# MOSQUITO RESISTANCE TO INSECTICIDES IN BULGARIA: WHAT DO WE KNOW AND SHOULD WE WORRY?

O. Mikov[1], S. Boneva-Bukovska[2], N. Ivanova-Aleksandrova[2], M. Nikolova[2], S. Stoitsova[2], A. Ivanova[1]

[1] Department of Parasitology and Tropical Medicine, National Centre of Infectious and Parasitic Diseases, Sofia
[2] Department of Epidemiology, National Centre of Infectious and Parasitic Diseases, Sofia

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## ABSTRACT

Mosquitoes are a group of blood-sucking insects that plays a major role in disease transmission in both humans and animals. They can pose a heavy nuisance, or serve as vectors of numerous viruses and parasites on most continents, including Europe. The strategy for successful control of the mosquito populations relies on the use of insecticides of five different categories: organochlorines, organophosphates, carbamates, pyrethroids, and biopesticides. The extensive use of the first four classes has resulted in the development of resistance originating in various mechanisms. The main ones are metabolic resistance and genetic resistance, manifested by point mutations in the insecticide targets, resulting in limited binding of neurotoxic substances. Much data is available on mosquito insecticide resistance in Bulgaria between 1948 and 1990, but only regarding organochlorines, organophosphates and carbamates. There is no data on pyrethroid resistance, which should raise an alarm for both public health authorities and private pest control companies since the only insecticides registered for professional mosquito control nowadays are pyrethroids. Therefore, there is an urgent need for the use of modern multidisciplinary approaches to study the resistance of native and invasive species of mosquitoes to insecticides, which should be a key contribution to elucidating their role in the circulation of pathogens of humans and animals and optimizing methods for controlling their populations.

**ADDRESS FOR CORRESPONDENCE:**
Ognyan Mikov
National Centre of Infectious and Parasitic Diseases
26, Yanko Sakazov Blvd.
1504 Sofia, Bulgaria
e-mail: mikov@ncipd.org

## INTRODUCTION

Control over infectious diseases is of paramount importance for the global economy and public health. Much of the world's recent pandemics have originated from wildlife zoonoses, and about a quarter of the pathogen invasions during the past century have been transmitted by blood-sucking insects acting as vectors and aiding in spreading the disease-causing agents (1).

During the latter part of the 20th century, the role of mosquitoes as vectors of diseases of public health concern was generally considered to be limited to the tropics. The disappearance and eradication of dengue and malaria in Europe by the 1950s saw a European landscape with minimal or no mosquito-borne disease. However, at the start of the 21st century, the picture is different. A world increasingly connected through travel, trade and tourism means that Europe will encounter regular transmission of mosquito-borne diseases, a trend confirmed by advances in pathogen detection. West Nile virus is now known to be transmitted to humans by native mosquitoes in several European and neighbouring countries each year. In recent years, Europe has witnessed the return of malaria, the emergence of the Usutu virus, as well as the ongoing transmission of the Sindbis virus, all transmitted by native European mosquitoes (2), not to mention the risks posed by the introduction and establishment of invasive mosquitoes and the associated outbreaks of Zika, chikungunya and dengue viruses. The changes in mosquito-borne disease epidemiology in Europe over recent years cannot be ignored, and risks to public health cannot be understated (3).

Successful control of mosquitoes is essential to prevent the spread and outbreaks of mosquito-borne diseases. This strategy relies mainly on the use of insecticides, both larvicides and adulticides in the case of mosquitoes. So far, five categories of insecticides have been recommended by the World Health Organisation (WHO) for use against adult mosquitoes in public health programmes (4). A recent global survey collecting data from 87 countries and analysing the use of vector control insecticides in spraying operations between 2010 and 2019 showed that organochlorines, organophosphates, carbamates, and pyrethroids are the most used classes of insecticides worldwide (5). Among them, pyrethroids are the most widely used, although recently disease control programmes have significantly reduced their dependence on pyrethroids due to the emergence of resistance (4). Pyrethroids offer several advantages over other classes of insecticides in terms of cost, human safety (low mammalian toxicity) and duration of residual action. They are widely used in agriculture and as household biocidal products. The frequent use of pyrethroids for impregnation of bednets and long-term use of some other classes, such as organochlorine (e.g. dichlorodiphenyltrichloroethane (DDT)) and organophosphate compounds, has probably contributed to the development of pyrethroid resistance among malaria vectors around the world and especially among African species. Since 2010, resistance to at least one class of insecticides has been reported in at least one type of malaria vector in 60 out of 96 malaria-endemic countries, and 49 countries have reported resistance to at least two classes of insecticides (6). Unfortunately, resistance to pyrethroids is increasingly observed in many mosquito species, which makes it difficult to control the vectors (7, 8).

Various mechanisms of insecticidal resistance have been observed in mosquitoes. The main ones are metabolic resistance, which is determined by three large enzyme families (glutathione S-transferases, multifunctional monooxygenases and carboxylesterases) and genetic resistance, manifested by point mutations in the insecticide targets, resulting in limited binding of neurotoxic substances. Several such target sites have been described: the *Rd1* gene encoding gamma-aminobutyric acid receptors, the *ace-1* gene encoding acetylcholinesterase (9), and the *kdr* gene encoding potential-dependent sodium channels (10). The main mechanism of resistance to DDT and pyrethroids in mosquitoes is the so-called knockdown resistance, which is manifested by a decrease in the sensitivity of the receptors for these substances in the potential-dependent sodium channels of nerve axons. This results from point mutations in the *kdr* gene (10, 11).

## THE MOSQUITOES IN BULGARIA

The blood-sucking mosquitoes (Diptera: Culicidae) are arguably the most well-studied group of blood-sucking insects in Bulgaria. The earliest data on species composition date back to the early 20th century in relation to malaria transmitted by them (12). Until the eradication of malaria in the country in 1965 (13), the prevailing part of the research was mainly related to malaria mosquitoes. In the coming decades, more and more attention has been paid to non-malarial mosquito species, and an increasing number of publications became purely faunistic (12). As a result, 39 species have been registered in the country by 1990 (14). Due to changes in taxonomy in recent years (15) and the finding of the invasive Asian tiger mosquito *Aedes albopictus* in Bulgaria (16), their number is somewhere between 47 (17) and 52 (18).

So far, all studies on the biodiversity of blood-sucking mosquitoes in Bulgaria have been based only on morphological methods of identification (12). This creates difficulties and raises a number of unresolved questions about the species composition of malarial mosquitoes from the *Anopheles maculipennis* s. l. complex, as well as non-malarial mosquitoes from the *Culex pipiens* s. l. complex. The potential significance of these species as carriers of malaria and West Nile fever, respectively, requires the conduct of studies using modern genetic methods.

## THE USE OF CHEMICAL INSECTICIDES FOR MOSQUITO CONTROL IN BULGARIA

As of 1948, Bulgaria began employing the use of the powerful organochlorine insecticide DDT against endemic malaria at that time (16). At the very beginning of its use, the studies focused on determining its effectiveness against pest insects and especially against malaria mosquitoes. In the first year following DDT application, significant results were observed (19). The discovery of insecticide-resistant mosquitoes in several European countries later in the 1950s directed the research to determine whether it has developed in Bulgaria. Numerous studies were conducted in different regions of the country - Ruse district (20, 21, 23), Varna district (22, 23), Burgas, Yambol, Plovdiv and Sofia districts (23, 24) on *An. maculipennis* and *An. sacharovi,* the two main vectors of malaria. During the experiments with DDT, hexachlorane, and dieldrin (all of them organochlorine insecticides) in field and laboratory conditions, the occurrence of physiological resistance in the studied species was not detected. An increase in tolerance to these insecticides was reported in overwintering mosquitoes at the beginning of the malaria season and in those entering diapause at the end of the season. *An. sacharovi*, considered a more dangerous vector along the Black Sea coast, was identified as slightly more sensitive than *An. maculipennis* (23).

Since 1965, Bulgaria has been officially declared a malaria-free country by the WHO (13). Since then, the attention of zoologists and physicians in the country has shifted from the study of mainly anopheline mosquitoes to the control of all blood-sucking mosquitoes.

According to WHO requirements, since 1961, no DDT or other insecticide has been used in the country to control malaria mosquitoes. However, because it has been applied for more than ten years, new studies began in 1963 on the occurrence of resistance to adulticides in malaria vectors. Between 1963 and 1965, 70 experiments with 8650 *An. maculipennis* mosquitoes were conducted in 23 settlements from Sofia, Pleven, Plovdiv, Pazardzhik, Stara Zagora, Silistra, Sliven, Ruse, Haskovo and Yambol districts. Employing standard WHO bioassay methods, a certain decrease in sensitivity to DDT has been detected, but no data regarding resistance was found (25). Between 1963 and 1975, longer experiments on the same species were conducted in the Varna district, and the occurrence of resistance to organochlorine insecticides (DDT and dieldrin) and tolerance to carbamates was detected. A high sensitivity of the species was reported to organophosphate formulations (26). A little later, resistance to DDT was established in the Silistra region, and to dieldrin in settlements in Pleven, Burgas, Vidin, Haskovo and Silistra districts (27, 28).

More attention at this time was paid to the use of larvicides. Between 1965 and 1967, field experiments with organophosphate larvicides (diazinon and dichlorvos) were conducted for the first time in places along the southern half of the Black Sea coast and near the city of Sofia. They involved *Cx. pipiens*, *Ae. caspius* and *An. maculipennis* and identified a stronger insecticidal effect than the control with DDT and lindane (29). In 1966 and 1967, experiments with DDT, lindane and dieldrin conducted in six districts of the country (Sofia, Varna, Dobrich, Burgas, Pazardzhik and Blagoevgrad) did not identify any resistance in larvae of *An. maculipennis, Ae. caspius, Cx. pipiens, Cx. theileri* and *Cx. hortensis* (30).

In 1967 and 1968, field experiments employing both organochlorine and organophosphate larvicides were performed on larvae and adults of *Cx. pipiens* and *An. maculipennis* in Sofia and Plovdiv. In both cases, resistance was not detected, but larvicidal treatments were found to be more effective than adulticidal ones (31, 32). No resistance to diazinon in larvae of *Cx. pipiens, Cx. modestus* and *An. maculipennis* was observed in biotopes near the Danube River in the Pleven region (33).

A comparison between the efficacy of chemical and biological means to control *Ae. caspius* larvae in rice fields was made for the first time in 1970 near Plovdiv. Several organophosphates (diazinon, fenitrothion, temephos and tetrachlorvinphos) were compared to Eastern mosquitofish (*Gambusia holbrooki*) and European carp (*Cyprinus carpio*). In comparison to chemical insecticides, fish species showed better and longer-lasting effects in keeping mosquito larvae in low densities (34).

Between 1968 and 1972, numerous experiments were performed in the Pleven district on adults and larvae of *An. maculipennis, Cx. pipiens* and *Cx. modestus* under field and laboratory conditions using several organochlorine and organophosphate insecticides. Resistance to dieldrin was detected in *An. maculipennis* (35, 36). During the same period, laboratory experiments were conducted with organochlorines and organophosphates on the larvae of *An. maculipennis, Cx. pipiens*, *Cx. theileri*, *Cx. hortensis* and *Ae. caspius* from Plovdiv and Sofia identified resistance to DDT and lindane in *Cx. pipiens* only (37). Similar to that, other laboratory and field trials with larvae of *An. maculipennis, Cx. pipiens* and *Ae. caspius* from Sofia demonstrated full susceptibility to organophosphates (38).

In 1971 and 1972, carbamates were employed together with organochlorines and organophosphates for the first time in field and laboratory experiments performed in Sofia, Varna, Burgas and Pleven districts. No resistance was detected in *Cx. pipiens* larvae (39).

Between 1978 and 1980, the first laboratory experiments using biocontrol agents other than fish species were conducted with three different strains of *Bacillus thuringiensis*. The larvae of *Cx. pipiens* showed no resistance to any of the strains (40).

No peer-reviewed articles on mosquito insecticide resistance exist between 1981 and 1991. Nevertheless, there are a few reports in the grey literature, showing resistance to DDT and susceptibility to organophosphates in Sliven, Sofia, and Montana districts (41, 42, 43, 44).

In the last 32 years since 1990, no studies have been conducted in Bulgaria on the insecticidal resistance of native mosquito species, either by classical WHO bioassays or by modern molecular genetic methods. Moreover, the availability of biocides for mosquito control drastically changed after banning organochlorines, organophosphates and carbamates, and nowadays only pyrethroids are registered as adulticides for professional use (45). Meanwhile, the susceptibility and vulnerability to malaria in the country are preserved (46), and *Cx pipiens* s. l. has been shown to transmit the West Nile virus (47). The entry into Bulgaria in 2011 of the invasive Asian tiger mosquito *Ae. albopictus* (16) creates an additional serious problem, as the species continues to expand its range on the territory of 25 of the 28 districts in the country (O. Mikov, own unpublished data), which shows the ineffectiveness of adulticidal methods to control it. In addition, resistance of this species to pyrethroids has already been reported in Europe and Asia (48, 49).

## CONCLUSIONS

The lack of studies on mosquito insecticide resistance in Bulgaria for more than 30 years should raise concern for both public health authorities and private pest control companies. Since all the adulticides registered for professional use in Bulgaria belong to the pyrethroid class, and only a few larvicides based on insect growth regulators and *Bacillus thuringiensis israelensis* are available (45), there is an urgent need for contemporary data on the populations of the most common native and invasive mosquito species in the country.

There is numerous data on the pyrethroid resistance of *Cx. pipiens* from Romania (50), Greece (51, 52, 53) and Turkey (54, 55, 56), most of them from regions bordering Bulgaria. A high level of resistance to pyrethroids was also observed in *An. maculipennis* in the European part of Turkey (57). The Asian tiger mosquito *Ae. albopictus* already showed possible incipient pyrethroid resistance due to the presence of *kdr* mutations in Greece (58), but also a spatial trend with the resistant *kdr* allele being mostly detected in two clusters in Europe. The first cluster, designated as the "Western Cluster," includes mainly Mediterranean coastal sites from Italy (Rome and Bari), France (Nice and Perpignan) and Malta (Luqa) but also sites in Spain (Basauri) and Switzerland (Basel). The second cluster, designated as the “Eastern cluster,” includes the easternmost sites on both sides of the Black Sea from Bulgaria (Burgas), Turkey (Istanbul and Igneada) and Georgia (Batumi) as well as one site from Romania (Bucharest) (59).

The application of modern multidisciplinary approaches studying the resistance of native and invasive species of blood-sucking mosquitoes to insecticides should be a key contribution to elucidating their role in the circulation of pathogens of humans and animals and optimizing methods for controlling their populations.

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