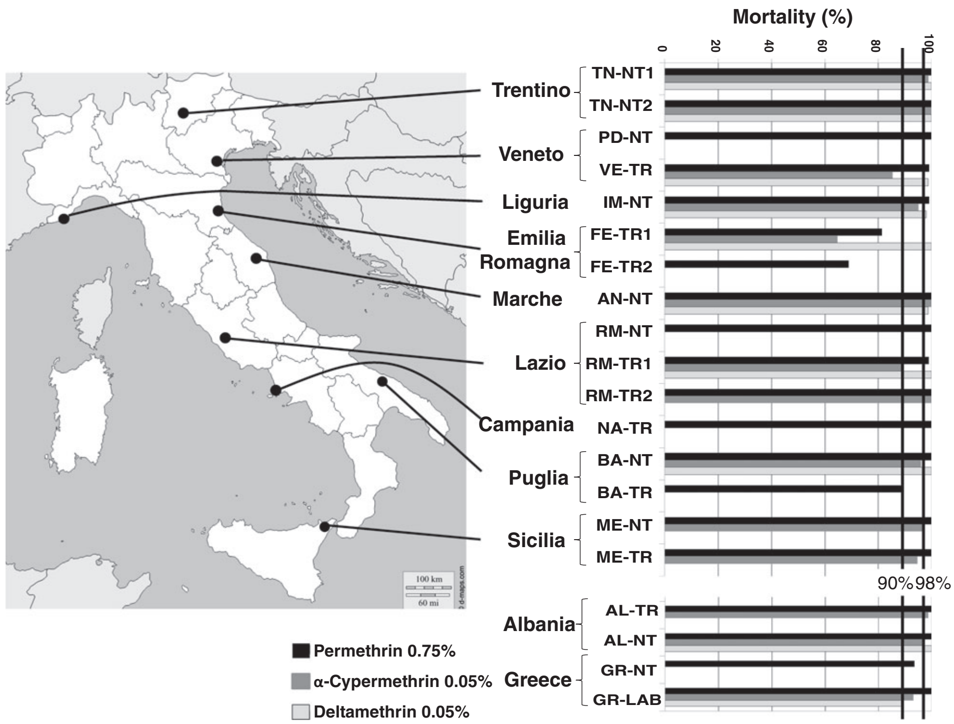

Figure 1. Distribution of *Aedes albopictus* tested populations and mortality (%) after 1 h of exposure to pyrethroids. 0.75% permethrin, black; 0.05% α-cypermethrin, dark grey; 0.05% deltamethrin, light grey. Vertical lines indicate 90% and 98% mortality thresholds.[34,68] Sites for which adulticide treatments were reported during the sampling season are labelled with 'TR'. Sites at which adulticide treatments were not carried out during the sampling season are labelled with 'NT'. GR-LAB, laboratory-selected temephos-resistant colony.

collected daily and transferred into 40-cm^3 cages. Emerged adults were identified as *Ae. albopictus* using morphological keys[51] and kept at the same temperature and RH as larvae until used for the bioassays. When samples from field-collected eggs were not sufficient to complete the experiments, adults were blood-fed and the progeny (F1) was used for bioassays (Table 1).

2.2 Insecticide susceptibility bioassays

Bioassays were performed according to WHO protocols[33,34] in WHO test tubes lined with filter paper impregnated with one of the following insecticides: permethrin (0.75%), α-cypermethrin (0.05%) or deltamethrin (0.05%) (Vector Control Research Unit, School of Biological Sciences, Penang, Malaysia). Insecticide concentrations were selected based on the dosages most frequently used for *Ae. albopictus* in order to allow comparison of results with previous studies.[40–43,45,52,53] The 0.05% concentration for deltamethrin was chosen based on data available for a candidate *Ae. albopictus* susceptible reference strain.[54] Insecticide-impregnated (and control) papers were discarded after being used in six bioassays.

Bioassays were performed in the insectary under the same conditions as for mosquito rearing (see above) using ~25 unfed *Ae. albopictus* females (3 to 5 days old), directly emerged either from field-collected eggs/larvae (F0) or from their progenies (F1) (Table 1). Mosquitoes were exposed to insecticides for 1 h and the number of knocked down mosquitoes (i.e. mosquitoes unable to stand or fly in a coordinated way[34]) was recorded every 10 min during the exposure time; after 1 h of exposure, the mosquitoes were transferred into tubes with untreated filter paper and allowed a 24-h recovery period, after which mortality was recorded. Depending on mosquito availability, three or four replicates per population per insecticide were performed, and for each population and insecticide, also a control tube (i.e. lined with filter paper impregnated only with the insecticide excipient and without the active ingredient) was set up and manipulated in the same way as the test tubes lined with treated filter papers.

Mean values of mortality were computed for each population. When mortality in control cages exceeded 5%, Abbott's correction for natural mortality was applied. According to WHO guidelines,[34] populations were considered "susceptible" if mortality at 24 h after exposure was ≥98%, 'possibly resistant' if mortality ranged between 90% and 97% and 'resistant' if mortality was ≤90%.

For knock-down assessment, a log time-probit statistical model was applied to compute knock-down (KD) curves for each population and to calculate 50% (KDT50) and 95% (KDT95) knock-down times. A binomial generalized linear model (GLM) was used to test the effect of insecticide control activities on mosquitoes and to evaluate if there was any significant difference between KD curves of populations from treated and untreated sites. Pearson's correlation coefficient was computed to evaluate the correlation between KDT values and percentage mortality. All analyses were carried out using R software version 3.3.3.[55] The R script used for computation can be provided by the authors upon request.

3 RESULTS

Susceptibility to permethrin, α-cypermethrin and deltamethrin as well as KDTs were assessed in 20, 14 and 10 *Ae. albopictus* populations, respectively (Figs 1 and 2; Table 1). Mortality in control tubes was always <5%, except for the permethrin bioassay of the Greek field population from Athens (mortality = 8%), for which Abbott-corrected values are reported. No knock-down was observed in control tubes during the 1-h exposure to insecticides.

Table 1. Results of WHO tube bioassays performed on *Aedes albopictus* populations from Italy, Albania and Greece.

Region/country	Site-code	Treatment	Tested generation	Number tested	Mortality % (95% CI)	KDT50 (95% CI)	KDT95 (95% CI)
			0.75% permethrin				
Trentino	**TN-NT1**	N	FO	74	100	12.9 (11.9–14.0)	26.5 (23.9–31.8)
	TN-NT2	Y	FO	82	100	27.9 (26.3–29.5)	55.1 (50.7–62.4)
Veneto	**PD-NT**	N	FO	74	100	15.5 (14.7–16.3)	22.7 (21.2–26.4)
	VE-TR	Y	FO	108	99.0	22.1 (21.1–23.2)	38.7 (36.1–42.8)
Emilia Romagna	**FE-TR1**	Y	FO	74	**81.3**	42.8 (38.4–47.6)	154.2 (121.8–260.9)
	FE-TR2	Y	FO	75	**68.9**	36.4 (33.2–39.9)	119.2 (98.2–172.1)
Liguria	**IM-NT**	N	F1	100	99.0	23.5 (22.2–24.8)	47.1 (43.5–52.9)
Marche	**AN-NT**	N	FO	75	100	19.5 (18.4–20.7)	33.8 (31.2–39)
	RM-NT	N	FO	122	100	21.1 (20.1–22.1)	39.3 (36.7–43.3)
Lazio	**RM-TR1**	Y	FO	96	99.0	25.2 (23.9–26.6)	48.9 (45.4–54.6)
	RM-TR2	Y	F1	77	100	21.5 (20.4–22.7)	35.5 (33–40.6)
Campania	**NA-TR**	N	FO	99	100	18.6 (17.9–19.4)	26.4 (24.9–30.1)
Puglia	**BA-NT**	N	FO	75	100	23.1 (21.9–24.3)	36.6 (34.1–41.6)
	BA-TR	Y	F1	77	**89.6**	31.3 (29.4–33.3)	66.7 (60.4–78.3)
Sicily	**ME-NT**	N	FO	50	100	18.6 (17.5–19.8)	29 (26.5–37.1)
	ME-TR	Y	FO	75	100	18.5 (17.5–19.6)	30.9 (28.5–35.8)
ALBANIA	**AL-TR**	Y	FO	77	100	22 (20.9–23.1)	33.5 (31.3–38.3)
	AL-NT	Y	F1	74	100	21.3 (20.2–22.4)	33.6 (31.2–38.5)
GREECE	**GR-NT**	N	F1	100	**93.5**	43.8 (41.2–46.5)	95.9 (85–119)
	GR-LAB	Lab	F1	100	100	27.9 (26.5–29.5)	57.1 (52.7–64.4)
			0.05% α-cypermethrin				
Trentino	**TN-NT1**	N	F1	78	98.7	25 (23.7–26.3)	39.9 (37.3–44.8)
	TN-NT2	Y	F1	90	100	22.2 (20.9–23.7)	53.5 (48.5–61.4)
Veneto	**PD-NT**	N	NA	NA	NA	NA	NA
	VE-TR	Y	FO	75	**85.3**	40 (37.7–42.3)	76.2 (68.9–91.5)
Emilia Romagna	**FE-TR1**	Y	F1	73	**64.8**	62.3 (54.2–71.6)	186.3 (142.7 –NA)
	FE-TR2	Y	NA	NA	NA	NA	NA
Liguria	**IM-NT**	N	F1	100	**95.0**	23.4 (21.8–25.2)	69 (60.9–82.7)
Marche	**AN-NT**	N	FO	75	100	28.5 (26.9–30.2)	53.6 (49.3–61)
	RM-NT	N	NA	NA	NA	NA	NA
Lazio	**RM-TR1**	Y	F1	74	**89.2**	39.1 (36.5–41.9)	89.8 (78.7–114.3)
	RM-TR2	Y	F1	78	100	26.6 (25.1–28.2)	51.2 (47.1–58.3)
Campania	**NA-TR**	N	NA	NA	NA	NA	NA
Puglia	**BA-NT**	N	FO	76	**96.1**	31.2(29.6–32.8)	50.9 (47.5–56.8)
	BA-TR	Y	NA	NA	NA	NA	NA
Sicily	**ME-NT**	N	F1	76	**96.7**	32.3 (30.6–34.1)	57.5 (53.2–65.1)
	ME-TR	Y	F1	75	**94.7**	33.7 (31.8–35.7)	64.9 (59.4–75)
ALBANIA	**AL-TR**	Y	FO	72	98.6	26.1 (24.7–27.6)	45.5 (42.2–51.3)
	AL-NT	Y	F1	75	**97.3**	30.3 (28.2–32.5)	73.6 (65.3–89.4)
GREECE	**GR-NT**	N	NA	NA	NA	NA	NA
	GR-LAB	lab	F1	100	**93.0**	32.7 (30.8–34.7)	76.3 (68.8–89.5)
			0.05% deltamethrin				
Trentino	**TN-NT1**	N	F1	78	100	15.7 (14.7–16.8)	30.7 (27.9–35.9)
	TN-NT2	Y	F1	75	100	18.3 (17.3–19.4)	30.3 (27.9–35.1)
Veneto	**PD-NT**	N	NA	NA	NA	NA	NA
	VE-TR	Y	F1	77	98.7	18.3 (17.1–19.6)	39 (35.4–45.4)
Emilia Romagna	**FE-TR1**	Y	F1	78	100	20.2 (19–21.5)	39 (35.7–44.8)
	FE-TR2	Y	NA	NA	NA	NA	NA
Liguria	**IM-NT**	N	F1	100	98.0	20.4 (19.3–21.6)	39.9 (36.8–44.8)
Marche	**AN-NT**	N	F1	77	98.7	19.2 (18.1–20.4)	35.8 (32.9–41.3)
	RM-NT	N	NA	NA	NA	NA	NA
Lazio	**RM-TR1**	Y	F1	74	100	25 (23.5–26.5)	46.4 (42.8–52.9)
	RM-TR2	Y	NA	NA	NA	NA	NA
Campania	**NA-TR**	N	NA	NA	NA	NA	NA
Puglia	**BA-NT**	N	FO	77	100	17.8 (16.7–18.9)	32.5 (29.8–37.7)
	BA-TR	Y	NA	NA	NA	NA	NA

Table 1. continued

Region/country	Site-code	Treatment	Tested generation	Number tested	Mortality % (95% CI)	KDT50 (95% CI)	KDT95 (95% CI)
Sicily	**ME-NT**	N	NA	NA	NA	NA	NA
	ME-TR	Y	NA	NA	NA	NA	NA
ALBANIA	**AL-TR**	Y	NA	NA	NA	NA	NA
	AL-NT	Y	F1	78	100	20.4 (19.2–21.6)	36.1 (33.3–41.3)
GREECE	**GR-NT**	N	NA	NA	NA	NA	NA
	GR-LAB	lab	F1	100	**89 .0**	25.8 (24.5–27.1)	47.8 (44.5–53.1)

Generation and number of female mosquitoes tested for pyrethroid resistance (i.e. 0.75% permethrin, 0.05% α-cypermethrin and 0.05% deltamethrin) are reported, as well as mortality (%) at 24 h after a 1-h exposure and times to knock-down (KDT) of 50% and 95% of the population [with 95% confidence intervals (CIs)]. Sites for which adulticide treatments have been reported during the sampling season are labelled with 'TR'. Sites at which adulticide treatments were not carried out during the sampling season are labelled with 'NT'. GR-LAB, laboratory-selected temephos-resistant colony. Results indicating resistance or possible resistance according to WHO[34,68] are highlighted in bold. NA = test not performed due to low sample size.

3.1 Permethrin

Bioassays suggested resistance to permethrin only in the treated populations (i.e. populations for which insecticide applications had been reported during the sampling season) from Ferrara Province in Emilia-Romagna (FE-TR1, mortality = 81.3%; FE-TR2, mortality = 68.9%) and from Bari Province in Puglia (BA-TR, mortality = 89.6%), while the field population from Athens (Greece) appeared to be possibly resistant (GR-NT, mortality = 93.5%). Correlation between KDT50, KDT95 and percentage mortality was significant ($r_{KDT50/mortality} = -0.71$; $r_{KDT95/mortality} = -0.85$), with populations from Ferrara and Bari Provinces showing the highest KDT50 and KDT95 values. A large variability of KDT50 and KDT95 values was observed across Italy (KDT50: 13–43 min; KDT95: 23–154min; Figure 2), with significantly higher values in populations from treated sites in Veneto and Puglia, when compared with populations from neighbouring untreated sites ($p < 0.05$; Figure S1).

3.2 α-Cypermethrin

Resistance to α-cypermethrin was suggested for the treated populations from Ferrara Province (FE-TR1, mortality = 64.8%), Venezia Province (VE-TR, mortality = 85.3%) and Rome (RM-TR1, mortality = 89.2%). These populations showed also the highest KDT50 and KDT95 values.

Results suggestive of possible resistance were obtained for several other tested populations (Figure 1), while full susceptibility was observed only for four Italian populations (TN-NT1, mortality =98.7%; TN-NT2, mortality = 100%; AN-NT, mortality = 100%; RM-TR2, mortality =100%), and one population from Vlore County in Albania (AL-TR, mortality = 98.6%). Correlation between KDT50, KDT95 and percentage mortality was significant ($r_{KDT50/mortality} = -0.96$; $r_{KDT95/mortality} = -0.96$) and large variability for knock-down times was observed across Italy (KDT50: 22 – 62 min; KDT95: 40–186 min). No significant differences were detected among populations in neighbouring treated versus untreated sites.

3.3 Deltamethrin

All the eight Italian populations tested, as well as the Albanian one, were fully susceptible to deltamethrin, while resistance was observed only in the Greek laboratory colony (mortality = 89.0%). KDT50 and KDT95 were highest in RM-TR1, but no significant differences were observed among treated and untreated sites and no strong correlation between KDT values and mortality was detected ($r_{KDT50/mortality} = -0.62$; $r_{KDT95/mortality} = -0.62$).

4 DISCUSSION

We report the first evidence of resistance to permethrin and α-cypermethrin in adult *Ae. albopictus* populations from Italy. The lowest mortality rates (<70%) were detected in populations from two sites along the Adriatic coast in the Comacchio area (Emilia-Romagna region, northeastern Italy). No detailed data on adulticide usage in Italy are available, but it is relevant to note that the two sites are highly touristic and insecticide spraying has been extensively conducted since 1991 during the summer seasons to reduce nuisance mostly caused by *Aedes caspius* and *Culex pipiens*.[56] In fact, preliminary results on sympatric *Cx. pipiens* showed mortality rates <20% after exposure to 0.75% permethrin (FE-TR2; data not shown), confirming that mosquito populations in that area are likely to be exposed to high selective pressure by adulticides. It would be interesting to test the susceptibility to pyrethroids of *Ae. albopictus* populations collected in neighbouring localities where no or scattered adulticide treatments are conducted.

Mortality rates suggestive of resistance (<90%) were also obtained for populations from Puglia (BA-TR) when exposed to permethrin, and Veneto (VE-TR) and Lazio (RM-TR1) when exposed to α-cypermethrin. Four additional populations from Italy (from treated as well as untreated sites) showed mortality rates indicative of possible resistance to α-cypermethrin (mortality <98%). Further tests on larger sample sizes are needed to confirm these preliminary results.

Evidence of lower susceptibility to both pyrethroids is also provided by the significant increase in the time to knock-down observed in some populations. The large variability observed across Italy for KDT50 and KDT95 values probably reflects differential adulticide usage. Nevertheless, both values showed a good correlation with mortality and confirmed the presence of some populations with strongly reduced susceptibility to permethrin and α-cypermethrin. In the case of permethrin, significant differences between treated and untreated sites were also found: populations collected at treated sites in Veneto and Puglia showed higher KDT50 and KDT95 values than populations collected in the same region at neighbouring but untreated sites, suggesting that adulticide spraying carried out at high frequency during the whole season at these sites lowered the species' susceptibility. This

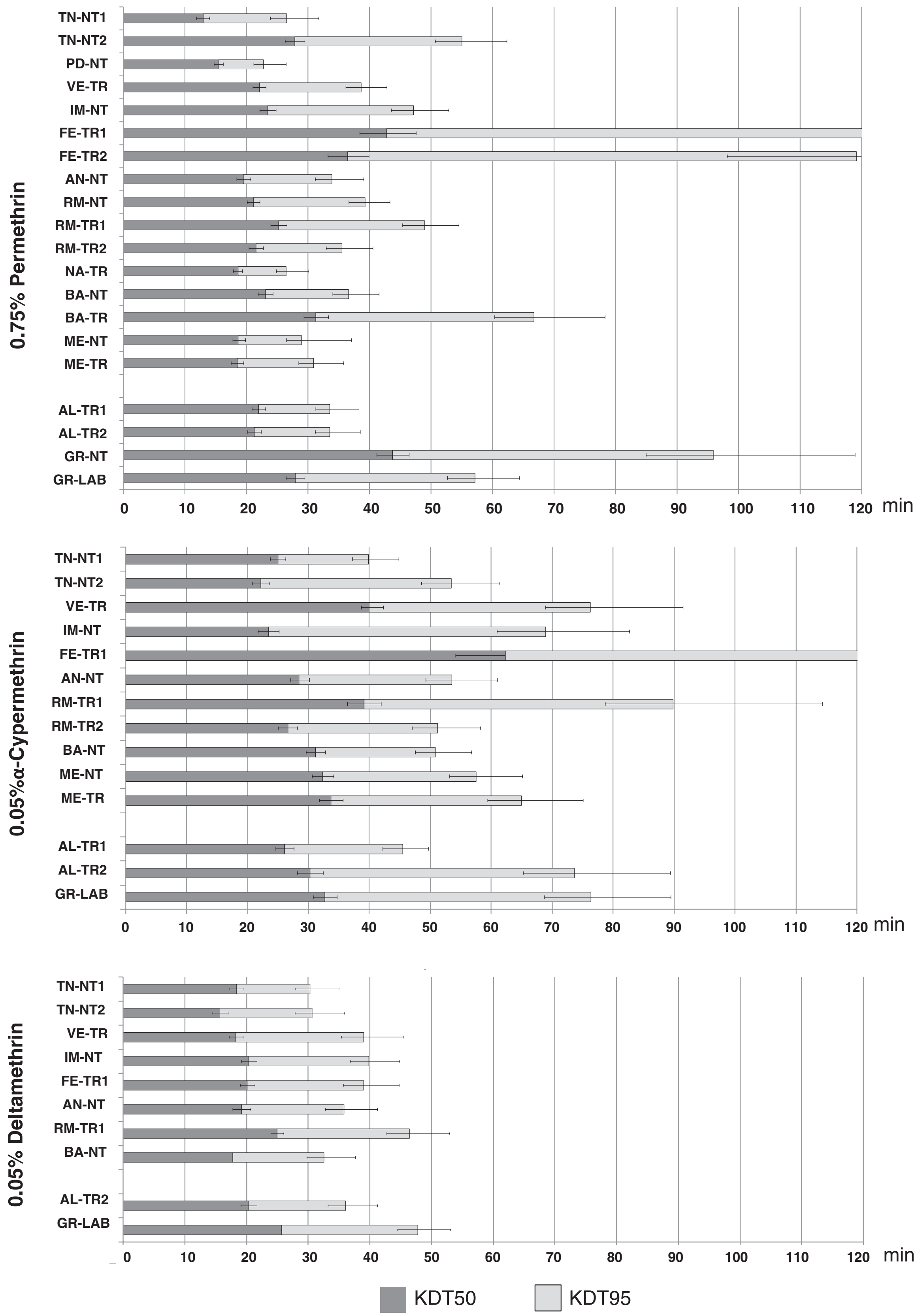

Figure 2. Knock-down times (and 95% confidence intervals) of 50% (KDT50; dark grey) and 95% (KDT95; light grey) of *Aedes albopictus* exposed to pyrethroids (0.75% permethrin, 0.05% α-cypermethrin and 0.05% deltamethrin). Sites for which adulticide treatments were reported during the sampling season are labelled with 'TR'. Sites in which adulticide treatments were not carried out during the sampling season are labelled with 'NT'. GR-LAB, laboratory-selected temephos-resistant colony.

appeared not to be the case in Lazio and Sicilia, possibly because of less effective or more recent adulticide treatments.

In contrast to observations for permethrin and α-cypermethrin, all Italian populations were susceptible to deltamethrin. Similar results were obtained in Greece,[36] Spain[47] and the USA.[54] This result is consistent with the hypothesis of a lower usage of this insecticide in Italy, but could also indicate that the deltamethrin dosage used was inappropriate (too high) for *Ae. albopictus*.

Mechanisms producing the permethrin/α-cypermethrin resistance phenotype in Italian populations will be evaluated in future studies. While target-site resistance mechanisms, which typically induce cross-resistance between pyrethroids,[57–60] are widespread and well known in anophelines,[31] far less information is available for *Ae. albopictus*. Several target-site mutations have been identified in this species, but their association with IR is still unclear[32,60] and appears to be less strong compared with those of other mosquito species. Also, the lack of cross-resistance to different pyrethroids in the Comacchio population suggests that multiple/other resistance mechanisms, possibly including detoxification pathways,[61,62] may be involved.

Aedes albopictus populations from Albania were found fully susceptible to all pyrethroids tested, with relatively low KDTs, despite being sampled in insecticide treated sites. On the other hand, the field population from Athens (which was shown to be susceptible to deltamethrin in 2009, (36)) did not show full susceptibility to permethrin and exhibited KDT95 values higher than all other tested populations, except those from Comacchio. Surprisingly, however, no public pyrethroid space-spraying has been carried out in Athens since 2007, although a selective pressure by intensive treatments performed by private citizens cannot be excluded. The lower susceptibility of the field-collected population from Greece to permethrin could be explained by a different origin of the Greek population compared to the Italian and Albanian ones, as supported by population genetic data [63,64], but also by cross-resistance between organophosphates and pyrethroids, as already reported for other mosquito species.[65,66] In fact the same amplified carboxyl esterase genes (CCEs) responsible for the temephos-resistance of the laboratory colony have been observed, as explained above, also in Greek field-populations[50] and could be associated with a reduced susceptibility to permethrin which can be hydrolysed by CCEs as shown in other insect species.[67]

The data presented herein need to be interpreted with caution, considering some limitations inherent to the study design and sampling efforts. First, WHO provides specific diagnostic dosages based on data available only for *Ae. aegypti*, *Cx. quinquefasciatus* and anopheline mosquitoes. The dosages used in this study were higher than those recently recommended as tentative for *Aedes* mosquitoes,[68] and this choice was made in order to obtain results that were comparable to those of previous studies (see Materials and Methods). This implies that our results certainly do not overestimate resistance levels, but may underestimate them. Further studies on a susceptible reference colony are needed to more precisely assess diagnostic dosages, the lack of which strongly limits the possibility of comparing and interpreting results across studies.[36] It should also be noted that, although the use of a reference colony is also recommended to demonstrate the effectiveness of insecticide-impregnated filter paper, the presence in our experiments of field populations showing 100% mortality to all three insecticides confirms the effectiveness of the WHO filter paper used. Secondly, we chose to perform bioassays with F0 females or, when not possible, F1 progenies, in order to avoid loss of selective pressure and inbreeding under laboratory conditions. This choice, however, meant that in some cases we could not test a minimum of 100 females per insecticide, as recommended by WHO[34] to confirm resistance. Thirdly, the classification of "treated site" in the study is heterogeneous as it reflects different mosquito control activities carried out in Italy, Albania and Greece, including differences in the used pyrethroid compounds, dosages, spraying methods, protocols and time schedules as well as different histories of pyrethroid applications in agriculture. Nevertheless, it is notable that only the populations from Trentino subjected to occasional adulticide spraying (Rizzoli AP, personal communication), together with populations from Marche (Ancona province; AN-NT), showed complete susceptibility to all the tested insecticides, while the highest resistance was observed at the Comacchio sites, where very intensive control activities following a well-defined monitoring plan were implemented even before the *Ae. albopictus* invasion to reduce nuisance caused by *Ae. caspius* (a very aggressive autochthonous species). In most other sites, adulticide treatments were adopted only after the colonization of the areas by invasive *Ae. albopictus*.

Overall, this first assessment of resistance to permethrin and α-cypermethrin in adult *Ae. albopictus* populations from Italy represents a first step in addressing a gap in knowledge about resistance to pyrethroids in invasive populations now fully established in Europe, where the species is becoming an increasing health threat. The results show that resistance to the most commonly used pyrethroids (i.e. permethrin and α-cypermethrin in Italy) is arising in areas where the species has been well established for several years, reaches high densities and has been the cause of substantial nuisance. Coupled with possible resistance observed recently in Spain[47] and the high levels of resistance found in the only Western European *Ae. aegypti* population from the island of Madeira,[69] the results should serve as a warning for Europe and encourage further efforts in monitoring this phenomenon and in standardizing protocols for IR detection and guidelines for IR management in temperate areas. Studies of this typology are in fact greatly needed to support local public health authorities in managing and planning effective control measures and to maintain effective insecticide-based vector control options. The large chikungunya outbreak[15] that occurred in central Italy in summer 2017 clearly highlights the urgency of more extensive studies to better understand and monitor the spread of resistance phenotypes with a larger spatial and temporal coverage, particularly in areas at risk of autochthonous arbovirus transmission,[32,70–72] as well as the implementation of synergic and coordinated actions aimed at controlling the mosquito population abundance at the larval stage.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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