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Effects of natural pyrethrum and synthetic pyrethroids on the tiger mosquito, *Aedes albopictus* (skuse) and non-target flower-visiting insects in urban green areas of Padua, Italy

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ABSTRACT

The tiger mosquito is a key vector of several human diseases and is considered a public health concern worldwide. The implementation of strategies aimed at maximizing mosquito control without affecting non-target insect groups is of major importance. In a field trial, we tested the efficiency of a natural pyrethrum-based vs. a synthetic pyrethroid-based insecticide in reducing tiger mosquito population and how they affect the diversity of non-target flower-visiting insects in green urban areas. Only the pyrethroid insecticide was effective in reducing mosquito abundance, although its effects disappeared nine days after application. The two adulticides did not significantly affect the diversity of flower-visiting insects, probably because of their large body size and the difference in flying and foraging activity. To effectively control mosquito populations while preventing intoxication of non-target flower-visiting insects, adulticide applications should be applied early in the morning and only on bushes and trees. Results from our small-scale applications cannot be extrapolate when larger areas are treated.

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Introduction

Mosquitoes (Diptera: Culicidae) are among the most annoying and dangerous insects in the world. They are vectors of many human diseases such as malaria (transmitted by mosquitoes belonging to the genus *Anopheles*), dengue fever (*Aedes* spp.) and encephalitis (*Culex* spp.). The incidence of mosquito transmitted diseases is increasing rapidly throughout the world due to climate change (Reiter 2001; Yi et al. 2014) and increased international trade and travelling that also promote the invasion of exotic diseases and vectors (Benedict et al. 2007). The Asian tiger mosquito *Aedes albopictus* (Skuse, 1894) (Diptera: Culicidae), in particular, is one of the major public health concerns (Kreß et al. 2014). This tropical species, native of South-East Asia, was introduced and has become invasive almost worldwide (Kraemer et al. 2015). The tiger mosquito is a known vector of many exotic arboviruses, among which dengue and yellow fewer, and is responsible for the recent outbreaks of chikungunya and Zika viruses in south Europe (Guzzetta et al. 2016). It is also a considerable nuisance because of its aggressive diurnal biting behaviour.

Many strategies have been implemented to control mosquito populations (Yi et al. 2014). Conventional control implies the application of broad spectrum insecticides formulated to kill adults (Nkya et al. 2013) or differently selective larvicides (Baldacchino et al. 2015). While the control of tiger mosquito is most efficiently accomplished at the larval stage, emergency situations may sometimes require the use of adulticides (Baldacchino et al. 2015; Fonseca et al. 2013). For instance, adulticides are important tools in case of disease outbreaks to quickly suppress the mosquito population and avoid the further spread of the disease (Bonds 2012). Among adulticides, pyrethroids are currently the most used insecticides in the world (Kreß et al. 2014; Liu and Weng 2013; Nkya et al. 2013; Schleier and Peterson 2010). They are widely applied to control insect pests in agriculture and urban areas (Liu and Weng 2013; Nkya et al. 2013; Pansa et al. 2015), and have a great value in the global insecticide market (Soderlund et al. 2002). As an alternative to conventional pyrethroid insecticides, natural pyrethrum has been also recently re-introduced in pest control strategies in urban areas due to environmental concerns (Boyce et al. 2007; Schleier and Peterson 2010). Natural pyrethrum is generally expected to have a lower environmental impact compared to pyrethroids, as it quickly degrades in

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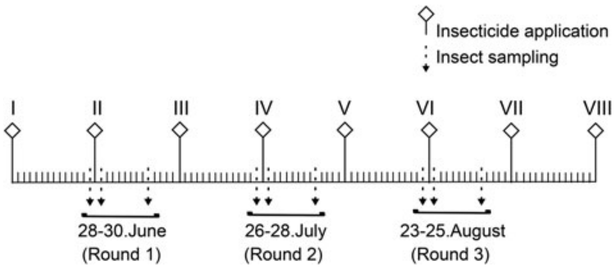

Figure 1. Eight adulticide applications (I-VIII) carried out in summer 2016, every two weeks from mid-June to the end of September. Abundance of mosquitoes and species richness and abundance of flower-visiting insects were sampled on three rounds (during the 2nd, 4th and 6th adulticide application: June, July and August). On each sampling round the survey was conducted three times: the day before, one and nine days after the adulticide application.

sunlight (Casida 1980). Compared to other insecticides, pyrethum-based adulticides have limited persistence in the open environments and negligible mobility in soil (Inglesfield 1989). Due to its high instability and short "knockdown" effect (rapid paralysis), natural pyrethrum may be not sufficient to control mosquito populations, especially during summer outbreaks (Schleier and Peterson 2011).

As both pyrethroid and natural pyrethrum insecticides lack selectivity, they can have negative effects on numerous animal groups that may be impacted by contact or ingestion of contaminated food (Davis and Peterson 2008; Hafsi et al. 2016; Kedwards et al. 1999; Soderlund et al. 2002). While most pyrethroid insecticides are only moderately toxic to mammals (Inglesfield 1989; Schleier and Peterson 2011; Soderlund et al. 2002), they can have strong negative effects on aquatic organisms and non-target insects, including many beneficial groups (e.g. pollinators) (Caron 1979; Ceuppens et al. 2015; Kedwards et al. 1999; Schleier and Peterson 2010, 2011; Yi et al. 2014). Laboratory studies shown that pyrethroid insecticides are highly toxic to bees (Ceuppens et al. 2015; Inglesfield 1989), while results from field studies are mixed revealing that negative effects can be weak in open environments (Boyce et al. 2007; Davis et al. 2007; Inglesfield 1989; Schleier and Peterson 2011). While the effects of pyrethroid insecticides on honey-bees have been intensively investigated (e.g. Boyce et al. 2007; Caron 1979; Inglesfield 1989), less attention has been paid to how these adulticides affect other flower-visiting groups. Here, we tested the efficiency of a natural pyrethrum-based insecticide, compared to the most often used pyrethroid-based insecticide, in controlling adult mosquito populations in urban areas, and how the two adulticides affected non-target flower-visiting insects. We tested both short-term effect after single applications and how the effects of the two insecticides changed over time due to repeated applications. Both formulations were tested using small scale applications of single green areas (treated areas < 0.1 ha).

Materials and methods

Study area

The study was carried out in 15 urban green areas situated in the eastern part of the city of Padua (Veneto, northern Italy) (Lat: 45°24'28.69" N; long: 11°53'9.10" E). Sampling sites have an average size < 0.1 ha and were covered mainly in spontaneous plants and flowers. The mean distance between sites was 2.54 km, with a standard deviation of 1.52 km.

Sampling design

Of the selected 15 urban green areas, five were treated with a conventional pyrethroid-based insecticide, five with natural pyrethrum, and five sites with no intervention measures (control sites). The treatments were intermixed in a completely randomized design. To limit mosquito populations, eight adulticide applications were carried out in the 10 treated green urban areas in 2016, every two weeks from mid-June to the end of September. Applications were carried out on the same day for all the sites. Conventional treatments with pyrethroids were compared with alternative treatments based on natural pyrethrum. Pyrethroid treatments were performed using Microsin® (INDIA, Padova, Italy) based on cypermethrin (10.0%) and tetramethrin (2.0%), synergized with piperonyl butoxide (5.0%) to prevent enzyme degradation. Natural pyrethrum treatments were performed using Piretrum Micro® (INDIA, Padova, Italy) based on pyrethrins (1.5%), synergized with piperonyl butoxide (11.5%). For Microsin the manufacturer reports a persistence on vegetation of 8–14 days, while no data is provided for Piretrum Micro. Both adulticides were applied using a mist sprayer (Citizen Kompact, 500 lt, 37 HP, produced by: TIFONE Ambiente s.r.l., Via Modena, 248/A - 44124 Ferrara, Italy) at the minimum recommended concentration (0.1% for Microsin and 2.0% for Piretrum Micro). All the applications occurred in the early morning, between 04:00 am and 06:00 am, when the weather was clear, dry, with little or no wind. The insecticide was applied on bushes and trees up to the canopy therefore the herbaceous vegetation was not directly sprayed. Applications were carried out always by the same operators following the manufacturer's instructions. Their use complied with all relevant regulations regarding the application of insecticides in the study area.

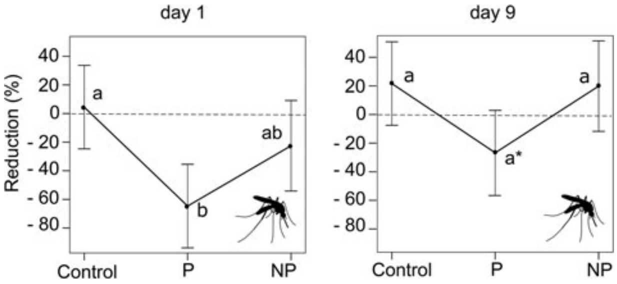

Figure 2. Percent reduction (%) in mosquitoes abundance one and nine days after adulticide application with a pyrethroid (P) and natural pyrethrum (NP) insecticide, and in control sites (control). Bars show confidence intervals at 95%. Groups that are significantly (P-value < 0.05) different are indicated with different letters. Marginally significant results (P-value between 0.05 and 0.10) are identified with an asterisk (*).

In each sampling site, we assessed the abundance of mosquitoes and the diversity of flower-visiting insects in three sampling rounds (during the 2^{nd}, 4^{th} and 6^{th} adulticide application, respectively) (Figure 1). In each sampling round, we conducted three surveys: the day before, one day after and nine days after the adulticide application.

Mosquito abundance

To estimate abundance of mosquitoes, we recorded the number of adults landing on exposed legs and arms of a field operator. The human landing catch is considered the most reliable existing measure of anthropophilic mosquito density (Almeida et al. 2005; Sikulu et al. 2009). To avoid double counting, mosquitoes were collected by a second operator using an aspirator. Mosquito collections and counting were carried out by the same operators as follows: three sessions of five minutes were performed, with a pause of approximately three minutes. Collections and counting took place, from 3:00 p.m. to 5:30 p.m., in the middle of each green area, in three randomly chosen points, spaced approximately 10 m from each other. The points were always the same for the entire study.

Flower-visiting insects diversity

In each green area, a cluster of three plastic pan-traps of different colors (one yellow, one blue, and one white; 750 ml, Ø 12.5 cm, h 4.5 cm) was placed to sample flower-visiting insects. Plastic cups were placed on the ground and filled with a solution of water and biodegradable dish detergent. At each sampling time, pan-traps were set out for 24 h. Samples were collected by pouring the liquid through a fine mesh and preserved in vials filled with 70% ethanol. The material was sorted and identified at the Entomology Laboratory of Sapienza University of Rome. Specimens are preserved at the Museum of Zoology, Sapienza University of Rome.

Statistical analyses

First, we tested the efficacy of pyrethroid-based vs. natural pyrethrum-based insecticides applied at day t in reducing mosquito abundance one $(t+1)$ and nine days $(t+9)$ after treatment, and if the effect of adulticide application was cumulative over sampling rounds. The percent reduction of mosquito density at time $t+i$ was calculated as follow:

$$\text{Reduction}_{t+i} = \left(\text{Density}_{t+i} - \text{Density}_{t-1}\right)/\text{Density}_{t-1}$$

where Density_{t+i} is the number of mosquitoes recorded i days after the treatment and Density_{t-1} is number of mosquitoes recorded the day before application. Linear mixed-effects models were used to explore how mosquitoes responded to the treatments. The response variable was the population reduction described above. The model included treatment (pyrethroid and natural pyrethrum insecticides, and control), the day after application $(t+1$ or $t+9)$, and sampling round (June, July and August) as fixed effects, and the sampling site ID as random factor. We also tested interactions between time and treatment and between sampling round and treatment. This model tested both the short-term and long-term effect (repeated treatments) of insecticides on population reduction compared to the control. One outlier was removed from the data-set based on Cook's distance (value ~ 1) and on diagnostic plot model residuals. In order to compare the effect of treatments separately by time $(t+1$ and $t+9)$, we computed a Tukey test for a family of 3 estimates with a significance level of alpha $= 0.05$.

Second, we tested for potential side-effects on the diversity of non-target flower-visiting insects. The response variables was the number of species (and morphospecies), ln-transformed to improve linearity. The model included treatment (pyrethroid and natural pyrethrum insecticides, and control), time (t-1, $t+1$ and $t+9$), sampling round (June, July and August), and the interactions between time and treatment and between sampling round and treatment as fixed effects. The sampling site ID was used as random factor.

All analyses were performed using R 3.2.2 (2015). The "nlme" (Pinheiro et al. 2014) package was used for the linear mixed-effects model analyses. For the Tukey test we used the "lsmeans" package.

Results

Mosquitoes

A total of 2,661 adult female mosquitoes were collected. Preliminary observations of the larval abundance in the catch basin showed that *Ae.*

Table 1. Results from linear mixed effect models testing the effects of treatment (Microsin, Piretrum Micro and control), time after treatment (t + 1 and t + 9), sampling round (June, July and August), and their interactions, on percent reduction of mosquito. Non-significant interactions and main effects (P-value > 0.05) were removed using a backward deletion procedure.

	df	F-value	P-value
Treatment	2, 12	5.519	0.020
Time	1, 65	14.289	<0.001
Round	–	–	–
Treatment × Time	–	–	–
Treatment × Round	–	–	–

Table 2. Results from linear mixed effect models testing the effects of treatment (Microsin, Piretrum Micro and control), sampling time (t-1, t + 1 and t + 9), sampling round (June, July and August), and their interactions, on species richness of flower-visiting insects. Non-significant interactions and main effects (P-value > 0. 05) were removed using abackward deletion procedure.

	df	F-value	P-value
Treatment	–	–	–
Time	–	–	–
Round	2,118	3.480	0.034
Treatment × Time	–	–	–
Treatment × Round	–	–	–

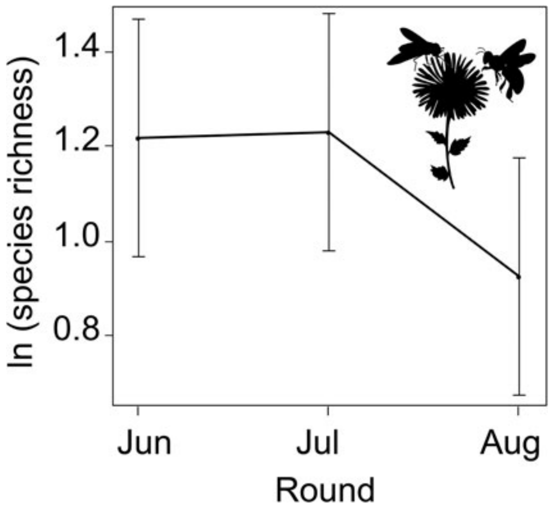

Figure 3. Species richness of flower-visiting insects over sampling rounds (June, July and August). Bars show confidence intervals at 95%.

albopictus was the most prevalent mosquito species followed by *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae). No other species were observed. The day after adulticide application, only the pyrethroid-based insecticide (Microsin) significantly reduced mosquito abundance, while the natural pyrethrum insecticide (Piretrum Micro) was not different from the control. Nine days after application the abundance of mosquito was similar between control and both adulticides (Figure 2; and Table 1). No effect of sampling round was found.

Flower-visiting insects

Five-hundred-twenty-four individuals, belonging to 87 species and morphospecies (i.e. taxa separated only by morphological characters), of flower-visiting insects were collected and identified: 41 morphospecies (364 specimens) of bees (Hymenoptera: Apidae), 24 species (66 specimens) of sphecids (Hymenoptera: Ampulicidae, Sphecidae, Crabronidae), 1 morphospecies (27 specimens) of asilids (Diptera: Asilidae), 4 morphospecies (13 specimens) of cuckoo wasps (Hymenoptera: Chrysididae), 2 morphospecies (15 specimens) of conopids (Diptera: Conopidae), 2 morphospecies (18 specimens) of soldier flies (Diptera: Stratiomyidae) and 13 species (21 specimens) of hoverflies (Diptera: Syrphidae). Fifty-six species (210 specimens) were found in control sites, 40 species (156 specimens) were found in sites treated with the pyrethroid insecticide and 48 (158 specimens) in sites treated with the natural pyrethrum insecticide. Species richness of flower-visitors significantly decreased over sampling rounds (Figure 3; and Table 2) but no effect of treatment and time was found.

Discussion

The efficacy of pyrethroid and natural pyrethrum insecticides in controlling population density of adult mosquitoes and how these insecticides impact non-target insects remain poorly studied in the field (but see Manica et al. 2017). Here, we found that Microsin (the synthetic pyrethroid insecticide) was more effective than Piretrum Micro (the natural pyrethrum insecticide) in reducing mosquito abundance. Our results confirmed that the high light instability and the short "knockdown" effect of natural pyrethrum compared to pyrethroids (Schleier and Peterson 2011) made the applications ineffective to control tiger mosquito populations even after 24 h. Despite laboratory bioassays suggests that pyrethrum may be a potential alternative candidate in areas where resistance to pyrethroid insecticides has become problematic (Duchon et al. 2009), its effectiveness in open field conditions appeared to be limited.

The relatively low population reduction in mosquito population after nine days with Microsin can be explained with a degradation of the active ingredient. The manufacture reports a persistence of 8–14 days on vegetation that is consistent with the population reduction observed. Other explanations might be related to the irritating effect of pyrethroid insecticide that often are not homogenously applied to the whole vegetation surface. The efficacy of adulticide application methods under field conditions may be further affected by mosquito behaviour. In particular, *Ae. albopictus* is mainly an exophilic and exophagic mosquito (Benedict et al. 2007) and the protection of some proportion of *Ae.*

albopictus population resting inside the vegetation may have a negative impact on adulticide efficacy (Marini et al. 2015). Finally, the small scale of the insecticide applications combined with the low persistence allow the mosquitos to quickly recolonize the green areas from the surrounding non-treated areas.

As adulticides target only adult individuals, repeated and frequent applications are needed to control population across generations (Bonds 2012). Here, eight applications were carried every two weeks to cover the period when adults were active. We found that the reduction in mosquito population was constant over sampling rounds and did not differed between treatments and control, suggesting that, due the low stability and persistence of pyrethroid insecticides in open environments, repeated treatments are needed to effectively control mosquito populations.

Although natural pyrethrum is generally expected to have a lower environmental impact compared to pyrethroid insecticides (Casida 1980), we found no difference between treatments when looking at the side-effects on non-target insects. The importance of a diverse and well-structured community of flower-visiting insects in the environment is well documented (Evans 2016; Kremer and Chaplin-Kramer 2007) and there is a general consensus that broad-spectrum adulticides can influence diversity and abundance of non-target insects (Boyce et al. 2007; Caron 1979; Ceuppens et al. 2015; Jensen et al. 1999; Kedwards et al. 1999; Schleier and Peterson 2010). We only found an effect of seasonality for all flower-visitor groups. However, despite the significant reduction of tiger mosquito abundance with the pyrethroid insecticide, we found that both Microsin and Piretrum Micro did not affect activity density of flower-visiting insects. Most insecticides are required to be tested for their side-effects on non-target organisms (ECHA, 2013). However, to our knowledge, there are a few field studies testing the effects of pyrethroids on non-target flower-visiting insects. Boyce et al. (2007) previously found that a pyrethrin-based adulticide limited mosquito population without influencing non-target large-bodied arthropods, among which the domestic bee (*Apis mellifera* L., 1758; Hymenoptera: Apidae), but had a negative effect on smaller arthropods. They attributed the results mainly to body size, suggesting that treatments aimed at controlling mosquitoes may not reach sufficient concentrations to be lethal to larger insects (Schleier and Peterson 2010). Moreover, it is now known that pyrethroid insecticides show repellent effects for honeybees (Rieth and Levin 1988) as well as other beneficial insects, reducing the exposure to insecticides (Desneux et al. 2007). Other studies (Caron 1979; Davis et al. 2007; Davis and Peterson 2008) suggested that observed differences in response between mosquitoes and other insects may be due to different time of activity between species. If treatments are applied when diurnally active non-target species are foraging (i.e. early morning, late evening and during the night), these insects should not be strongly affected by adulticide applications. In our study, treatments were targeted at *Ae. albopictus* and, despite the diurnal mate- and host-seeking activity, were applied very early morning (between 04:00 am and 06:00 am) when this species rests on woody vegetation (Allan et al. 2009; Lima-Camara 2010). Adult mosquitoes, and in particular *Ae. albopictus*, can be found in open habitats when host-seeking, but are more often resting in dense vegetation, presumably to avoid high light intensity, high temperatures, low humidity and strong wind (Jaenson 1988; Marini et al. 2015; Veronesi et al. 2012). The propensity for *Ae. albopictus* to rest on trees and bushes provides an excellent opportunity for targeted control, as it can maximize the efficiency of adulticides on mosquitoes and prevent intoxication of diurnal flower-visiting species. Adulticides were sprayed on bushes and trees up to the canopy. Hence, the herbaceous vegetation was not directly sprayed reducing the potential negative impact of the treatments on the non-target organisms visiting herbaceous flowering plants. The different spatial distribution of adult mosquitoes and flower-visiting insects may reduce the risks of intoxication for these groups (Davis et al. 2007; Davis and Peterson 2008).

Control against adult mosquitoes is quite expensive (Marini et al. 2015), and finding the more efficient control measure to limit mosquito population without affecting other groups is of major importance (Kreß et al. 2014). We showed that Microsin was more effective in reducing mosquito abundance and that Piretrum Micro was not sufficient to control mosquito populations even in the short term. Both adulticides were synergized with piperonyl butoxide to prevent enzyme degradation. However the the effect of both pyrethroids and natural pyrethrum on mosquitoes was not cumulative over sampling rounds. Since repeated applications are needed to control the mosquito population, this may cause insecticide resistance. The development of novel and complementary control strategies against mosquitoes may help to reduce the risk of insecticide resistance (Liu et al. 2006). Adulticide applications should be conducted taking into account the flight activity of target mosquito species (Bonds 2012). For most *Culex* and *Anopheles* species, after-sunset applications may be effective in both reducing population density and conserving non-target insects (Caron

1979; Veronesi et al. 2011). However, *Ae. albopictus* is a diurnal biter (Lima-Camara 2010), and for this species early-morning or sunset applications are needed. According to our results, we suggest to carry out the applications early in the morning focusing on bushes and woody vegetation avoiding contamination of flowering vegetation and grasslands. We showed that these simple recommendations can prevent intoxication of large-bodied non-target flower-visiting insects. Our study only tested the lethal effects of Microsin and Piretrum Micro insecticides sprayed at small spatial scale (generally < 0.1 ha) and our results might be different if insecticides are sprayed at larger spatial scales or if different formulations are tested. Moreover, treatments can have sub-lethal negative effects that we were not able to assess. Further studies evaluating the long-term effects of insecticide applications are, therefore, needed to fully understand how mosquito treatments affect flower visiting communities and other smaller-bodied organisms.

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