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# Analysis of population structure and insecticide resistance in mosquitoes of the genus *Culex*, *Anopheles* and *Aedes* from different environments of Greece with a history of mosquito borne disease transmission

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ABSTRACT

Greece has been recently affected by several mosquito borne diseases with the West Nile Virus (WNV) outbreak in 2010 being one of the largest reported in Europe. Currently at the epicenter of an economic and refugee crisis and visited by over 16 million tourists a year the integrated management of diseases transmitted by mosquitoes is a public health and economic priority. Vector control programs rely mainly on insecticides, however data on insecticide resistance and the mosquito fauna is essential for successful applications.

We determined the mosquito species composition and population dynamics in areas of increased vulnerability to vector borne disease transmission, as well as investigated the resistance status of major nuisance and disease vectors to insecticides. High mosquito densities were recorded in Thessaloniki and Evros, with *Aedes caspius*, a nuisance species, *Culex pipiens*, a known vector of WNV and *Anopheles hyrcanus* a potential vector of malaria being among the most prevalent species. Both vector species populations reached their peak in late summer. *Aedes albopictus* was recorded at high densities in Thessaloniki, but not in Evros. Notably, *Cx. pipiens* hybrids, which show an opportunistic biting behavior and are suspected to be involved in the transmission of the WNV, were recorded in considerable numbers in Thessaloniki and Attica. *Culex pipiens* and *An. hyrcanus*, but not *Ae. caspius* mosquitoes, showed moderate levels of resistance to deltamethrin. The presence of resistance in areas not exposed to vector control indicates that other factors could be selecting for resistance, i.e. pesticide applications for agriculture. Both L1014F and L101C kdr mutations were detected in *Cx. pipiens* populations. *Anopheles hyrcanus* resistance was not associated with mutations at the L1014 site. The Ace-1 mutations conferring insensitivity to organophosphates and carbamates were detected at low frequencies in all *Cx. pipiens* populations. Increased activity of P450s and esterases was found in *Cx. pipiens* individuals from Thessaloniki. Our study contributes evidence for sustainable and efficient vector control strategies and the prevention of disease outbreaks.

## 1. Introduction

After having been, to a large extent, eliminated from Southern Europe more than three decades ago, Vector Borne Diseases (VBDs) have recently re-appeared sporadically in this region due to several factors, such as climate changes, colonization of invasive vector species (i.e. *Ae. albopictus*), large numbers of tourists travelling annually into endemic areas and immigration of infected individuals (Danis et al., 2013). Greece is one of the European member states mostly affected by mosquito borne diseases with several important outbreaks reported across the country. For example, autochthonous malaria transmission reappeared in certain prefectures of Greece in 2009, and peaked in 2011 with 42 locally acquired malaria cases (HCDCP, 2015). West Nile Virus (WNV) poses also an important threat to human and animal health because of its capacity to cause large and unpredictable epidemics such as the Greek outbreak in Central Macedonia in 2010 with

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262 human clinical cases and 35 fatalities (Chaskopoulou et al., 2013; Chaskopoulou et al., 2016). Although the last large Dengue epidemic in Europe was observed during 1927–28 in Greece, with more than 1000 reported deaths (Papaevangelou and Halstead, 1977), the possibility of reintroduction of Dengue virus (DENV), as well as the new introduction of other tropical arboviruses such as Zika currently exists, due to the presence of competent mosquito vectors in the region.

Some of the most important competent vectors in Europe, also present in Greece, are members of the *Cx. pipiens* complex, which are the most important vectors of WNV. A detailed molecular analysis of species within this complex, during the 2010 WNV outbreak identified an increased frequency of hybrid *Cx. pipiens pipiens/molestus* biotypes (with a putative opportunistic biting behavior, i.e. feeding both on birds/WNV reservoirs and humans) in WNV infected regions (N. Greece) which was hypothesized to be associated with the epidemics (Gomes et al., 2013). From then onwards no follow up studies on biotype distribution and dynamics have been conducted. Other competent vectors of disease present in Greece are members of the *Anopheles maculipennis* complex, transmitting malaria, and *Aedes albopictus* transmitting Chinkungunya, DENV and Zika. To date, there is little information available on the seasonal activity profiles, of some of the major disease vectors. Furthermore, some remote areas along the borders of Greece, such as the agricultural region of Thrace along the Evros river between Turkey and Greece remain understudied in relation to their mosquito fauna. This cross-border region in particular is of major public health significance due to the high movements of immigrants and refugees towards Europe. The collection of entomological information on vector species and their seasonal abundance becomes of crucial importance to the understanding of, risks for introduction of pathogens in Europe, the risks these vulnerable populations face from potential local vector borne disease transmission, and the measures that need to be implemented to alleviate those risks (ECDC, 2015).

The prevention of mosquito-borne diseases, as well as the alleviation of nuisance caused by mosquitoes which negatively affects the quality of life and the development of tourism, largely relies on the implementation of vector control programs that utilize chemical, mechanical, and biological interventions to manage vector populations (Becker et al., 2010). In Europe chemical interventions are most commonly used and have relied on the use of biocides (public health insecticides) against mosquito larvae, such as organophosphates (i.e. temephos, although currently suspended in several countries), microbial insecticides (i.e. Bti), and insect growth regulators (i.e. diflubenzuron), applied in places where the immature mosquito stages are found, such as rice-fields. Biocides for the control of adult mosquitoes, such as pyrethroids (i.e. deltamethrin) are also available and are applied as household treatments or via outdoors residual and/or ultra low volume applications in places where adult mosquitoes rest or seek for a food source. Biocides are a necessary tool for disease prevention but a major problem associated with their intensive use is the development of insecticide resistance. Furthermore, many biocides share the same active ingredient (i.e. pyrethroids) with insecticides used in agriculture, which adds selection pressure for insecticide resistance (Reid and McKenzie, 2016) as vectors become exposed to agricultural chemicals from contamination of nearby breeding sites.

Insecticide resistance is primarily conferred by two molecular mechanisms: the target site resistance and the metabolic resistance. Target site resistance is linked to specific point mutations on the insecticide's target molecule, which render it less sensitive to inhibition. Well studied cases are the mutations F290V and G119S on acetylcholinesterase (Ace-1), linked to resistance against organophosphates and carbamates and mutations on the voltage gated sodium channel of mosquitoes, like the L1014F of *Cx. pipiens*, associated with pyrethroid resistance. An insecticide resistance monitoring study that was conducted in 2008–10 on *Cx. pipiens* populations from five regions in Greece found the mutation L1014F at frequencies ranging from 28,5% to 63% with the majority of the resistant alleles detected in heterozygocity (Kioulos et al., 2014). In a recent study in neighboring Western Turkey (Taskin et al., 2016) both the L1014F and the less common and less characterized in terms of its precise contribution to the resistant phenotypes L1014C mutation were detected in *Cx. pipiens* populations. The majority of the mosquitoes were homozygous for the L1014C mutation with allele frequencies ranging from 53% to 83%. Metobolic resistance, the second major insecticide resistance mechanism involves the increased activity of detoxification enzymes like P450s, esterases and glutathione-S transferases, which either metabolize or sequester the insecticide and to our knowledge, there are no monitoring studies on the metabolic resistance status of mosquito vector field populations from Greece.

To increase our understanding on the ecology of important mosquito vectors in Europe and the mechanisms of insecticide resistance we designed a field study in Greece that aimed to a) determine species composition and population dynamics of mosquito populations in areas of increased vulnerability to disease transmission, and b) investigate the resistance status of major nuisance and disease vectors to insecticides and the principal resistance mechanisms (target site, metabolic resistance).

## 2. Materials and methods

### 2.1. Study regions and mosquito surveillance

Two major agricultural regions in North Greece (Region of Central Macedonia − Thessaloniki Regional Unit and Region of East Macedonia with part of Thrace − Evros Regional Unit bordering with Turkey) and one major urban center (Attica Region) in Central Greece were surveyed in 2014 (Evros, Thessaloniki) and 2015 (Attica) with a total of nineteen sampling sites (Fig. 1.) All three regions have prolific mosquito breeding sites, a history of vector borne disease (VBD) transmission (Chaskopoulou et al., 2013; Chaskopoulou et al., 2016; HCDCP, 2013a), varying levels of organized vector control (VC) and they often host large numbers of economic and political refugees/immigrants. Thessaloniki and Evros represent major agricultural ecosystems with similar characteristics (river-based irrigation sources) and crops. Rice is one of the major crops with approximately 15–20,000 ha in Thessaloniki (Region of Central Macedonia) and at least 30,000 ha in Thrace both in the Greek and Turkish province (Sürek, 2001) right adjacent to the east and west side of the Evros river. Cotton is also a dominant crop ranging approximately from 9000–14,000 ha in Thessaloniki (Region of Central Macedonia) and 16,000-20,000 ha in Evros (Lemonakis and Chatzioglou, 2002). Pyrethroid insecticides are one of the most dominant classes of insecticides used in Greece (Hellenic Ministry of Rural Development and Food, 2017). The agricultural regions of both Evros and Thessaloniki are exposed to pyrethroid spraying for agricultural purposes, e.g. for the control of cotton pests (Chaudhry, 1996). Furthermore, the agricultural area of Thessaloniki was exposed to pyrethroid spraying associated with mosquito control (Chaskopoulou et al., 2016). The area of Attica that was sampled could be characterized as suburban with the majority of mosquito breeding sites being drainage canals and artificial containers. Attica is exposed to both household related and outdoors pyrethroid spraying for mosquito control.

Thessaloniki and Evros were sampled for adult mosquitoes biweekly using CDC light traps baited with dry ice (Chaskopoulou et al., 2016) from May to October from 18.00 p.m-8.00 a.m. Overall 9 sampling events were conducted per sampling site. In Attica *Cx. pipiens* larvae were specifically sampled bi-monthly from July to September 2015. Overall two sampling events were conducted per sampling site. The standard dipping technique was used where a 400 ml in volume plastic cup with an extensible handle was partially submersed in water for at least 5 times per breeding site. The larvae captured per dip were collected in vials for further analysis. The CDC light traps were deployed outdoors at ca. 1.5 m height and were distributed evenly within the agricultural zone (Thessaloniki) or following the Evros river pattern within a distance of 400 m–2 km from the river bed. Climate data