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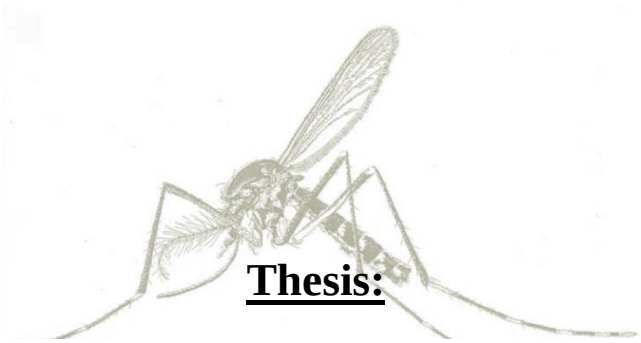
SCHOOL OF SCIENCE

FACULTY OF BIOLOGY

POSTGRADUATE PROGRAM IN MICROBIAL BIOTECHNOLOGY



Thesis:

Application of diagnostics for mosquito vector incrimination & innovative biotechnology- based methods for disease control

by Sofia Balaska, Master's student

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Περίληψη

Τα κουνούπια, ειδικότερα είδη των γενών *Aedes*, *Anopheles* και *Culex,* είναι οι πιο κοινοί φορείς ασθενειών παγκοσμίως, προκαλώντας περισσότερα από 500 εκατομμύρια ανθρώπινα κρούσματα ετησίως, εξαιτίας της μετάδοσης παθογόνων, όπως το παράσιτο της ελονοσίας, ο Δάγκειος ιός, ο ιός του Δυτικού Νείλου, ο ιός Zika, κ.λπ. Η πλειοψηφία των κρουσμάτων συμβαίνει στην Αφρική, την Κεντρική και Λατινική Αμερική και την νοτιοανατολική Ασία, ωστόσο, τις τελευταίες 2 δεκαετίες, ορισμένες ασθένειες έχουν (επαν)εμφανιστεί και στην Ευρώπη.

Η καταπολέμηση των εντομομεταδιδόμενων ασθενειών βασίζεται σε μεγάλο βαθμό στον έλεγχο του εντόμου-φορέα, κυρίως με τη χρήση εντομοκτόνων (π.χ. πυρεθροειδή, οργανοφωσφορικά, ρυθμιστές ανάπτυξης). Όμως, ο περιορισμένος αριθμός εγκεκριμένων εντομοκτόνων σε συνδυασμό με την αυξανόμενη εμφάνιση ανθεκτικότητας σ'αυτά τονίζουν τη σημασία της συνεχούς εντομολογικής επιτήρησης. Ο αριθμός τέτοιων μελετών για την Ελλάδα είναι μικρός. Το είδος *Aedes albopictus*, γνωστό ως Ασιατικό κουνούπι τίγρης, αποτελεί σημαντικό κίνδυνο για τη δημόσια υγεία, με παγκόσμια διασπορά. Η παρουσία του στην Ελλάδα είναι ευρεία και, πιθανώς, συνδεδεμένη με τις πρόσφατες επιδημίες του ιού του Δυτικού Νείλου. Το πρώτο μέρος της παρούσας μεταπτυχιακής μελέτης αφορά στην ανάλυση της ανθεκτικότητας σε εντομοκτόνα πληθυσμών *Ae. albopictus* από διαφορετικές περιοχές της Ελλάδας, με τη χρήση μοριακών διαγνωστικών εργαλείων. Η μεταλλαγή F1534C στο γονίδιο που κωδικοποιεί για το κανάλι νατρίου, που προσδίδει ανθεκτικότητα στα πυρεθροειδή, βρέθηκε σε όλες τις δειγματοληπτικές περιοχές, με υψηλότερη συχνότητα στην Αθήνα, ενώ η I1532T στο ίδιο γονίδιο εντοπίστηκε για πρώτη φορά στη χώρα μας, με σχετικά χαμηλή συχνότητα. Ανθεκτικότητα στο temephos, λόγω υπερέκφρασης των καρβοξυλεστερασών 3 και 6, βρέθηκε επίσης διαδεδομένη. Τέλος, δεν ανιχνεύθηκε κάποια μεταλλαγή σχετιζόμενη με ανθεκτικότητα στο προνυμφοκτόνο diflubenzuron.

Οι περιορισμοί που προκύπτουν από την εκτενή χρήση εντομοκτόνων υποδεικνύουν την ανάγκη εφαρμογής ολοκληρωμένης στρατηγικής ελέγχου των κουνουπιών-φορέων. Στο δεύτερο τμήμα της συγκεκριμένης εργασίας, μελετήθηκε μια μέθοδος γενετικού ελέγχου μεταδιδόμενων ασθενειών: πολυ-αμιδο-αμίνες (PAAs) αξιολογήθηκαν ως πολυμερικοί φορείς δίκλωνου RNA (dsRNA) στο κουνούπι *An. gambiae*, με σκοπό τη σίγηση γονιδίων (RNAi) του φορέα που υποβοηθούν τη διέλευση του παρασίτου της ελονοσίας από το έντερό του. dsRNA εναντίον των γονιδίων *CACTUS* και *ESP* του κουνουπιού συζεύκτηκαν με PAAs και εξετάστηκε η σταθερότητα των νανοσωματιδίων σε αναγωγικό περιβάλλον. Εν συνεχεία, τα νανοσωματίδια χορηγήθηκαν σε ενήλικα κουνούπια μέσω τροφής ή εμβολίου. Η σίγηση των γονιδίων-στόχων στο έντερο, ήταν ικανοποιητική, ως πρώτη ένδειξη. Αυτά τα προκαταρκτικά αποτελέσματα θα αξιοποιηθούν ως θεμέλια στα επόμενα πειραματικά βήματα με ορισμένες κάποιες βελτιώσεις/ τροποποιήσεις.

Summary

Mosquitoes, especially species of the genera *Aedes*, *Anopheles* and *Culex,* are the most common disease vectors worldwide, responsible for more than 500 million human cases annually, due to malaria, Dengue virus, West Nile virus, Zika virus, etc., transmission. Mosquito-borne diseases occur mainly in Africa, Central and Latin America and southeastern Asia, but during the last two decades they have (re-)emerged also in Europe.

Prevention of mosquito-borne diseases depends to a great extent on vector control; insecticides (e.g. pyrethroids, organophosphates, growth regulators, etc) are the most powerful tool. However, the limited number of available formulations along with the increasing occurence of insecticide resistance highlight the importance of continuous surveillance and evidence-based control. There is a small number of such recent studies in Greece. *Aedes albopictus*, commonly known as the Asian tiger mosquito, is a major risk for public health, being globally dispersed. Its presence in Greece is also wide and potentially associated with West Nile virus circulation, over the last years. Therefore, we conducted an extended analysis on the insecticide resistance status of *Ae. albopictus* populations throughout Greece, using molecular diagnostic techniques. Target-site resistance mutation F1534C in the *vssc* gene leading to pyrethroid resistance was spread across the country, with the highest frequency in Athens, while mutation *vssc* I1532T was reported for the first time in Greece, in low frequencies. Metabolic resistance to temephos attributed to carboxylesterases 3 and 6 overexpression was also recorded extensively in many sampling areas. No mutation correlated with resistance to the larvicide diflubenzuron was detected.

Limitations arisen by the extended use of insecticides determine the necessity of integrated vector control methods. In the second part of the present study, RNAi was used as a gene silencing mechanism to block malaria parasite transmission to humans; polyamidoamines (PAAs) were evaluated as polymeric carriers of dsRNA molecules in *Anopheles gambiae*, against mosquito genes that promote *Plasmodium* invasion of the midgut. dsRNAs against *CACTUS* and *ESP* genes were conjugated to PAAs. Nanoparticle stability was estimated, and then they were administered to adult mosquitoes both by injecting and feeding them. A moderate knock-down efficiency of the nanoparticles against the genes expression in the mosquito midgut was measured. Many experiment modifications are required and our findings are still preliminary, but of fundamental importance for the next steps.

Contents

INTRODUCTION

CHAPTER 1:

Mosquito Taxonomy and Biology

1.1 Mosquito Taxonomy

Mosquitoes are insects belonging to the Diptera class and the Culicidae family (Meigen, 1818). The family composes of approximately 3.601 described species and subspecies (http://www.mosquitocatalog.org/) organized in two subfamilies: the Anophelinae and the Culicinae, including more than 480 and 3.100 species, respectively. Among all the mosquito genera, *Anopheles* is the best-known of the Anophelinae subfamily and *Aedes* and *Culex* of the Culicinae subfamily, due to their medical importance and widespread geographical distribution. *Mansonia, Culiseta, Haemagogus* and *Coquillettidia* are other common mosquito genera [54, 136].

1.2 Mosquito Ecology

All mosquito species need an aquatic habitat to complete their development and life cycle [54]; water-bodies, permanent or temporary, clean or highly-polluted, natural or artificial, including small-water accumulations, like buckets, flower pots, tyres, etc. can be used as breeding sites. Common habitats colonized by mosquitoes are flooded areas, swampy woodlands, tree-holes, coastal areas, etc. Mosquitoes display high ecological flexibility as they are capable of adapting to a wide variety of urban and rural environments in both tropical and temperate climates, demonstrating a global spread, apart from Antartica [54]. Moreover, the eggs of many mosquito species are tolerant to desiccation and can survive for more than a year [54].

1.3 Mosquito Life Cycle

All the mosquito species share a common life cycle, consisting of 4 stages: egg, larva, pupa and adult **(Image 1).** Depending on the mosquito species and the environment and breeding conditions (temperature, larvae density, food availability, etc), the life cycle needs from 1 to 2 weeks to complete [33]. Despite the fact that almost every natural or man-made collection of water can support mosquito production [37], different mosquito species flourish in different habitats.

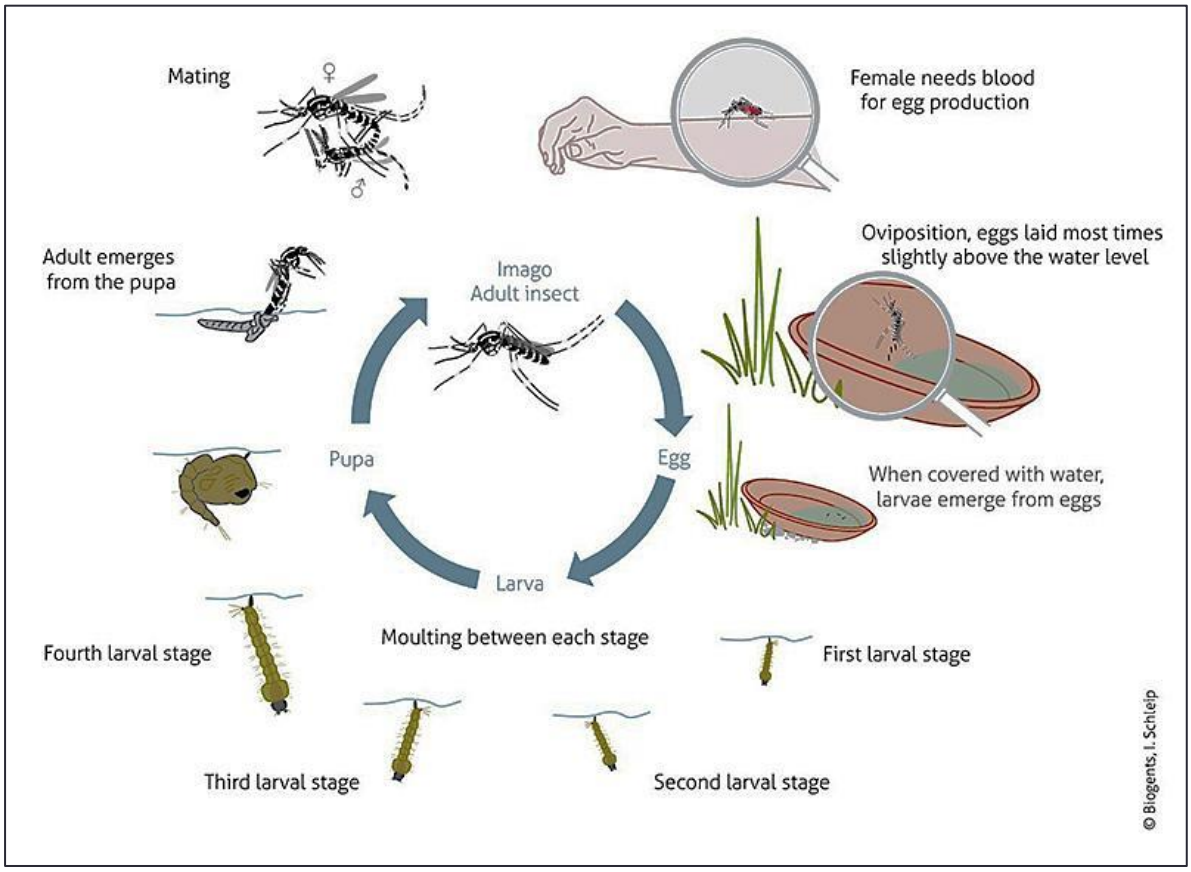


Image 1: Generalized life cycle of mosquitoes [33].

Following mating, female mosquitoes lay their eggs either individually (*Anopheles* and *Aedes* species) or in attached groups, called rafts (*Culex* species) [23] **(Image 2)**, directly on the surface of standing water, in treeholes or in containers flooding from rain or irrigation. Females can oviposit either once during their lifetime or at intervals, but at least one blood-meal is required for this process. They usually produce 30-300 eggs at a time [33]. Initially, eggs are transparent, but gradually they darken to brown or black [34].

Image 2: Eggs laid **(A)** in rafts by *Culex* mosquitoes and **(B), (C)** individually by *Aedes* and *Anopheles* mosquitoes, respectively. Note the float on both sides of each Anopheles egg (C). (Sources: University of Hawaii Reed Lab, [11]; bei RESOURCES, Supporting Infectious Diseases Research, [2]; PIXELS [28])

The time that eggs need to hatch depends on the temperature and the presence of water; for some species, this will happen within a few days after eggs have been laid on water. Frequently, mosquitoes overwinter in the egg stage, but some species also as larvae or adults [54].

The length of the larval stage is determined by the species, the temperature of water and the food availability and it can range from 4 to 14 days. The aquatic habitat is necessary through the whole larval development. Larvae feed on microorganisms the water contains [34]. They hang suspended from the water surface, breathing air through a siphon extended from their posterior **(Image 3A)**; *Anopheles* larvae lack this siphon tube [52]. Larvae development goes through a series of stages, called instars, in which they molt and enlarge **(Image 3B)**, they are active and light- and movement-sensitive, until they reach the comma-shaped pupal stage [34].

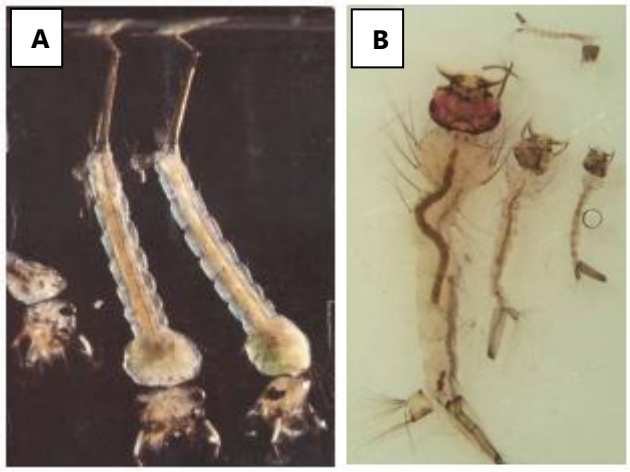


Image 3: **(A)** Larvae hanging from the water surface and breathing using their siphon. **(B)** Larvae skin discarded upon molting and transition to the serial instars [34].

The pupal stage requires no food, but still breaths air through a pair of tube-like organs, called trumpets, from the water surface **(Image 4)**. Pupae are sensitive to light, temperature, shadows and any disturbances. They are physically active, demonstrating a rolling/ trumbling action. This stage lasts from 1 to 4 days [34].

Image 4: *Culex sp*. pupa breathing from the water surface using its trumpets [34].

Once a pupa is mature and the adult tissues have developed, the skin splits letting a fully developed adult mosquito to emerge above the water surface, where it rests for a short period of time in order to let its wings and body to dry before flying off.

Usually, male mosquitoes emerge first and "wait" near the breeding site for the females. Adult mortality rates are high; hence, mating occurs soon after the adult emergence. Female mosquitoes usually mate once [14]. Male mosquitoes live 6-7 days on average, feeding primarily on plant nectars or other sugar sources. Females' estimated average lifespan is 6 weeks, but if the food supply is enough they can live for up to 5 months or more [34]. Apart from plant nectars, female mosquitoes require blood-meal(s), usually from mammals, birds, reptiles or amphibians, for nourishment and egg development. They are attracted to their host by CO_2, temperature patterns and a variety of other volatile substances they produce. Female mosquito gonotrophic cycle includes the search for a host of preference and blood-feeding, the digestion of the blood-meal, egg maturation and oviposition in a suitable site; under ideal conditions, this procedure takes approximately 3 days [52].

CHAPTER 2:

Mosquito Vectors of Infectious Diseases

2.1. Why are mosquitoes a problem?

2.1.a. Nuisance caused by Mosquitoes

The mosquito bite causes a common allergic reaction to humans, including redness and itchiness, as an immunological response to proteins of the mosquito saliva. According to the American Academy of Asthma, Allergy and Immunology [1], a mosquito must have a contact for 6 seconds or longer in order to produce this reaction. In most cases, symptoms disappear after 48 hours. Larger mosquito bites may cause a larger area of itching, bruises or hives at or around the site of bite. Interesting is the case of Skeeter syndrome, a condition in which a mosquito bite induces a large local inflammatory reaction followed by fever [12].

Added to that, mosquito bites compose an important nuisance problem, especially during the summer. Some mosquitoes, such as *Aedes albopictus*, a day-time bitter, adapted to urban areas, are aggressive bitters and the majority of them have a preference on human blood. These traits have an impact in areas or countries that rely their economy on agriculture and/or tourism. The problem is more intense in countries with temperate climate. Increased density of mosquito populations is a discouraging factor for livestock, too; apart from transmitting pathogens to animals (e.g. West Nile virus to horses), they also cause irrigation and blood loss, extensive weight loss and decrease in milk production, specifically in cattle and goats [39]. Besides, in urban and sub-urban residencies, the nuisance associated with mosquitoes affects the quality of life and the daily outdoor activities of the local populations [82].

2.1.b. Public Health Threat: Mosquito-borne Diseases

Vectors are living organisms that have the ability to transmit infectious diseases from human to human or from animals to humans. Vector-borne diseases constitute more than 17% of total infectious diseases, causing at least 700.000 deaths per year (WHO, 2017; https://www.who.int). The majority of vectors are insects that after having

ingested an infective microorganism, virus or worm obtained via a blood-meal from an infected host, they inject it to a new host during the next blood-meal. Mosquitoes (mainly *Anopheles, Aedes* and *Culex* species) compose major disease vectors among other insects, including flies (*Chrysops, Simuliidae, Glossina* species), sand flies (*Phlebotomus* species), bugs (subfamily Triatominae) and ticks (*Ixodes, Amblyomma, Dermacentor* species).

Mosquitoes, the deadliest animals to humans and the most common disease vectors, causing approximately 2.7 million dealths and 500 million human cases annually (http://www.mosquitoreviews.com/mosquito-statistics/) are in the epicenter of the entomological research interest. More than half of world's population lives at risk of an infected mosquito bite, carrying malaria, Dengue virus, West Nile virus, Zika virus, lymphatic filariasis, etc (WHO).

The majority of mosquito-borne disease cases occur in Africa, Central and Latin America and southeastern Asia, in humid tropic and sub-tropic regions, where almost three quarters of all mosquito species are found [94]. Some of them are included in the so called "neglected" tropical diseases (e.g. malaria, lymphatic filariasis among other tropical diseases, such as Chagas disease, leishmaniasis, schistostomiasis, etc.), as they predominantly affect poor populations in low income countries, lacking access to clean water, adequate sanitation, medical treatment, safe housing conditions and, thus experiencing the cycle of poverty [128]. However, due to the changing climate conditions and globalization, the introduction and establishment of mosquito vectors in new developed countries is a realistic public health threat.

2.2. Ecology and Distribution of Major Mosquito Vectors

2.2.1. *Aedes* Mosquitoes

The genus *Aedes* consists of more than 900 mosquito species [5], originally found in tropical and sub-tropical areas, but currently dispersed all over the world, including temperate and cooler habitats. *Aedes* mosquitoes are day-time bitters, with an activity peak at dawn and dusk. Some species bite aggressively humans, causing a serious nuisance. They can breed in any kind of natural or man-made water-body and their eggs are tolerant to long periods of dryness and cooler temperatures.

Aedes mosquitoes have gained the interest of the entomological research as they transmit several arboviruses. Especially, *Aedes (Stegomyia) aegypti* and *Aedes (Stegomyia) albopictus* species are of major public health significance, being key vectors of more than 22 arboviruses, like Dengue virus (DENV), Chikungunya virus

(CHIKV), Zika virus (ZIKV), Yellow Fever virus (YFV), etc. (ECDC; https://ecdc.europa.eu/en/). *Ae. aegypti* is the primary vector of these diseases, but *Ae. albopictus* is also considered a competent (secondary) vector. Both species were originally classified as forest mosquitoes, but their ecological plasticity and competitive behavior, facilitated by globalization and human activities, have led to their widespread distribution [112]. Other *Aedes* species that potentially play a role in arboviruses circulation (even as enzootic or bridge vectors) are *Ae. japonicus, Ae. atropalpus, Ae. koreicus* and *Ae. triseriatus* (ECDC; Mosquito Fact Sheets).

Aedes aegypti, the Yellow Fever mosquito*,* is a species native to Africa, with an intense current presence in the Sub-Saharan Region, the Middle-East, northern Australia, south-eastern United States and a large part of Latin America. Between 300-500 years ago, *Ae. aegypti* invaded south-eastern Asia and over the last 25 years it has colonized most of the tropic and subtropic areas of all six continents. Between the 18^{th}-20^{th} century, *Ae. aegypti* was established in southern Europe, but later disappeared from the Mediterranean and Black Sea. Now, populations have been re-introduced in some European countries, like Georgia, Turkey and Russia (ECDC), but its presence in southern Europe is now limited in the Portuguese Island of Madeira, since 2005 [98], being responsible for the DENV-1 outbreak there with 2.000 notified cases, between 2012-2013 [45]. Out of Europe, to date, it has been involved in all CHIKV outbreaks in Africa, southeastern Asia, Pacific and Carribean Islands, in DENV epidemics in America and southeastern Asia, in YFV transmission in Asia, Sub-Saharan Africa and in tropical regions of Central and South America and in the recent ZIKV epidemic between 2015-2016 in Brazil (ECDC).

Ae. albopictus, commonly known as the Asian tiger mosquito, originates from South-East Asia. Progressively, it underwent through a domestication process, feeding alternatively on humans and animals and exploiting a broad variety of water habitats for breeding. Today, it occurs in rural areas or human and suburban environments, demonstrating rapid adaptation and competitiveness. *Ae. albopictus*, listed among the 100 most invasive animal species worldwide (ISSGroup- Global Invasive Species Database; [17]), has spread to all continents apart from Antartica. It is present in southern Asia, in many Islands of the Pacific and Indian Ocean and in Australia, but also in New Zealand. Additionally, *Ae. albopictus* is recorded in many countries of Central Africa and widely in Central and Latin America and the Carribean Islands. In Europe, it was first reported in Albania, in 1979 [43] and so far it has been well-established in Mediterranean (southern Spain, southern France, Italy, Greece) and some Balkan Countries (**Image 5**; ECDC 2019). Lately, populations of the Asian tiger mosquito have also been introduced in northern European countries.

The contribution of *Ae. albopictus* to pathogen transmission and epidemics worldwide is not negligible. Apart from many recent CHIKV and DENV outbreaks in

Europe, it was also implicated in DENV outbreaks in Hawaii, Mauritius, China, Japan and Seychelles, in CHIKV epidemics in the Indian Ocean and in transmission of dirofilaria in Asia, North America and Europe (ECDC). Its rapid expansion and adaptation to new habitats driven by both human activites and climate changes increases the risk for disease transmission in additional countries around the world [89]. *Ae. albopictus* ability to tolerate cold climates or undergo winter diapause makes the potential danger of longer disease transmission cycles and introduction to new countries even stronger (ECDC, [112]).

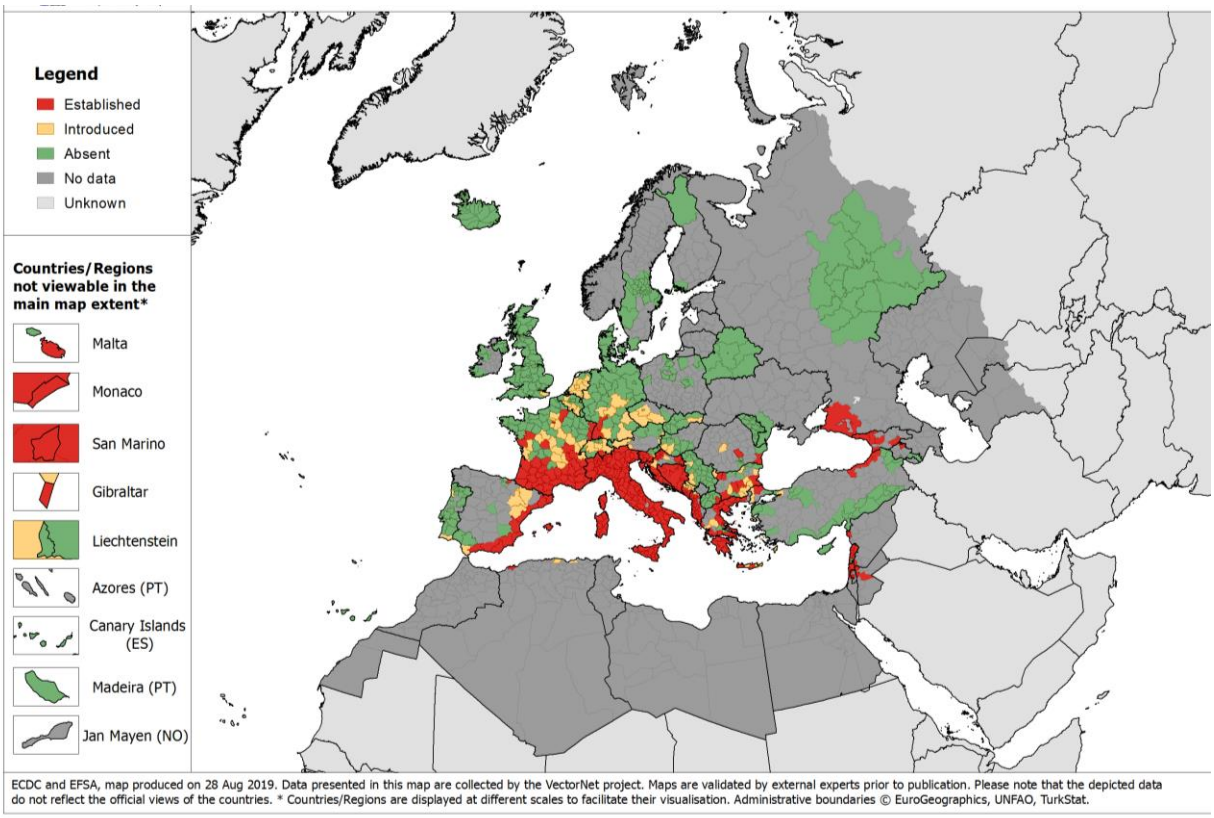


Image 5: The map shows the current known distribution of *Aedes albopictus* in Europe at 'regional' administrative level, as of August 2019. (Source: ECDC & EFSA. Stockholm: ECDC; 2019.: https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps)

2.2.2. *Anopheles* Mosquitoes

There are more than 450 *Anopheles* mosquito species; approximately 50 of them are medically important due to their capacity to transmit malaria parasites and filariasis worms (CDC). *Anophelines* are distributed worldwide, apart from Antartica (CDC).

Interestingly, fertilized females may overwinter in caves, resulting in a longer malaria-cycle over the year. Eggs can also survive in cold, but not freezing, temperatures. Depending on the species, *Anopheles* mosquitoes can be active at dusk, dawn or even during the night, feed and rest indoors or outdoors. These mosquitoes may display anthropophillic (prerefence on human blood meal) or zoophilic behaviour, but often they feed opportunistically depending on host availability [54]. The primary malaria vectors in Africa, *An. gambiae* and *An. funestus*, are anthropophillic and, therefore, very important vectors of the malaria parasites (CDC).

Anopheles gambiae complex comprises of at least seven reproductively isolated and morphologically similar sibling species including *An. gambiae s.s., An. arabiensis s.s, An. melas, An. merus, An. quadriannulatus s.s*, *An. coluzzii*, collectively called *Anopheles gambiae sensu lato*. Some of them are amongst the most efficient malaria vectors in Sub-Saharan Africa [32]. Different species/ species complexes are the dominant malaria vectors in different regions; *An. sacharovi, An. labranchiae, An. plumbeus* and *An. atroparvus* are the prevalent malaria vectors in Europe, *An. gambiae, An. funestus* and *An. arabiensis* in the African Region, *An. quadrimaculatus* and *An. darling* in America and *An. superpictus, An. stephensi* and *An. culicifacies* in Asia [90].

2.2.3. *Culex* Mosquitoes

The genus *Culex* is a large and diverse group of mosquitoes with more than 20 subgenera and over 750 species; new species are frequently described [54]. Generally, they are distributed worldwide, in tropical and temperate regions. The genus includes the common house-mosquitoes *Cx. pipiens* and *Cx. quinguefasciatus* that develop in urban or rural/agricultural areas.

Typically, they bite during the night on humans and animals (e.g. birds, horses), and during the day they rest indoors or outdoors, around structures and vegetation [54]. Several tropical *Culex* species originating from Asia and Africa are well known vectors of viral diseases, e.g. West Nile Virus (WNV), Japanese encephalitis, St. Louis encephalitis, etc., and lymphatic filariasis (ECDC). Moreover, in temperate latitudes, *Culex* female mosquitoes in reproductive arrest can overwinter in protected places (e.g. sewers, crawlspaces, basements), posing a public health risk, as diapaused

females could serve as reservoir hosts for arboviruses, when cold climate hinders viral replication [104].

Culex pipiens complex (subgenus: *Culex)* consists of several morphologically similar species, subspecies and biotypes, with variable ecology, behavior and vectorial capacity. *Cx. p. pipiens*, *Cx. p. quinquefasciatus*, *Cx. p. pallens*, *Cx. torrentium* and *Cx. australicus* are some of them, widely spread across Europe, Asia and America [54]. Particularly, *Cx. p. pipiens* (biotype: *pipiens*, *molestus* or *pipiens/molestus*) is the most abundant mosquito species in southern Europe and the primary vector of WNV, while *Cx. quinquefasciatus* is the predominant vector in southeastern and western parts of the United States [57].

2.2.4. Other Mosquito Vectors

Field evidence and laboratory tests have proved that some species of the genera *Culiseta, Mansonia, Coquillettidia*, *Haemagogus* and *Sabethes* also have an implication in transmission of filarial nematodes, *Plasmodium* parasites, West Nile virus, Yellow Fever virus, Chikungunya virus, Western Equine encephalitis, Eastern Equine encephalitis, Zika virus, etc. to humans and domestic or wild animals [54]. Usually, their contribution to human infections and outbreaks globally is minor.

CHAPTER 3:

Biology & Epidemiology of Mosquito-borne Diseases

3.A. Pathogens Transmitted by Mosquitoes

3.A.1. Arboviruses

Arbovirus (arthropod- borne virus) is a term used to characterize a group of viruses transmitted to humans or animals by arthropod vectors, such as mosquitoes, sandflies, ticks or migdes. There are 6 taxonomic families including more than 130 different RNA viruses (single-stranded positive- or negative- sense RNA or double-stranded RNA) able to infect humans: *Togaviridae, Flaviridae, Bunyaviridae, Rhabdoviridae, Orthomyxoviridae* and *Reoviridae*. The majority of mosquito-borne human viruses belong to the three first families [76].

All arboviruses maintain a transmission cycle between vertebrate animals (amplifying/ reservoir hosts) and insects (primary vectors). Representative arbovirus transmission cycles of West Nile virus (WNV) and Dengue virus (DENV) are shown in **Image 6.** Female mosquitoes, during a blood-meal on an infected animal, acquire the virus. Arboviruses replicate in the mesenteronal epithelial cells of the arthropod vector and, after being released, they infect its salivary glands. In the subsequent blood-meal, arboviruses from the salivary glands of the mosquito are transmitted to a new vertebrate host [76].

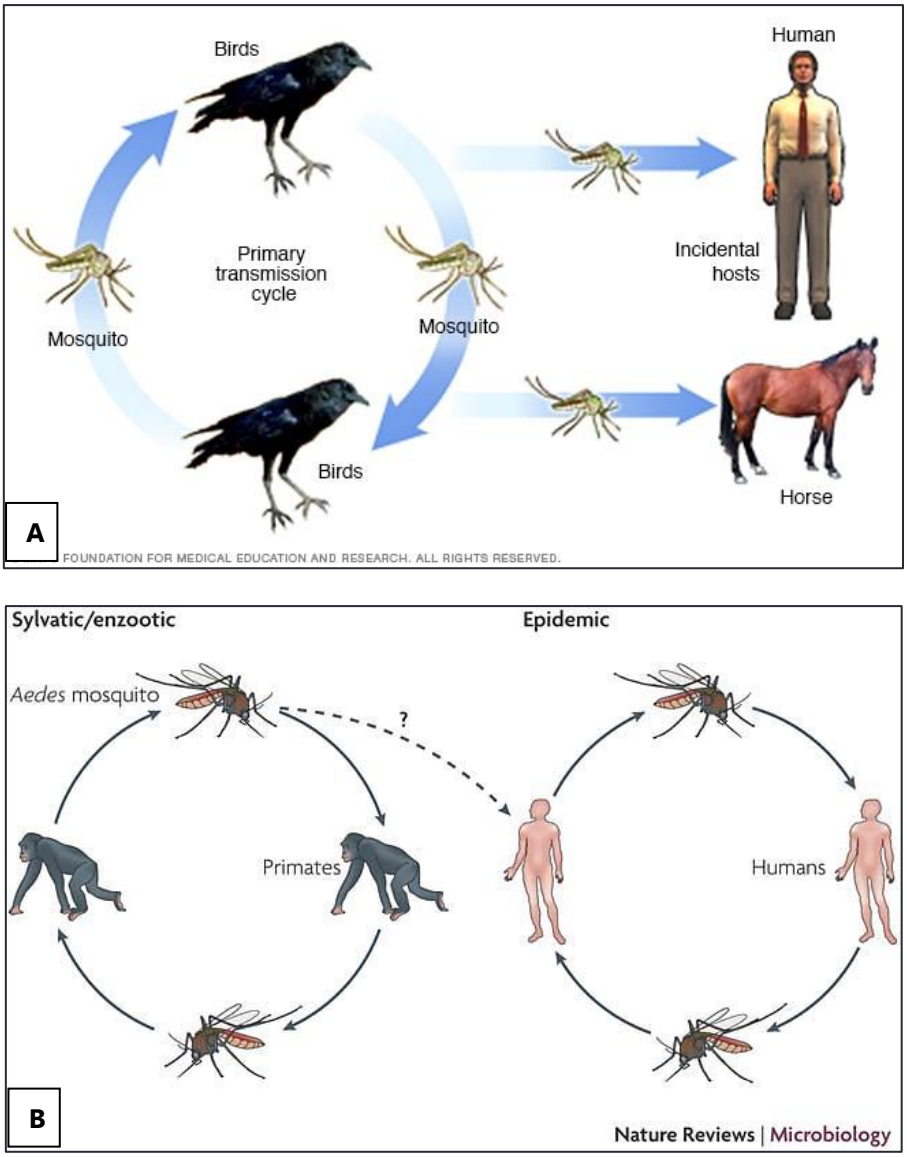


Image 6: **(A)** WNV transmission cycle [22]. **(B)** The two DENV transmission cycles: epidemic cycles involve the human host and viruses are transmitted by *Ae. aegypti* (primary vector) and *Ae. albopictus (*secondary vector). The sylvatic transmission cycle involves monkeys and several different *Aedes* mosquitoes [135].

Mosquito-borne viruses are responsible for millions of infection cases annually and thousands of deaths worldwide (WHO, http://www.mosquitoreviews.com/mosquito-statistics/). The major arboviral diseases transmitted by mosquitoes are caused by Dengue virus (DENV), Yellow Fever virus (YFV), West Nile virus (WNV), Chikungunya virus (CHIKV), Zika virus (ZIKV), Japanese encephalitis virus (JEV), Rift Valley Fever virus (RVFV), etc. Africa is considered to be the ancestral home of most of them [101].

DENV (*Flavivirus, Flaviviridae* family) is the most dominant arbovirus spread in tropical and sub-tropical countries, transmitted by *Aedes* mosquitoes (*Ae. aegypti*:

primary vector, *Ae. albopictus*: secondary vector) and causing more than 390 million infections every year, with more 20.000 of them being fatal (WHO, 2019; Dengue and severe Dengue virus Fact Sheets). During the last decades, DENV incidence demonstrates an increasing tendency; today about half of world's population is at risk of DENV infection. More than 100 countries in Africa, America, Eastern Mediterranean, South-East Asia and the Western Pacific are endemic for the disease. Over the last ten years, multiple locally acquired DENV infections have been reported in new areas (previously not affected by the disease), including a number of DENV outbreaks recently recorded in European countries (WHO, ECDC).

CHIKV (*Alphavirus, Togaviridae* family) is transmitted to humans principally by *Aedes aegypti* and *Aedes albopictus* mosquitoes. Originating from Africa, to date the disease has been introduced in more than 60 countries of Asia, Africa, America and Europe, but it mainly occurs in Africa, Indian and Pacific regions. The distribution of the mosquito vectors is at large responsible for the current worldwide distribution of CHIKV. In 2015, 240 deaths out of 1.675.387 human cases were recorded in 45 countries across the world [113]. Moreover, over the last 15 years, CHIKV epidemics have occurred also in Europe, particularly in Italy, France and Spain (WHO, CDC, [64]).

WNV (*Flavivirus, Flaviviridae* family) emerged from Uganda, Africa in 1937 [23] and was firstly identified in birds. The virus is transmitted to vertebrate hosts primarily by *Culex* mosquitoes, but *Aedes albopictus* is also a competent vector. It is one of the most widely distributed arboviruses, with intense presence in Africa, Middle-East, parts of Europe, but also Asia, Australia and America (WHO); the geographic range of WNV transmission is increasing over the last years [60]. In the European Region, large WNV outbreaks occurred in Bucharest, Romania, Russia, Greece and Israel until 2000. Added to these, since 2010, Greece experiences WNV epidemics almost in every transmission season, included among the deadliest in Europe [111].

ZIKV (*Flavivirus, Flaviviridae* family) was first discovered in Uganda, Africa in 1947 in rhesus monkeys; a few years later, it was identified in humans in Tanzania and sporadic outbreaks across the world followed. Large recent outbreaks occurred in the Pacific Islands in 2013-2014 and in Brazil, in 2015-2016 [3]. Currently, ZIKV transmission is ongoing in America, western Pacific region, South-East Asia and Africa; particularly, 87 countries distributed across these four WHO Regions report evidence of autochthonous ZIKV transmission cases (WHO). In America, the highest burden of ZIKV was recorded in 2016, followed by a significant decline in 2017 and 2018. There are data gaps concerning the epidemiological situation of ZIKV for Africa, SE Asia and the Pacific Region. However, the risk of ZIKV introduction in additional areas is intense, since the primary vector *Aedes aegypti* is well established in 61 countries and territories in six WHO regions (WHO; Zika Epidemiology Update, 2019).

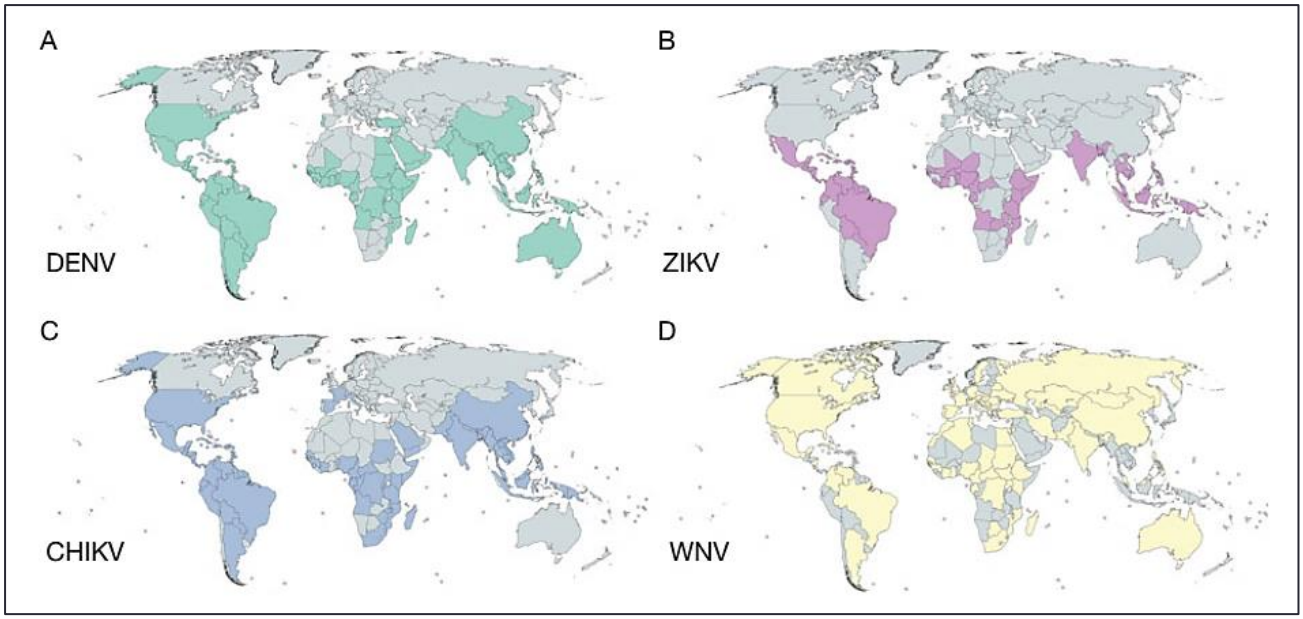


Image 7: Global distribution of some arboviruses. The colored regions consist countries or territories with current or previous transmission of **(A)** Dengue virus, **(B)** Zika virus, **(C)** Chikungunya virus and **(D)** West Nile virus [138].

3.A.2. Malaria Parasites

Malaria is one of the most severe infectious diseases worldwide: over 219 million cases and 435,000 reported deaths in 87 countries, during 2017 (WHO, 2019; Malaria Fact Sheets). The most vulnerable group are children under 5 years old, holding more than 50% of the total malaria deaths. The vast majority of malaria incidence in 2017, corresponding to 92% of infection cases and 93% of related deaths, occurred in sub-Saharan Africa, followed by SE Asia (7%; in India, Cambodia, Vietnam, Thailand, Laos) and eastern Mediterranean Region (2%; mostly in Saudi Arabia and Yemen). The highest burden countries reporting almost half of the malaria cases worldwide are Nigeria (25%), Democratic Republic of Congo (11%), Mozambique (5%), India (4%) and Uganda (4%). Concerning America, an increase in malaria transmission has been recorded in Brazil, Nicaragua and Venezuela (WHO; World Malaria Report 2018).

This life-threatening disease is caused by parasites transmitted to humans through infected female *Anopheles* mosquitoes bites. These apicoplexan parasites belong to the genus *Plasmodium,* that includes more than 100 species; species that cause the disease to humans are *Plasmodium falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi*, with the first two species posing the greatest threat (CDC). Indicatively, in 2017, *P. falciparum* was responsible for 99,7% of the estimated cases in the African

Region, 62,8% in SE Asia, 69% in the Eastern Mediterranean and 71,9% in the Western Pacific, while *P. vivax* is the predominant malaria parasite in America, causing 74,1% of total malaria cases there (CDC data, 2017).

The vectors of *Plasmodium* parasites are *Anopheles* mosquitoes. Dominant vector species vary depending on the region: *An. sacharovi, An. labranchiae* and *An. atroparvus* are prevalent malaria vectors in Europe, *An. gambiae, An. funestus* and *An. arabiensis* in the African Region, *An. quadrimaculatus* and *An. darling* in America, *An. superpictus, An. stephensi* and *An. culicifacies* in Asia [106, 90].

The lifecycle of the malaria parasite involves the invertebrate (mosquito) and the vertebrate (human or other mammals, birds, reptiles) host (CDC, [100]) **(Image 8)**. During a blood-meal by an infected female *Anopheles* mosquito, sporozoites are injected into the human bloodstream and infect the liver cells, where they turn into schizonts. Mature schizonts rupture and vesicles containing merozoites travel to the heart and the lungs; in the lung capillaries, merozoites are released to the bloodstream, where they invade the erythrocytes. Merozoites multiply asexually inside the erythrocyte (erythrocytic schizogony), until it bursts; then, they invade more erythrocytes. Some merozoites leave the asexual replication cycle and develop into gametocytes, the sexual form of the parasite that circulates in the human blood. Male gametocytes (microgametocytes) and female ones (macrogametocytes) are ingested by a female *Anopheles* mosquito during a next blood-meal. The sporogonic cycle happens inside the mosquito midgut; during this process the microgametocytes penetrate the macrogametocytes, producing zygotes. The actively moving and elongated form of a zygote is called ookinete, which during its transition through the mosquito midgut wall develops into an oocyst. Inside the oocyst, there are thousands of active sporozoites developing, which upon the oocyst burst/rupture will translocate to the mosquito salivary glands. The new cycle begins with the inoculation of these sporozoites in a new human host, after a mosquito bite [21].

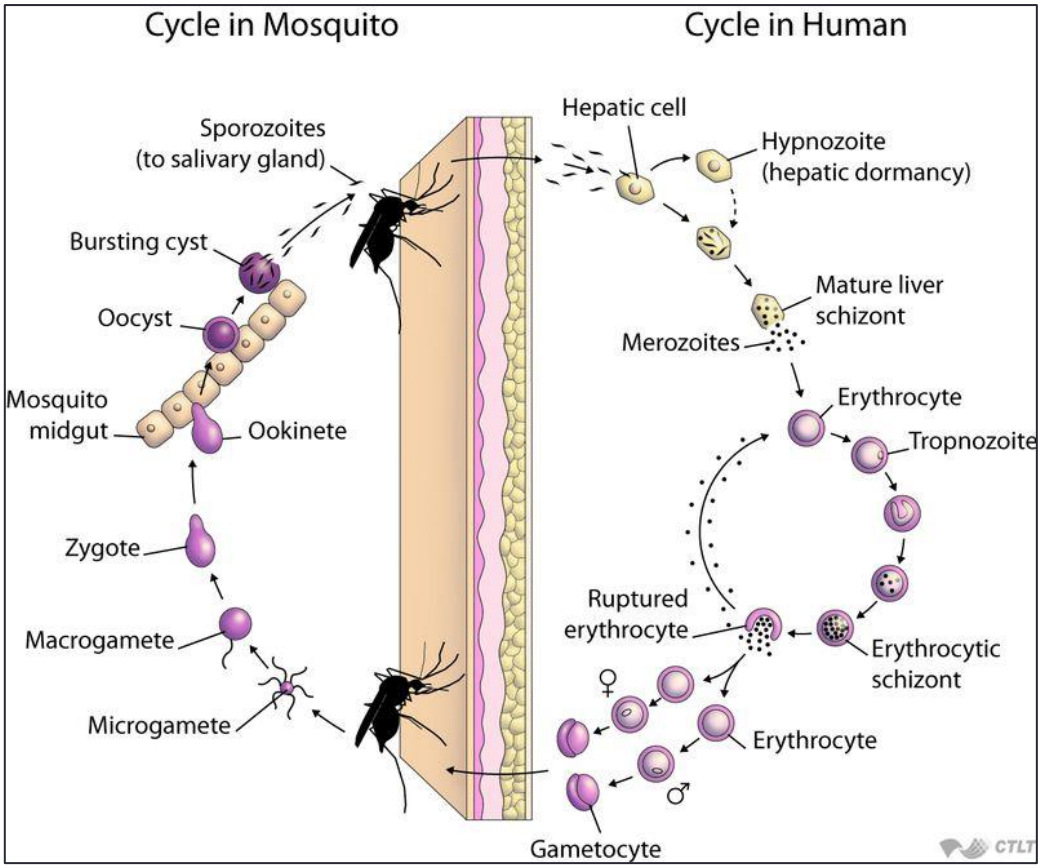


Image 8: Life cycle of Malaria parasite [26].

Following the infective bite by an *Anopheles* mosquito, there is an incubation time between 7-30 days until the first symptoms appear: they are non-specific and flu-like. Generally, malaria infection symptoms may range from mild headache to severe implications leading to death, especially in *P. falciparum* infection (CDC). Depending on the combination of symptoms observed, malaria cases are categorized either as uncomplicated (influenza, cold or other common infection symptoms) or severe/complicated (serious organ failures, abnormalities in the patient's blood or metabolism, kidney injuries, abnormal behavior, etc). All the clinical symptoms are linked to the asexual erythrocytic or blood stage parasite (WHO, [53]).

Since 2000, there is an important decline of 18% in malaria incidence and 28% in mortality, totally, in every WHO region; in 2017, the largest decline in mortality compared to 2010 was reported in SE Asia (54%), Africa (40%) and eastern Mediterranean (10%) (WHO; World Malaria Report 2018). Indicatively, between 2000-2015, the global malaria death toll has become almost half: from 839.000 deaths in 2000 to 438.000 in 2015 **(Image 9)**. The African Region, except for some areas where the burden of malaria is unchanged or even increased, has managed to reduce malaria deaths from 764.000 to 395.000 [105]. This decrease arises as a result of many factors including vector control efforts utilizing insecticides, extended indoor residual

spraying (IRS), provision of insecticide-treated nets (ITNs), rapid diagnostic tests, antimalarial drugs, chemoprevention in pregnant women and children, larval source management and improvement of housing and sanitation conditions. Despite the hopeful overview, the global mortality reduction rate is decreasing since 2015 and, generally, the number of deaths, also in children under 14 years old, remains almost unchanged ([63], WHO; World Malaria Report 2018).

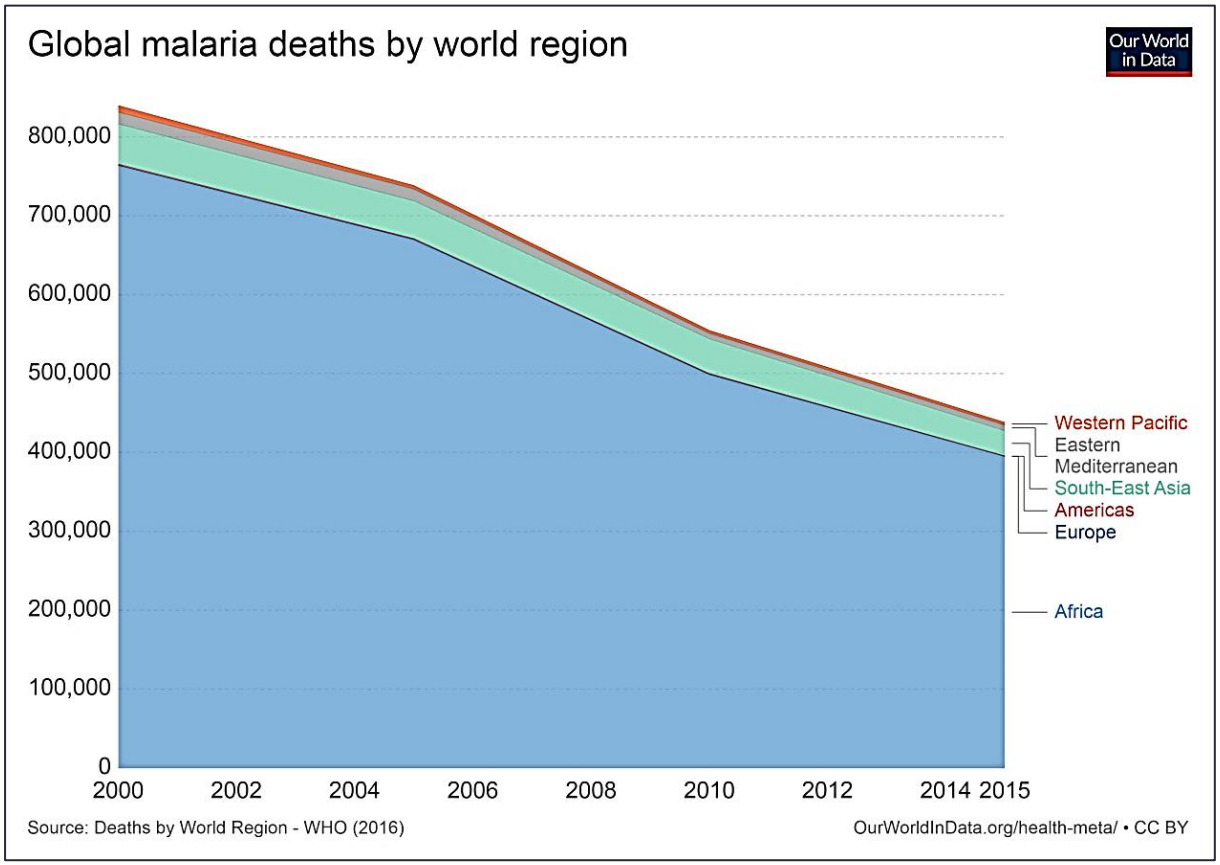


Image 9: Global malaria deaths by world region, over the last 19 years [27].

3.A.3. Filarial Nematodes

There are three species of parasitic and microscopic worms, nematodes,that can cause lymphatic filariasis to humans: *Wuchereria bancrofti*, *Brugia* malayi and *Brugia timori*. The first one is responsible for the majority of the global infections, while the two latter are more common in Asia (CDC).

The parasite transmission from human to human is mediated by mosquito bites. Depending on geographical distribution, vectors of these nematodes can be

mosquitoes of the following genera: *Culex* (mainly *C. quinquefasciatus* and *C. pipiens*) in the Americas, *Anopheles* (mainly *A. arabinensis*, *A. funestus*, *A. gambiae*) in Africa, *Aedes* (mainly *A. aegypti*, *A. darling*) and *Mansonia* in the Pacific Islands and in Asia.

During a human blood-meal, mosquitoes transmit pathogen-larvae to the host, which migrate to the lymphatic vessels and lymph nodes and grow, in a process that lasts at least 6 months. Here the pathogen larvae develop to adult worms, mate and produce millions of microfilariae. An adult worm can live for up to 7 years in the human lymphatic system. When the infected person is bitten by a mosquito the microfilariae enter and develop within the mosquito vector (**Image 10**). Following the infection, the worm damages the lymph system of the person, but most of the patients will not have any clinical symptom. Only a small percentage will develop lymphedema, caused by fluid collection that results in swelling of the breasts, arms, legs and genitalia (CDC).

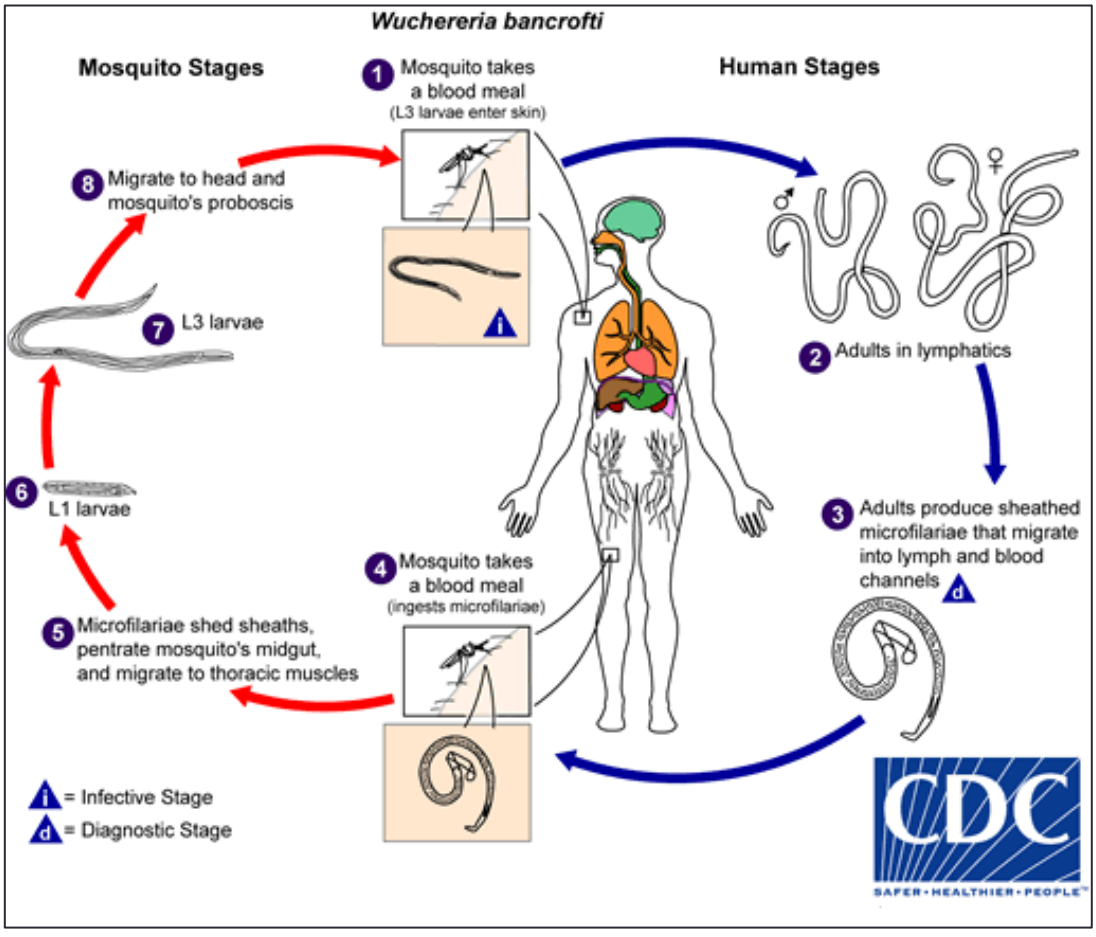


Image 10: Life cycle of filariasis nematode *Wuchereria bancrofti* (CDC).

According to CDC, over 120 million people from 72 countries live at risk of the filarial nematodes. The disease is spread throughout the tropics and sub-tropics of Asia, the

Western Pacific, Sub-Saharan Africa and parts of the Caribbean and South America. Almost two thirds of global lymphatic filariasis infections occur in Asia (mostly in China, India, Malaysia, the Philippines and Indonesia) [91]. Fortunately, over the last 10 years, there has been an important decrease in lymphatic filariasis infections due to provided chemotherapy; more than 96 million cases were prevented or cured in the America, Asia and the Pacific Ocean [117].

3.B. Mosquito Immunity to Pathogens

Mosquitoes lack an adaptive immune system, but have conserved innate immunity, comprising of both cellular and humoral response to pathogens. Apart from the hard chitin exoskeleton, that poses a first physical barrier to any infection, parasites, abroviruses, bacteria, etc, have to overcome the physiological and molecular barriers in three key mosquito components during their "journey" inside the vector **(Images 11, 12)**:

- the midgut
- the haemocoel
- the salivary glands

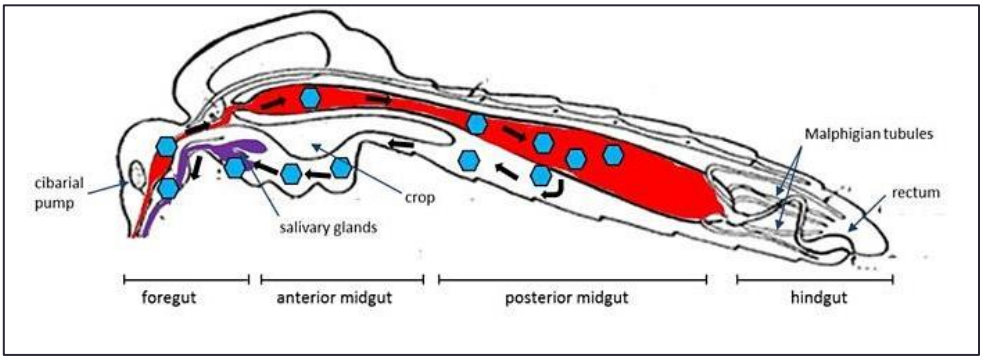


Image 11: Schematic representation of arbovirus tropism in a mosquito vector [72].

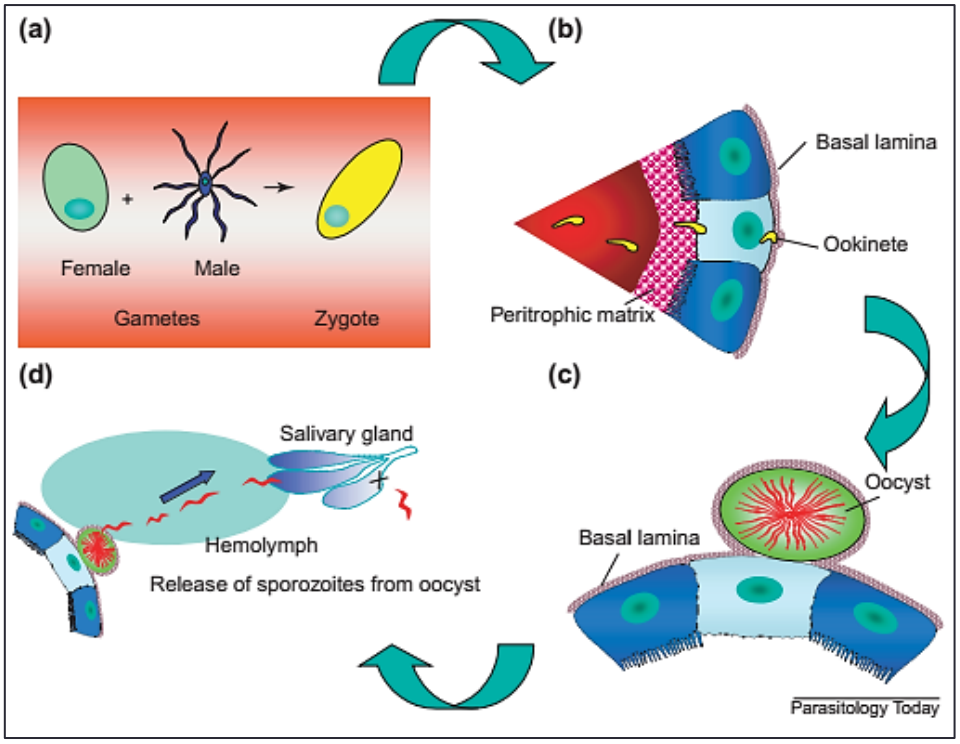


Image 12: Development of *Plasmodium* in the mosquito: **(a)** Fertilization, **(b)** Migration of the zygote to the peripheral of the blood bolus,and invasion of the midgut epithelium, **(c)** Rupture of the oocyst and release of thousands of sporozoites into the haemolymph, **(d)** Sporozoites invasion of the salivary gland [75].

Mosquito proteins involved in the interaction with the pathogen can be categorized either as **restriction factors** (pathogen antagonists), that suppress the infection and promote host survival, or as **host factors** (pathogen agonists), that facilitate the development and replication of the pathogen within the vector [122].

After being ingested by the female mosquito, *Plasmodium* parasites and viruses enter the midgut, the former in order to fertilize and develop from ookinetes to oocysts and the latter in order to replicate. In both infections, both host factors and mosquito's immunity mechanisms act. Microbiota, *e.g. Proteus sp., Enterobacter sp., Beauveria bassiana,* etc., stimulate the immune system and actively restrict the infection in the midgut lumen. Interestingly, there have been some symbiotic microorganisms reported to increase susceptibility of mosquitoes to pathogens (for example, *Serratia odorifera* increases CHIKV susceptibility). Secondly, reactive oxygen species (ROS) and nitrogen species (RNS) induce oxidative stress that burden parasites/arboviruses survival. The presence of the peritrophic matrix, a chitinaceous glycoprotein membrane, complicates *Plasmodium* parasites invasion of the midgut epithelium; they secrete chitin-degrading enzyme and exploit host chitinases and

other factors, like fibrinogen-related protein 1 (FREP-1), to integrate into the *Anopheles* peritrophic matrix [122].

After exiting the midgut lumen, pathogens are spotted in the midgut epithelium. *Plasmodium* ookinetes pass through epithelial cells intracellularly (upon invasion, cells die via apoptosis) and they develop into oocysts at the basal lamina. Epithelial serine protease (ESP) is expressed in *An. gambiae* midgut epithelium and salivary glands and is highly induced during ookinete invasion, promoting *Plasmodium* entry into the midgut lamina [122]. The parasite also takes advantage of the mosquito lipid system to survive inside the host; for example, lipophorin in the midgut epithelium acts as *Plasmodium* agonist [122]. Viruses enter the midgut epithelial cells via clathrin-mediated endocytosis associated with many cell-surface factors (e.g. cadherins) and, after unpackaging, they replicate inside the cells. Studies, mostly focusing on DENV, have shown that the majority of host factors for mosquito transmitted viruses act in the midgut.

Exiting the midgut epithelium, pathogens are released into the haemolymph. Haemocytes are the main components of the cellular response, killing pathogens via phagocytosis, encapsulation or melanization. Thioester-containing protein 1 (TEP-1) of the *Anopheles* mosquito is an antipathogen effector circulating in the haemolyph (but also expressed in the midgut), that binds to the ookinete surface, as tag for parasite lysis or melanization [122]. Melanization is a major process of the arthropod immune response; melanin-producing prophenoloxidases are induced by many pathogen recognition receptors (PRRs) and deposit melanin on the surface of invaded pathogens. In *An. gambiae,* C-type lectin 4 (CTL-4) and CTL mannose binding-2 (CTLMA-2) protect *Plasmodium* ookinetes from melanization [122].

When the infection proceeds to the salivary glands, parasites exploit host factor ESP expressed on the basal side. Silencing of ESP has been reported to decrease the number of sporozoites invading the tissue [122]. Mosquito- virus interactions in the salivary glands are not well understood; DENV infection induces the production of antimicrobial peptides (AMPs) and miRNAs [122].

In *Aedes* and *Anopheles* mosquitoes, it has been shown that there is a tissue-specific regulation of effector molecules that compose the humoral response against pathogens, regarding genes encoding for/relative to hydrolytic enzymes, peritrophic matrix, extracellular matrix proteins, iron-responsive proteins, lipid metabolism, antioxidant response proteins, antimicrobial peptides (AMPs), pathogen recognition receptors (PRRs), components of the phenoloxidase cascade, etc [92]. The molecular backgroung underlying all the pre-mentioned immune responses is relative to three major signaling pathways **(Image 13)**, activated by the presence of a pathogen:

- the Toll pathway
- the Immune Deficiency (IMD) pathway
- the JAK-STAT pathway

[92, 122]. The initial step after a pathogen invades a mosquito is the binding of host-derived PRRs to pathogen-associated molecular patterns (PAMPs). PRRs are secreted proteins found all over the mosquito body, mainly in the midgut and the haemocele. In *An. gambiae* and *Ae. aegypti*, TEPs and FREPs function as major PRRs [122].

The interaction between PRRs and PAMPs activates the Toll pathway. Signaling through adaptor proteins leads to phosphorylation and degradation of Cactus protein, an inhibitor of Rel-1; this allows Rel-1, an NF-kB transcription factor, to be translocated to the nucleus and activate the transcription of relative genes. The Toll pathway is typically activated by Gram-positive bacteria, by the malaria parasite and is the main pathway regulating *Ae. aegypti* immunity against ZIKV and DENV [92].

The IMD pathway is the most effective pathway against malaria parasite infection in mosquitoes. It is induced in a similar way as Toll pathway. Rel-2 transcription factor is a known IMD agonist; Caspar is a negative regulator of Rel-2, demonstrating a role similar to Cactus protein [92].

The JAK-STAT signaling pathway is typically linked to viral infection (including DENV, ZIKV) and stimulated by interferons. The binding of Upd protein to the Dome receptor triggers the cascade. SOCS and PIAS are both antagonists, the former inhibiting STAT dimerization and the latter preventing the production of JAK-STAT – induced effectors [92].

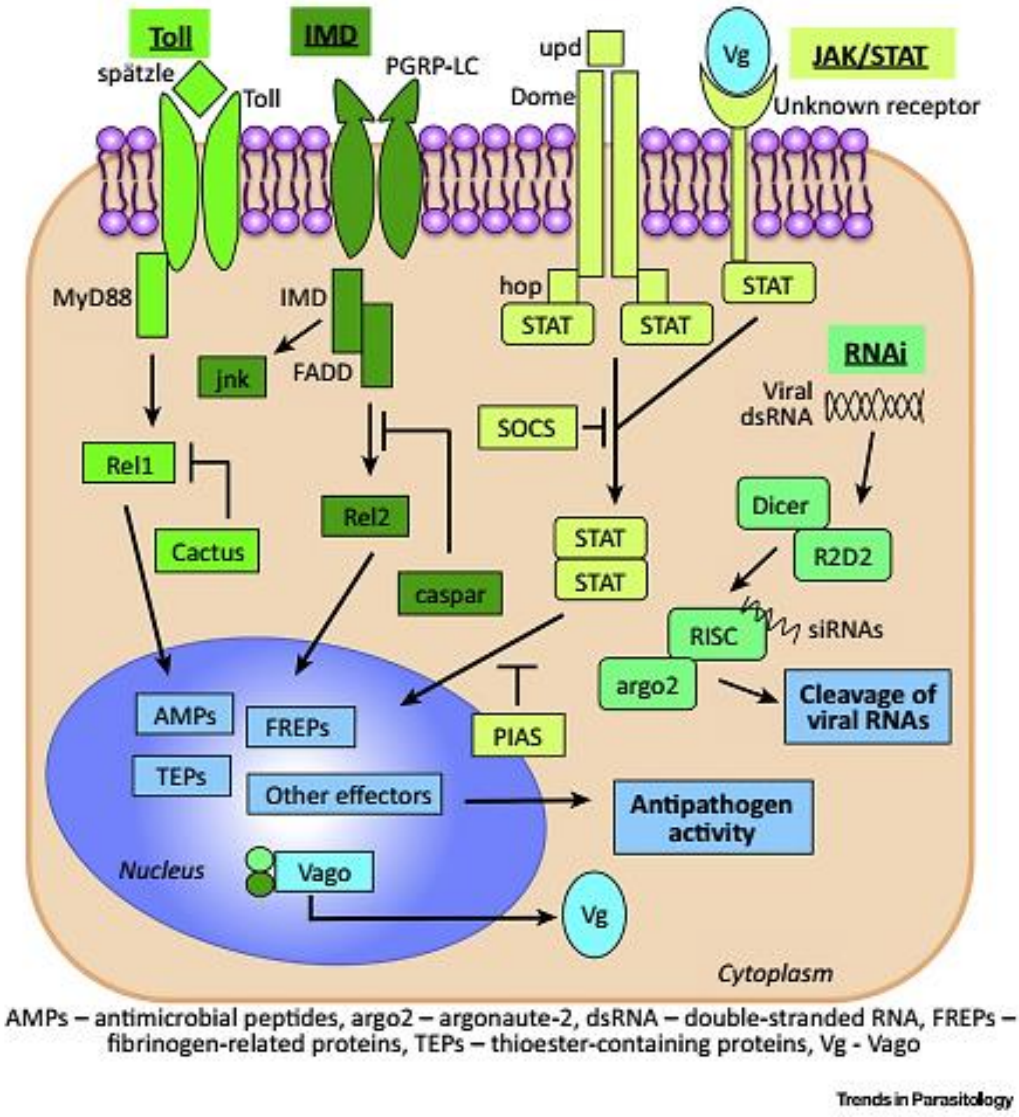


Image 13: Mosquito innate immunity pathways [122].

In mosquito innate immunity responses against viruses, RNA interference (RNAi) is the major pathway; it is a conserved sequence-specific gene-silencing mechanism, also implicated in many other cellular processes upon pathogen infections. During the invaded viral genome replication, the produced double-stranded RNAs (dsRNAs) are processed by Dicer-2 and cleaved to 21 nt virus-derived small interfering RNAs (vsiRNAs). Dicer-2 and R2D2 mediate the incorporation of vsiRNAs to the RISC compound, so that, after this process, any matching viral RNA entering the cell will be cleaved by the RISC-associated argonaute-2 protein (AGO2), hindering the replication of the virus [80, 122].

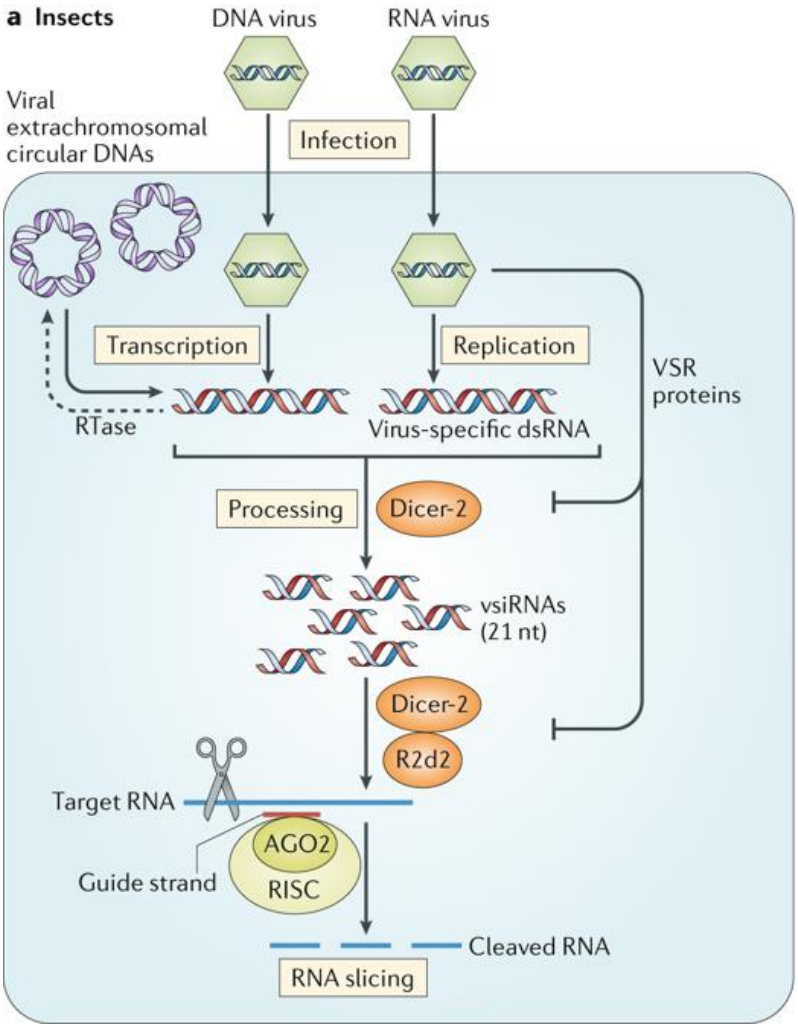


Image 14: Antiviral RNA interference (RNAi) in insects., RTase: reverse transcriptase, VSR: viral suppressor of RNAi, RISC: RNA-induced silencing complex [80].

CHAPTER 4:

The Re-emergence of Mosquito-borne Diseases in Europe

4.1. Historical Perspective and Current Situation in Europe

The vast majority of mosquito-borne diseases (MBDs) originate from Africa and are more prevalent in tropic and subtropic areas (Sub-Saharan Africa, South-East Asia, Cental and Latin America) and countries under poor socio-economic conditions. Europe was endemic for malaria, until the end of the Second World War, with the Mediterranean and Eastern part of the continent being mostly affected [58]. After 1955, there has been an important decline in malaria cases in Europe and the disease was finally eradicated in 1975 (WHO, 2016; History of Malaria Elimination in the European Region).

Between 18th and 20th century European port cities had experienced more than 20 epidemics of haemorrhagic fever attributed to Yellow Fever virus (YFV) and Dengue virus (DENV). Indicatively, in Greece, a DENV outbreak caused more than 1.000 fatal human cases, in 1927-1928 [58]. Port cities have historically been more vulnerable to infectious diseases, since mosquitoes arriving on ships could find abundant breeding sites there, introducing epidemics in naïve populations.

During the last two decades, Europe faces a (re)-emergence of MBDs by old and new pathogens. Factors associated with this situation include globalization and the expansion of trade (products and animals) and travel, mass movement of displaced populations (refugee crisis since 2015), direct transportation by airplanes, ships or cars, climate change and landscape modifications; all these have potentially facilitated the importation of mosquito-borne pathogens and the introduction of new invasive mosquito species.

The most representative paradigm of invasive mosquito species is *Aedes albopictus*; it is well established across all southern European countries, but in the recent years, populations have also been introduced in northern European countries (e.g. northern France, Germany, the Netherlands, Czech Republic, Belgium, Austria; ECDC 2019)

(Image 5), showing signs of adaptation to colder climates and raising public concern about the introduction of mosquito-borne viruses to new countries [58].

Within the WHO European Region, most of the malaria cases currently occurring are imported by travelers to endemic countries or immigrants and are reported in Western and Mediterranean European countries, mainly Germany, UK, France, Italy and Greece [115]. According to ECDC (ECDC; Malaria- Annual Epidemiology Report for 2017), during 2017, 8.401 malaria cases have been reported in Europe, 99.8% of them were travel-related and 21 confirmed cases were acquired locally (ECDC; Malaria- Annual Epidemiology Report for 2017). *Anopheles atroparvus, An. sacharovi* and *An. labranchiae* are the most important malaria vectors distributed in Europe [115]. Hence, the risk of malaria resurgence in Europe exists and is region specific, depending on vector presence, its sensitivity to *Plasmodium* strains, climate conditions and other factors.

West Nile virus (WNV) is endemic in Europe; the virus first circulated in the 1950s and until 1996 sporadic cases and discontinuous outbreaks occurred in southern France and Romania [58]. Over the years, cases have been reported in southern, eastern and western European countries. WNV lineage 1 was responsible for occasional outbreaks in humans and animals in Europe, prior 2004. WNV lineage 2 appeared for the first time in Hungary, in 2004, and spread in Central Europe and eastern Mediterranean countries, causing outbreaks mainly in Greece, Italy, Romania, Serbia and Hungary [57]. Since 2010, consecutive WNV outbreaks occur almost every year in Europe, with the two deadliest in 2010 and 2018 having happened in Greece. According to ECDC Surveillance Data, since the beginning of 2019 WNV transmission season until September 5th, 241 human infections have been reported in Greece (93,8% of them), Romania, Cyprus, Italy, Hungary, etc. and 20 deadly cases (https://ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/disease-data-ecdc). In Europe, *Culex pipiens*, *Cx. modestus* and *Cx. pipiens modestus* hybrids are the dominant vectors of WNV; however, *Aedes albopictus* is a secondary vector of WNV with a significant importance in virus local transmission.

Ae. albopictus is a competent vector of more than 20 arboviruses, and during the last 20 years it has been responsible for repeated CHIKV and DENV outbreaks in the WHO European Region. The first European CHIKV outbreak occurred in Italy in 2007, caused by an infected traveler coming back from India; the local *Ae. albopictus* populations facilitated the transmission of the disease, resulting in 217 confirmed cases (https://ecdc.europa.eu/en/disease-vectors/facts/mosquito-factsheets/aedes-albopictus). In 2010 and 2014, 13 autochthonous cases in total were reported in southeastern France (https://ecdc.europa.eu/en/chikungunya/facts/factsheet). Regarding DENV, autochthonous cases linked to *Ae. albopictus* presence were recorded in France and Croatia in 2010, and further in France in 2013, 2014 and 2015;

the most recent cases happened in Spain and France during 2018. Moreover, between 2018-2019, 82 human Rift Valley fever cases have been reported across rural areas in Mayotte, France (ECDC; Rapid Risk Assessment of RVFV Outbreak in Mayotte, France).

Europe receives hundreds of millions of tourists annually. Travel-associated mosquito-borne disease cases along with the presence of competent vectors increase the possibility of outbreaks. Among others, there is increasing concern about the potential transmission of Zika virus in Europe. In detail, since 2015, 22 EU/EEA countries reported 2.398 ZIKV imported cases in total, almost 99% of them were mosquito-bite acquired during travel outside Europe, mainly in the Carribean ([127], ECDC; Zika Risk Assessment 2019). *Ae. albopictus* is considered to have vector competence for ZIKV, but much lower than *Ae. aegypti*.

6.2. The Case of Greece

Greece is an eastern Mediterranean country, located at the junction of Europe, Africa and western Asia, with intense shipping and trade activity. Since 2009, the country witnesses an extended financial recession; added to that, is among the European countries most widely affected by the ongoing refugee crisis, since 2015. According to UNCHR (https://data2.unhcr.org/en/situations/mediterranean/location/5179), during the last 4 years more than 1 million refugees/immigrants have arrived in Greece and more than 100.000 are now hosted in the country. Displaced populations, coming mainly from African and Asian countries, live and travel under poor hygiene conditions, with limited access to primary health care and services. Besides, the country relies its economy principally on tourism, receiving millions of visitors every year.

In 1974, Greece was declared as a malaria-free area. Over the next years until 2009, there have been a few malaria cases in the country [115]. Since then, autochthonous reports of *P. vivax* infection occurred every year, resulting in a peak of 42 locally acquired cases during 2011 ([107], ECDC data; Rapid risk assessment: Update on autochthonous *Plasmodium vivax* malaria in Greece, 2011), followed by a decline in the subsequent years, with only some sporadic imported infections. Between 2016-2017, there were 7 autochthonous cases reported in Greece (ECDC data, 2017). In the Peloponnese region of Greece, *Anopheles maculipennis s.s., An. sacharovi, An. hyrcanus* and *An. superpictus* have been identified as competent vectors, while throughout eastern Greece, *Anopheles sacharovi and Anopheles superpictus* are dominant [124, 129].

Additionally, since 2010, Greece witnesses consecutive WNV-lineage 2 outbreaks, with the deadliest one in 2018, resulting in 211 cases and 47 deaths (ECDC; Epidemiological update: WNV transmission season in Europe, 2018). Since the beginning of the 2019 transmission season and as of October 3rd 2019, there had been 215 WNV cases recorded, with 27 out of them being fatal (ECDC Weekly updates: 2019 WNV transmission season). These WNV epidemics occurring in Greece are attributed to the primary vector *Culex pipiens* (3 biotypes: *pipiens*, *molestus* and *pipiens/molestus* hybrids) [62, 99].

Aedes albopictus first record in Greece occurred in Thesprotia and Corfu Island between 2003-2004. According to mosquito surveillance data for LIFE CONOPS project (2017), until 2016 *Ae. albopictus* dispersed all over the country, apart from a few areas in the northern mainland and some Aegean Islands [47]. Presence of this mosquito species increases the risk for local transmission of infectious diseases, regarding the fact that since 2013, a small number of imported arbovirus infection cases were reported: in detail, 1 case of CHIKV in 2014, 10 cases of DENV between 2013-2017 and 5 ZIKV cases between 2015-2017 (ECDC; Dengue virus and Zika virus Annual Epidemiological Reports for 2016 and 2017). Moreover, it could have an additional key-role in WNV circulation over the years, regarding its vector competence for this virus (secondary vector).

The presence of mosquito vectors of diseases in Greece (*Cx. pipiens, Ae. albopictus, Anopheles sp.*) along with the mass influx of populations (tourists and refugees/immigrants) every year from countries probably endemic for vector-borne diseases are primary factors favoring disease transmission in the country, which is already burdened by its intense recent mosquito-borne disease history.

CHAPTER 5:

Mosquito-borne Disease Control

5.1. Vaccines against Mosquito-borne Diseases

Since exposure to mosquitoes is often unavoidable, the development of preventive medicine, at the context of chemoprophylaxis, prompt treatment and control of the problems sequel to the infection, is indispensable for disease control.

Yellow fever and Japanese encephalitis are two vaccine-preventable infectious diseases. In both cases, vaccination has led to reduced morbidity and mortality linked to the disease, and it is strongly recommended for travelers visiting countries with an ongoing YFV or JEV outbreak [44]. Concerning DENV, until now, a vaccine (CYD-TDV) is licensed against it, which has been introduced in Mexico and Philippines, while WHO recommends to be implemented in countries with high epidemiological burden of the disease [44].

Malaria prevention is principally based on chemoprophylaxis and protection against mosquito bites. Early diagnosis of malaria infection is the key to successful treatment and avoidance of disease transmission. The artemisin-based combination therapy is the best treatment currently available, especially against *P. falciparum* infection (CDC). However, there is still much effort on developing an effective vaccine against malaria, but there are difficulties to address, associated with the complex parasite life cycle: a) many antigenic proteins produced by parasites during human infection vary between individual parasites within an infected person, b) individual malaria parasites may switch the selection of proteins appeared on the surface of infected erythrocytes to evade the host's antibodies [31]. RTS,S/AS01 is the first malaria vaccine provided at the beginning of 2019 to children in three sub-Saharan African countries for a pilot implementation program, shown to protect partially against the disease [21, 44].

No vaccines are yet available for the prevention of WNV, ZIKV and CHIKV. Various clinical and preclinical trials for WNV vaccines are ongoing (e.g. HydroVax-001WNV vaccine) [44]. Additionally, the development of a ZIKV vaccine consists an active area of research, with efforts focused on purified inactivated vaccines, DNA vaccines and viral vectored vaccine [109].

Interestingly, since 2017, the National Institute of Allergy and Infectious Diseases (NIH) elaborates research on a universal vaccine that will protect against mosquito-borne diseases, called AGS-v. This proposed vaccine, being still in clinical trials,

contains 4 synthetic proteins from mosquito salivary glands, that will induce antibodies production in a vaccinated individual and cause an allergic reaction, in a way that will prevent the infection during an infective mosquito bite [13].

5.2. Vector Control Strategies

Despite the efforts on development of vaccines and medication against mosquito-borne pathogens, disease control relies largely on a variety of vector control tools. In 2004, WHO adopted the Integrated Vector Management (IVM) approach globally to optimize the use of sources for vector control. This process focuses on the combination of strategies to achieve more efficient, cost effective, environmental friendly and sustainable vector control. It requires a local evidence base (e.g. identification, seasonality and abundance of native mosquito species, their behavior and potential breeding sites, analyses of their insecticide resistance status, epidemiological data of the area, etc) for the selection of appropriate chemical, biological and environmental methods and collaboration within health sector and among all sectors, communities and households (WHO, 2012; HandBook for IVM).

5.2.a. Chemical Control

4.2.a.i. Insecticides: Main Classes and Mode of Action

The primary strategy for mosquito vector control is the use of insecticides. There has been a variety of insecticides extensively used against mosquitoes, either as larvicides or as adulticides: larvicides are used to treat larval habitats, while insecticides targeting adult mosquitoes have an impact on mosquito density and longevity, applied either as residual surface treatments or as space treatments. The main classes of insecticides are:

- Organochlorines
- Organophosphates
- Carbamates
- Pyrethroids

Organochlorine (OC) pesticides were used all over the world both in the chemical industry and in agriculture. They are chlorinated hydrocarbon derivatives, like cyclodienes and dichloro-diphenyl-trichloroethane (DDT). DDT was synthesized in 1984, and it was banned by the early 1970s in many countries due to its high toxicity to humans and wildlife, slow degradation and bioaccumulation [87]; however, it is still used in some African, Asian and south American countries at risk of malaria.

Pyrethroids are synthetic insecticides, similar to the natural pyrethrins produced by the chrysanthemum flowers. In contrast to organochlorines, the insecticidal activity of pyrethroids is combined with low toxicity and fast biodegradation. Their increased efficacy and limited effects on mammals are determinant factors for their extensive use [119]. Pyrethroids are the most common adulticides used for *Aedes* mosquito vectors and the only insecticides recommended for Insecticide Treated Nets (ITNs) [125]. Based on the absence or presence of an α-cyano group and on other chemical characteristics, pyrethroids are divided into type I (e.g. permethrin) and type II (e.g. deltamethrin, cypermethrin), respectively [119].

Organophosphates (OPs) are organic derivatives of phosphorus, used as pesticides and neurotoxic agents, performing high levels of toxicity not only to insects, but also to mammals [29]. Some commonly used OPs are temephos, malathion, parathion, etc. Organophosphorus insecticides need to be metabolized to the compound paraoxon in order to act in a toxic way (WHO, 2011; Specifications & Evaluations for Public Health Pesticides- Temephos). Temephos is used as larvicide to control mosquitoes in domestic water containers, however, since 2007, it has been officially banned for mosquito control in Europe (EU Pesticide database; [9]).

Carbamates are esters of N-methyl carbamic acid; aldicarb, bendiocarb, terbucarb are some of them. This class of insecticides acts in a similar way to OPs, but displays easier degradability and lower toxicity [121].

The four insecticide classes pre-mentioned, comprising the vast majority of insecticides used for mosquito control yet, have only two different modes of action **(Image 15)** [61, 66]:

1. **Inhibition of the Voltage-Gated Sodium Channel (VSSC)**

Pyrethroids and DDT (OC) bind to the VSSC in mosquito nerve cells, prolonging its opening. Sodium ions enter the nerve axon continuously, producing excessive potential followed by a hyper-depolarization of the membrane. Consequently, the charge in the axon membrane disappears. At a neuron level, this leads to uncontrolled neurotransmitter release and, gradually, to ultimate loss of the electrical activity, paralysis of the insect and death.

2. **Inhibition of Acetylcholinesterase (AchE)**

AchE is an enzyme with a key role in the termination of neurotransmitter signaling by hydrolyzing acetylcholine to acetic acid and choline. OPs phosphorylate AchE irreversibly, while carbamates bind to the enzyme reversibly, causing carbamylation of it. In either case AchE inhibition leads to the accumulation of acetylcholine at neuronal synapses and neuromuscular junctions and, finally, paralysis of the insect.

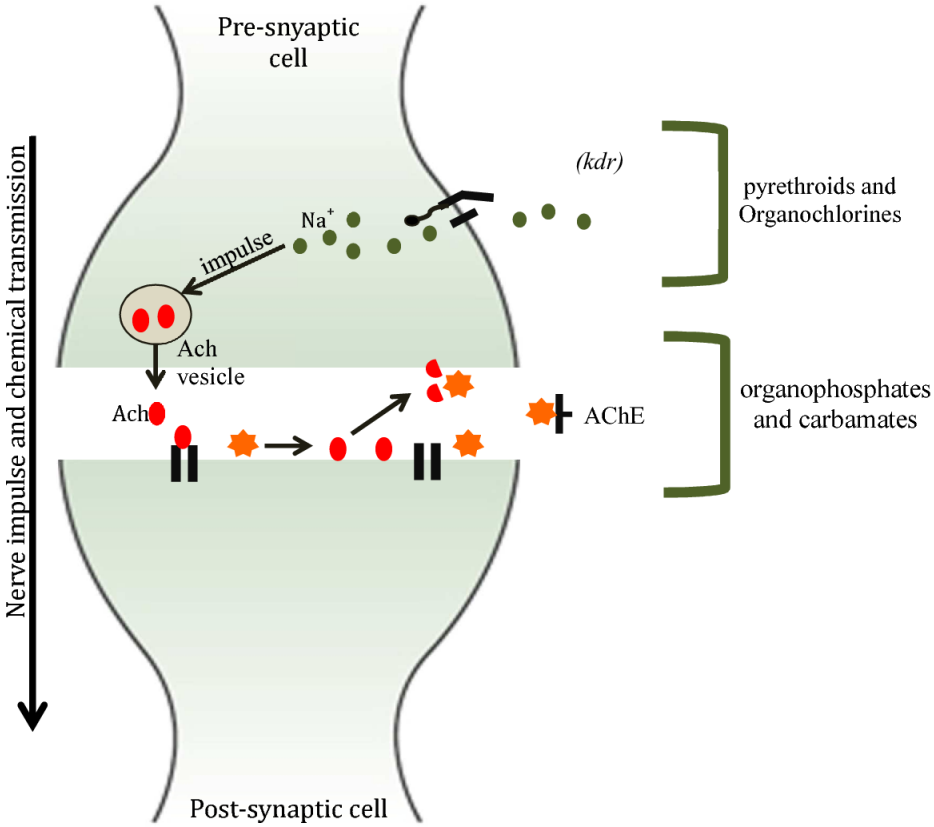


Image 15: Biochemical target sites of synthetic insecticides. Pyrethroids and DDT block the closing of VSSC (Vg-Na^+). Organophosphate and carbamates inhibit AChE, which terminates nerve impulses. Kdr: knock-down resistance, Ach: acetylcholine *(Hughes, University of the Pacific, 2017*).

Insect growth regulators (IGRs) consist a group of insect control agents that interfere with the insects' normal growth and development, regulating or inhibiting selectively a growth/development related biochemical pathway. IGRs have very low toxicity to other animals and to the environment, in general, being more compatible with pest management using biological control methods. The most well-known IGRs are: **(a) Hormonal IGRs** and **(b) Chitin Synthase Inhibitors**. Hormonal IGRs, like juvenile hormone or ecdysosteroid analogs, inhibit the acquisition of the final morphology and the moulting of the insect. Pyriproxifen and methoprene are hormonal IGRs. Diflubenzuron is an insecticide of the benzoylureas (BPUs) family that directly interacts with chitin synthase 1 (CHS-1), which is the enzyme responsible for chitin synthesis in the insect cuticle, preventing the proper formation of exoskeleton [68]. It is among the most effective larvicides, also of very low acute toxicity, widely used for agriculture and public health purposes (WHO, 2008; Guidelines for Drinking-Water Quality).

5.2.a.ii. Insecticide Application Techniques

The method of insecticide application varies based on several factors, like the type of insecticide used, the target mosquito species, the environment that mosquito (larva or adult) thrives in, the purpose of chemical control (e.g. agricultural or public health issue), the available and affordable equipment, etc.

Space spraying relies on the production of a large number of small insecticide droplets delivering a lethal insecticide dose to the target- insect upon contact. This is considered to be an effective method for adult mosquito population reduction, especially in epidemics (commonly used in ZIKV and DENV outbreaks), but it must be repeated at intervals and coincide with the period of time that mosquitoes have the maximum outdoor activity [38]. The traditional methods of space spraying are: **a) Thermal fogging/ spraying:** a thermal power-operated machine produces a dense cloud of insecticide droplets [15] and **b) Ultra-low volume (ULV) spaying:** a cold mechanical aerosol-generating machine that produces small insecticide droplets at low pressure (WHO, Geneva 2003; Space spraying application of insecticides for vector and public health control: A practitioner's guide).

The selection of equipment for space spaying primarily depends on the size and accessibility of the treated area. The most commonly used machines for thermal or cold fogging are portable foggers, vehicle-mounted foggers **(Image 16A,B)** or aircraft application of fogs. It should be mentioned, than in 2009 European Union prohibited aerial spraying of pesticides, due to its potential adverse impact on human health and environment, with some exceptions concerning public health emergencies (https://eur-lex.europa.eu). Following the regulations, pyrethroids and some organophosphates (e.g. malathion) are the only insecticides used in fogging applications.

Indoor residual spraying (IRS) is a fundamental tool of vector control programs. IRS refers to the application of long-lasting, residual insecticides on all interior surfaces (walls, ceilings, etc) of houses, structures, animal shelters, etc. targeting resting adult mosquitoes **(Image 16C)**. When a resting mosquito vector contacts a treated surface, it absorbs a lethal dose of the insecticide, reducing its lifespan (WHO, Geneva 2015; Indoor Residual Spraying, An Operational Manual for IRS for Malaria Transmission Control and Elimination, 2nd Ed.). IRS is more effective against indoor-feeding and indoor-resting mosquitoes. It reduces adult mosquito density and longevity and it has been proven a very powerful tool for control of malaria transmission, being the core control method during the Global Malaria Eradication Campaign (1955-1969). (CDC, [30], WHO, Geneva 2015; Indoor Residual Spraying, An Operational Manual for IRS for Malaria Transmission Control and Elimination, 2nd Ed.). Insecticides currently

recommended by WHO for IRS applications belong to all 4 main classes (pyrethroids, OCs, OPs, carbamates) (http://www.who.int/whopes/quality/en), with pyrethroids and DDT being the most widely used.

Insecticide- treated Nets (ITNs) act as physical barriers, protecting individuals from mosquito bites either by killing or repelling them. They take advantage of the indoor -resting or –feeding behavior of some *Anopheles* species. ITNs are considered one of the most powerful tools for malaria prevention in sub-Saharan Africa **(Image 16D)**, where *An. gambiae* complex and *An. funestus*, the dominant malaria vectors, bite mainly during the night when people are sleeping. In the African Region, extended utilization of ITNs has led to a 20% decrease of malaria deadly cases in children under 5 years old and a 60% aversion of malaria cases between 2000-2015, under ideal conditions ([30], CDC, WHO; Global Malaria Programme- ITNs: a WHO Position Statement). ITNs have been proven to be effective also against vectors of lymphatic filariasis, Japanese encephalitis, Chagas disease, leishmaniasis, etc (WHO; Global Malaria Programme- ITNs: a WHO Position Statement). It should be mentioned, that pyrethroids are the only active compound used in ITNs, according to human safety requirements.

Apart from the conventional ITNs, **long-lasting insecticide-treated nets (LLINs)** retain their insecticidal activity for at least 3 years and after many WHO-standard washes. Given the emerging pyrethroid resistance in Africa, it has been suggested to incorporate piperonyl butoxide (PBO) along with pyrethroids in ITNs and LLINs; PBO acts as a synergist inhibiting metabolic enzymes involved in pyrethroid detoxification, increasing in this way pyrethroid efficacy [120]. Additionally, there are insecticide-treated clothes that can be worn during the day, at work, at school or outdoors [42].

Autodissemination is an approach of treating habitats with insecticides, where dispersal and transfer of the toxic compound is carried out by contaminated adult mosquitoes; this requires the mosquito to come in contact with an insecticide-treated material or dissemination station, such as ovitraps, in order to spread the insecticide in every subsequent contact with any other surface [42]. This method has been recently applied against *Ae. aegypti* and *Ae. albopictus*, targeting their container breeding sites which are often difficult to identify, reach and treat in large scale mosquito control programs. Under this approach female mosquitoes following contact with a larvicide treated surface transfer themselves a lethal concentration of the insecticide (e.g. IGRs, OPs) to the larval habitat during oviposition **(Image 16Ea)**, leading to a decrease in larval survival. Autodissemination method for *Aedes* mosquitoes control is also facilitated by the fact that they breed in multiple containers during a single gonotrophic cycle and share the oviposition sites with many female mosquitoes; thereby, gravid females are easily contaminated by the insecticide [131]. Furthermore, males demonstrating a polygamic behavior can

contaminate multiple females during subsequent mating **(Image 16Eb,c)**. Preliminary studies suggest that autodissemination could also reduce *Anopheline* larval populations [96, 131].

Attractive Toxic Sugar Baits (ATSBs) rely on the use of toxic sugar meals sprayed on plants or in bait stations to attract mosquitoes to feed on them, causing mortality. Bait solutions contain sugar, an attractant and an oral toxin. Sugar and attractants are usually products of local fruits, juices, etc. Mosquito baiting methods are fundamentally based on mosquito biological requirements, ecology and feeding behavior. ATSBs can set up either outdoor or indoor and mortality comes as a result of direct feeding and/or dissemination. Some insecticides and toxic compounds having been incorporated into ATSB for testing purposes, so far, are carbamates, OPs, pyrethroids, neonicotinoids, pyriproxyfen, fipronil, boric acid, spinosad, dsRNA, etc [42]. The main disadvantage of this application is the possible side-effect on non-target insects [42,71].

Spatial repellents are products designed to release airborne chemicals that cause alterations in insect behavior, e.g. moving away from the chemical stimulus, inhibiting host attraction, interfering with feeding response, etc., thus reducing human-vector contact and, consequently, pathogen transmission. Such products can be applied at a household level (e.g. mosquito coils, vapourizing strips) or as personal protection (e.g. sprays, stickers). Spatial repellents contain either natural or synthetic compounds [41]. The exact mechanism of how spatial repellents induce mosquito avoidance behavior and how this behavior is maintained in the population remain unclear [42].

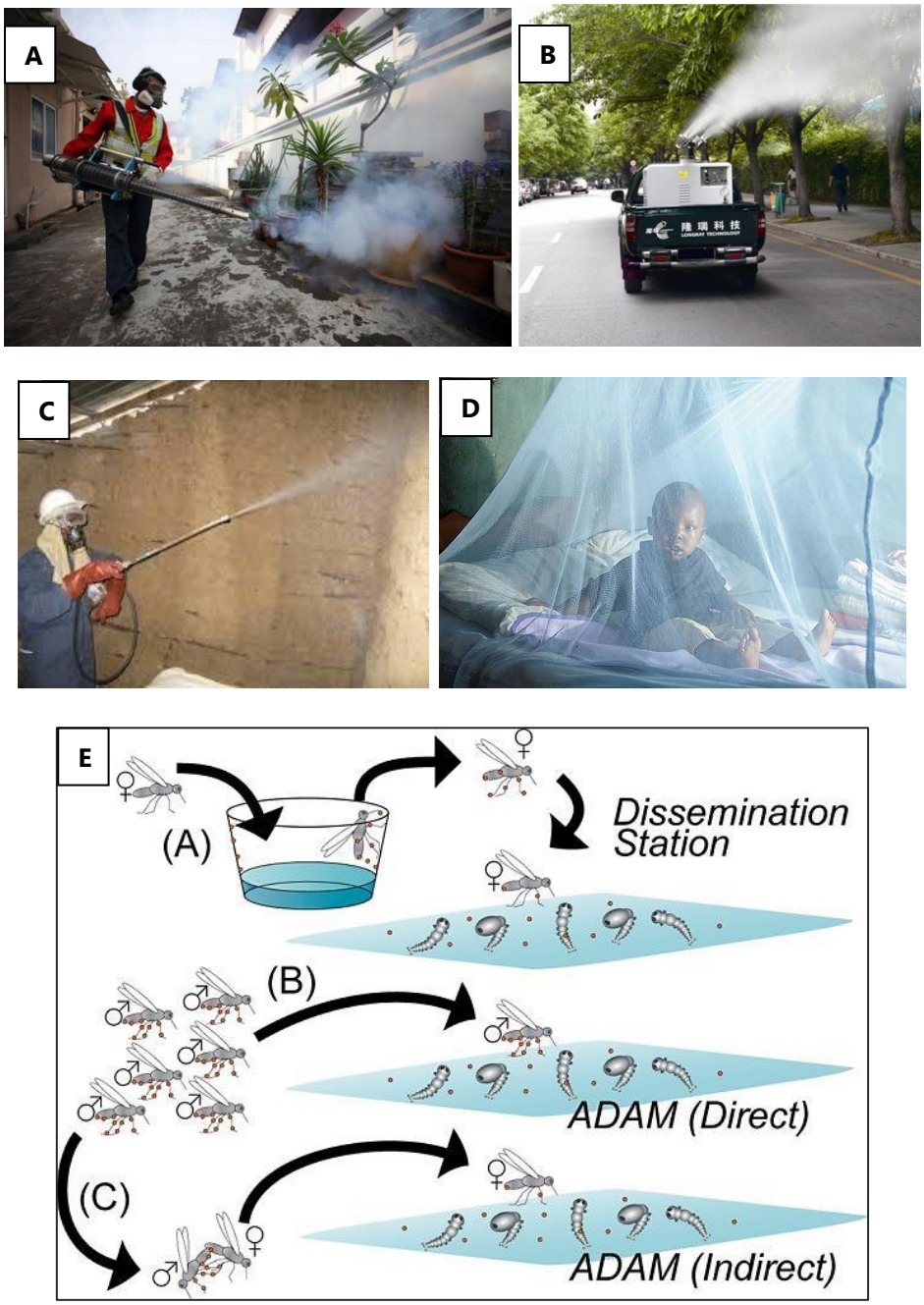


Image 16: Insecticide application techniques: **(A)** Portable thermal fogger, **(B)** Truck-mounted ULV cold, **(C)** Indoor residual spraying (IRS) in Africa, **(D)** Children protected under an insecticide-treated bednet in Africa, **(E)** Autodissemination approach [20, 35, 36, 96].

5.2.b. Biological Control

Mosquito biocontrol makes use of predators, competitors, parasites, microorganisms, and toxins produced by microorganisms to reduce the mosquito population at an "acceptable" level without disturbing the ecosystem irreversibly (Woodring & Davidson, 1996).

For this purpose, entomopathogenic fungi, such as ascomycetes *Beauveria bassiana* and *Metarhizium anisopliae,* have been suggested for control of both larval and adult mosquito stages, as they produce infective conidia that attach to and penetrate the mosquito cuticle, releasing toxins [51]. Moreover, *Bacillus thurigiensis* var. *israelensis* (*Bti*) is increasingly used in Europe as a microbial larvicide in mosquito control; *Bti* is a Gram-positive bacterium, that forms spores and produces insecticidal toxins and virulence factors, targeting the larval mosquito stages. It can be used in combination with another microbial larvicide, *Lysinibacillus sphaericus* (*Lsph*). Once the larvae feed on the microbial larvicides in a treated breeding site, toxins are activated in the gut of the larva and act by disrupting the cell membranes. However, *Bti* has a short-term efficacy, in comparison to the chemical insecticides, and it does not significantly reduce the vector density, when applied alone in field-studies [51, 55].

Another biocontrol strategy proposes the exploitation of natural enemies that feed on larvae and pupae stages in aquatic environments. Larvivorous fish of the genera *Cambusia* and *Poecilia* have been proven effective at reducing mosquito larval populations in more than 60 countries; however, as invasive species, these fish could be a threat to the native aquatic fauna. Cyclopoid copepods, such as *Cyclops vernalis, Megalocyclops aspericornis, M. edax*, can also pray on mosquito larvae; the first introduction of copepods for mosquito control was performed in a village in northern Vietnam, in 1993, and by 2000 the primary vector of DENV, *Ae. aegypti*, was eradicated by the surrounding area and disease transmission was eliminated [51, 55].

An alternative strategy for mosquito population replacement and, gradually, suppression is the release of mosquitoes, implementing the *Wolbachia*-induced cytoplasmic incompatibility (CI). *Wolbachia*, a natural endosymbiotic α-proteobacterium in multiple insect species (including *Cx. pipiens* and *Ae. albopictus*), is known to cause alterations in the host's reproductive phenotype; it infects the gonads and is maternally transmitted to the next generation of eggs [42]. One *Wolbachia*-induced reproductive phenotype is CI, where mating between *Wolbachia*-infected males and uninfected females produces unviable offsprings **(Image 17A,B)**. Regular release of male mosquitoes infected with a *Wolbachia* strain that is not present in the wild-type mosquito population is expected to suppress the population in the field. Moreover, some *Wolbachia* strains could affect the vectorial capacity of the mosquito due to the upregulation of the mosquito immune system and direct interference with the pathogen (virus) or by shortening the mosquito longevity **(Image 17C)** [42, 130].

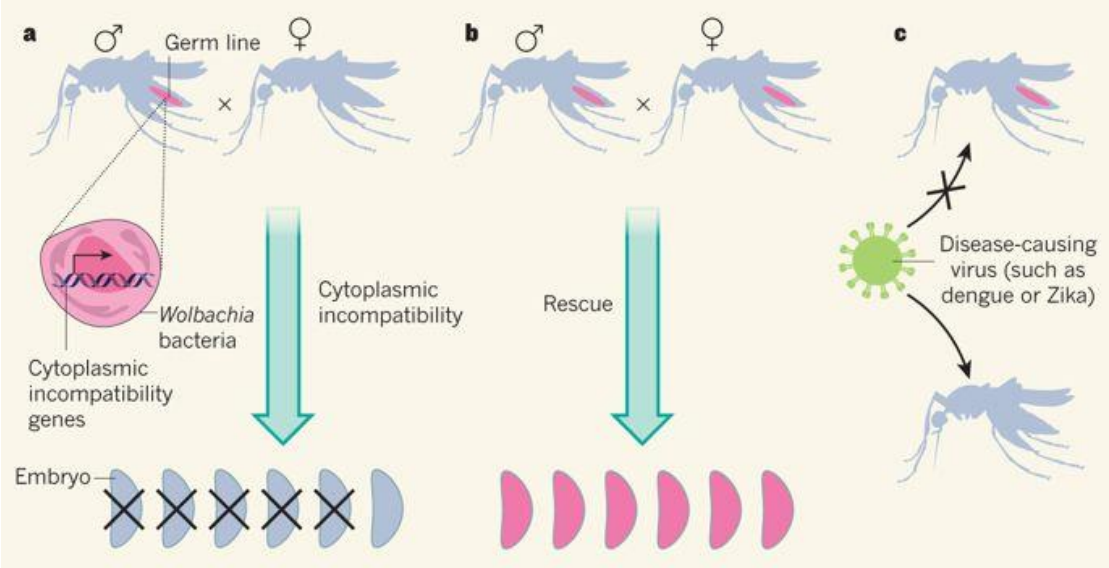


Image 17: Cytoplasmic incompatibility and *Wolbachia* in disease control: **(A)** Matings between *Wolbachia*-infected males and uninfected females results in embryo lethality or low hatch rates (insect populations suppression). **(B)** CI can be prevented in rescue matings between *Wolbachia*-infected males and *Wolbachia*-infected females. **(C)** *Wolbachia* infection can prevent insects from being infected by some viruses that cause human disease [130].

5.2.c. Physical or Mechanical Control

Traps are powerful tools for mosquito surveillance and control, in recent years. The sensitivity and specificity for target species determine traps efficacy for vector elimination, both in larval and adult stages. They mostly target *Aedes* mosquitoes, especially gravid females seeking for a host or an appropriate oviposition site.

Sticky traps with adhesive surfaces or lethal ovitraps with egg-laying strips treated with insecticides (mostly pyrethroids, e.g. deltamethrin) are two types of traps extensively used **(Image 18)** [42, 51]. Other traps released in the market mimic the smell of humans, by producing CO_2, UV or visible light, heat, octenol, or a combination of them to attract mosquitoes in, where they die. However, it is doubtful whether this method can lead to a measurable reduction in mosquito bites in a wider area surrounding the trap [18].

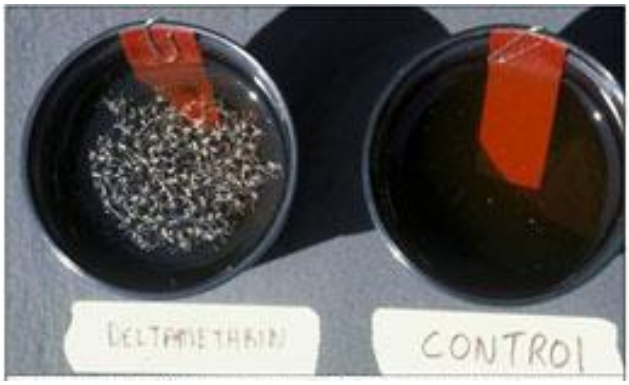


Image 18: A lethal ovitrap (left cup) with a paper strip treated with deltamethrin (adulticide).

5.2.d. Environmental Control- Larval Source Management

Larval source management (LSM) is the manipulation of aquatic habitats, that can be occupied by larvae, aiming to limit the presence of potential breeding sites and prevent the completion of the immature stages development. LSM includes **a)** habitat modification (e.g. drainage of vegetation), **b)** habitat manipulation (e.g. removing or cleaning artificial water containers), **c)** application of biological (e.g. *Bti*) or chemical (e.g. diflubenzuron) insecticides and **d)** biological control, introducing natural predators (e.g. larvivorous fish) into water bodies (WHO, Geneva 2013; Larval source management: a supplementary measure for malaria vector control: an operational manual).

LSM is an important part of Integrated Vector Management. So far, it has been widely implemented for malaria control in Africa, supplementing IRS and ITNs. Besides, it is fundamentally applied against *Aedes* mosquitoes, but may also affect the distribution of *Culex* mosquitoes in a locality, by destroying potential oviposition sites.

Environmental management of mosquito larval sources partially relies on the consistent collaboration between sectors and between public authorities and houseowners, as private residences are important sources; thereby it is difficult to implement in an effective way. Community programs and campaigns organized by professionals to educate and sensitize the public could have a positive impact on this limitation [51].

5.2.e. Genetic Control

Genetics can provide new, species-specific and environmental friendly approaches for mosquito control, aiming either towards population suppression or replacement by

introducing a specific trait. All approaches rely on the mate-seeking behavior of the genetically modified mosquito.

The sterile insect technique (SIT) is based on the sterilization of large numbers of male mosquitoes using DNA damaging means, mostly γ-radiation, and the release in the field in order to suppress the population by antagonizing with wild male mosquitoes. Irradiation induces random lethal mutations in the male germ cells that prevent fertilization; consequently, mating with wild-type females will not produce any offspring. The results of SIT for *Ae. albopictus* field-populations were encouraging, but it remains a cost-effective method, still difficult to be implemented at a large-scale [42, 51, 55].

In recent years, release of genetically modified mosquitoes for population suppression or replacement gains more scientific interest. The release of insects carrying a dominant lethality (RIDL) is a strategy that reduces the vector population through individual (mostly male mosquitoes) that carry a repressible transgenic construct acting in any chosen time of developmental cycle to prevent the mosquito survival [55].

Gene drive is a recently developed technology, involving genetic elements inherited from parents to offsprings at a higher frequency (almost 100%) than Mendelian-inheritance, achieved by CRISPR/Cas9 mediated homologous recombination **(Image 19).** Thereby the desired trait is spread through the population very quickly. Gene drives may aim to suppress or replace mosquito populations, e.g. inheriting a trait that affects mosquito fertility or provides resistance to a virus/parasite, etc [64].

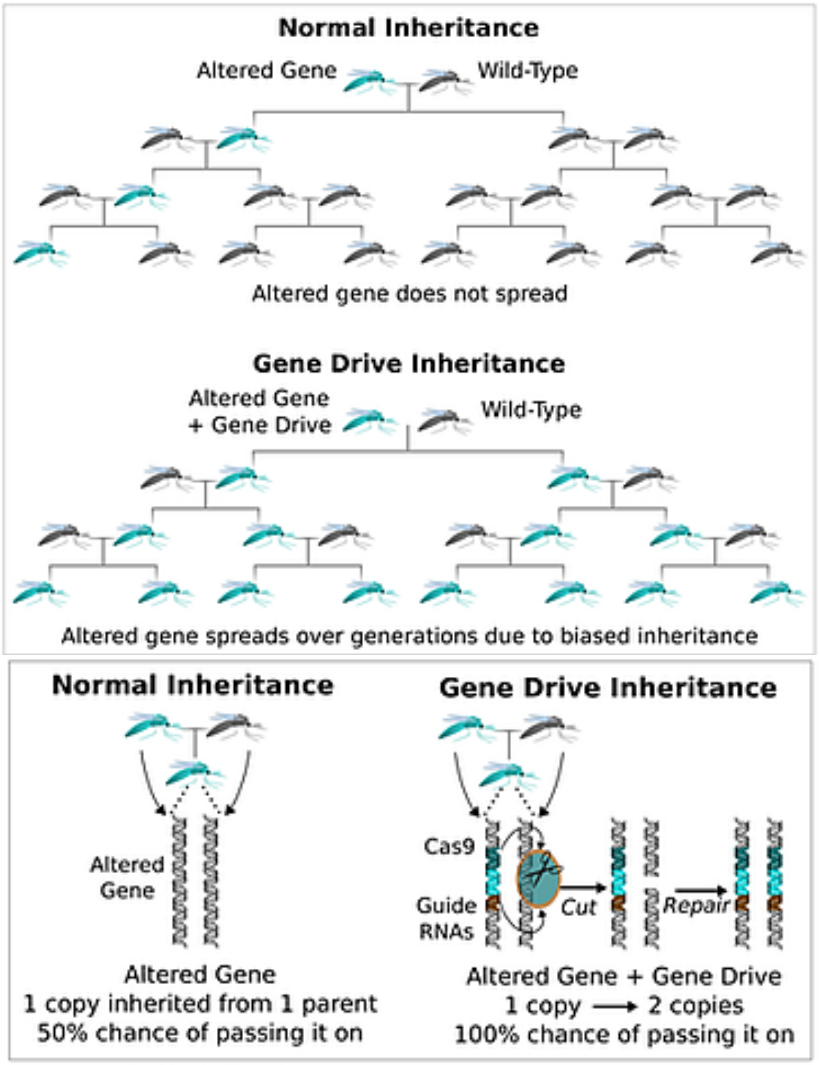


Image 19: Comparison of normal Mendelian inheritance with Gene Drive inheritance [4].

The RNA interference (RNAi) method is another approach used to modify mosquitoes, by enhancing the endogenous RNAi immune response of the insect to viruses. To date, this strategy has been implemented in *Ae. aegypti* mosquitoes genetically modified by RNAi against a specific sequence of DENV-type 2 RNA genome, that finally gave female mosquitoes resistant to DENV-2 [51].

5.2.f. Mosquito Control in Greece

In Greece, larvae control composes the major strategy against *Cx, pipiens*, *Ae. albopictus* and *Anopheles spp*. vectors (http://www.ecodev.gr), focusing on elimination of larval habitats, biological control (*Bti)* and chemical control using approved IGRs (diflubenzuron, pyriproxyfen, methoprene, spinosad), in large scale mosquito control programs. Larvicides are

mainly implemented in rice-fields, swamps, irrigation channels, streams, ponds, wells, etc. via spraying from the ground using portable foggers or, rarely, via air-spraying (e.g. drones, helicopters) (https://eody.gov.gr). Targeting larval sources in urban areas requires the collaboration of the community to remove artificial water containers.

Adult mosquitoes control by the use of pyrethroids (e.g. permethrin, deltmethrin) is supplementary, for example in public health emergencies, through IRS and/or ULV foggers, after relative authorization (https://www.moh.gov.gr).

CHAPTER 6:

Insecticide Resistance

6.1. Insecticide Resistance Mechanisms

The prolonged and intense use of a limited number of insecticides in both mosquito and agricultural pest control has led to the development and spread of insecticide resistant mosquito populations, posing a serious problem for their efficient control.

The Insecticide Resistance Action Committee (IRAC) defines resistance as "*the selection of a heritable characteristic in an insect population resulting in the repeated failure of an insecticide product to provide the intended level of control when used as recommended*" (https://www.irac-online.org/about/resistance/mechanisms/). There are four different mechanisms of insecticide resistance documented: **(a)** target-site insensitivity, **(b)** metabolic resistance, **(c)** penetration resistance and **(d)** behavioral resistance **(Image 20)** [46]. The first two mechanisms are the most well-studied. Multiple resistance mechanisms can coincide in individual specimens.

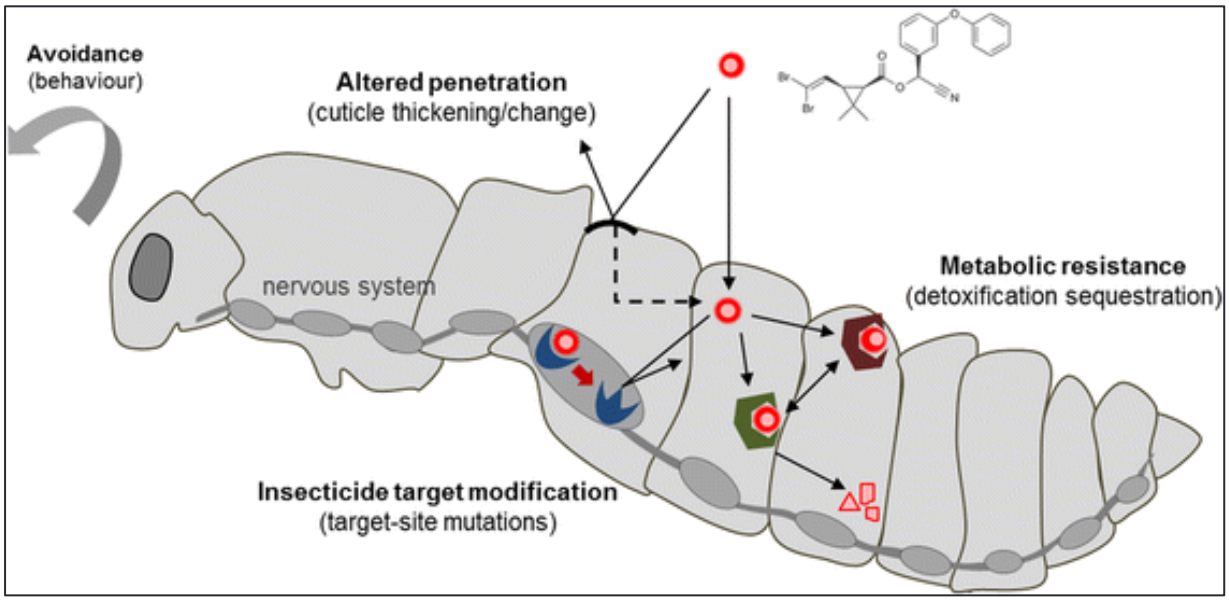


Image 20: Insecticide resistance mechanisms in mosquitoes [6].

- **Target-Site Insensitivity**

Target-site resistance results from genetic modifications of the insecticide target site of action (mutation in the gene encoding for the insecticide target) reducing or eliminating the binding of the insecticide at that site [46].

Mutations in the voltage sensitive sodium channel gene (*vssc*) are the most common causes of target-site resistance to pyrethroids and DDT, often referred as knock-down resistance (*kdr*) mutations. The intense use of pyrethroids and DDT globally has led to the development of several *kdr* mutations in many mosquito species (including the disease vectors of genera *Aedes*, *Culex* and *Anopheles*), varying in frequency, geographical distribution and effect on resistance levels. The level of the *kdr* effect is defined by the change in affinity between the insecticide and the binding site on the VSSC due to single or multiple substitutions in the *vssc* gene.

Synonymous *kdr* mutations identified in mosquitoes and their location on the VSSC are presented in **Image 21**. Six of the shown mutations have been functionally characterized through heterologous expression in *Xenopus* oocyte system: S989P, I1011M/V, L1014F/S, V1016G/I, F1534C and D1763Y (the numbering of mutations corresponds to the amino acid positions on the house fly VSSC-1). Multiple combinations of these mutations could act synergistically, co-conferring various levels of resistance to different pyrethroids (mostly, permethrin and deltamethrin) and, some of them, also, to DDT.

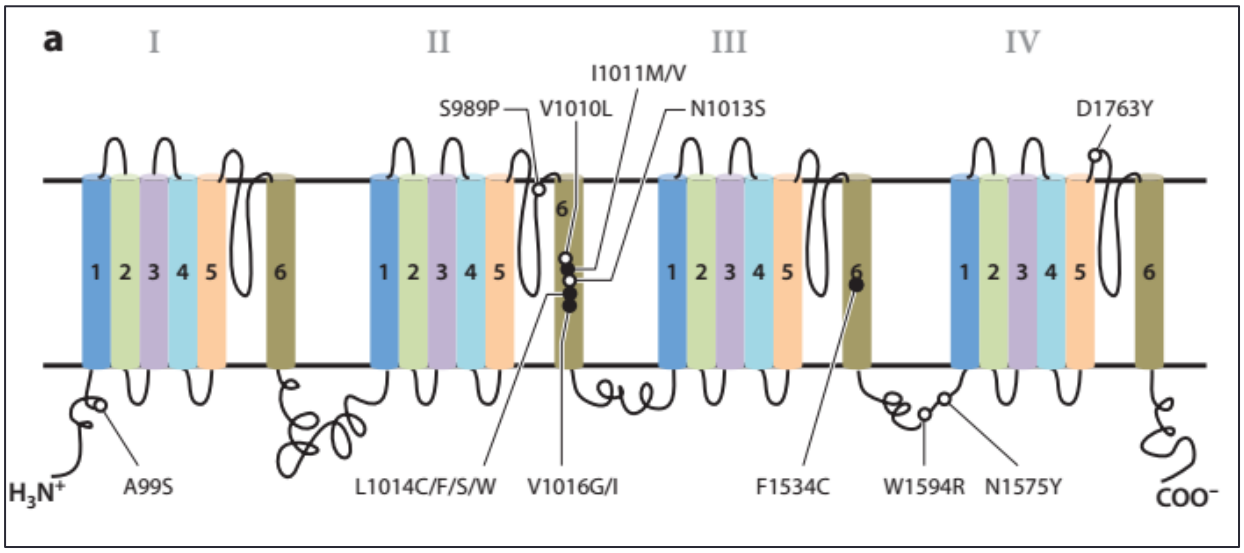


Image 21: Location on the VSSC of some *kdr* mutations-associated with pyrethroid resistance, detected in several mosquito species. [95].

Acetylcholinesterase 1 (AChE1) is involved in organophosphate (OP) and carbamate resistance in mosquitoes; mutations in *ace-1*, the gene encoding for AChE1, F455W and G199S have been identified in many mosquito species, including *Cx. tritaeniorhynchus*, *Cx. pipiens, Cx. quinquefasciatus, An. gambiae* and *An. albimanus*, and confer resistance or reduced sensitivity to OP and carbamates [95].

Substitutions I1043L/M in the chitin synthase gene 1 (*CHS1*) have been associated with high levels of targer-site resistance to diflubenzuron. These mutations are located in the diflubenzuron putative binding site of the enzyme and were recently identified in *Cx. pipiens* field-caught populations from Italy [68, 79, 142].

- **Metabolic Resistance**

Metabolic resistance occurs as a result of the enhanced detoxification of the insecticide molecule caused by overexpression or conformational change of an enzyme that is normally involved in the metabolism, sequestration or excretion of the insecticide. Enzymes undertake such processes are mainly P450-monooxygenases, glutathione S-transferases (GSTs) and carboxycholinesterases (CCEs) [46]. This resistance mechanism is very common among mosquitoes and has been reported for all the insecticides used for public health and agricultural purposes.

Mosquito genome encodes for over 100 P450s [46]; these enzymes are primarily associated with resistance to insecticides, when overexpressed. Elevated P450s activity is frequently reported to mediate mainly pyrethroid, but also DDT, resistance in many mosquito species: CYP6P3, CYP6P9, CYP6M2, CYP6Z1 are validated pyrethroid and/or DDT metabolizers, associated with resistance in African Anopheline mosquitoes, while CYP9J32, CYP9J24 and CYP9J28 mediate pyrethroid resistance in *Ae. aegypti* and CYP6M10 and CYP4H34 in *Culex* mosquitoes. Esterases and GSTs also contribute partially to pyrethroid resistance. Additionally, increased P450s activity could potentially confer cross-resistance to carbamates [66, 102].

Studies on metabolic resistance to temephos (OP) mostly concern *Aedes* mosquitoes. Transcriptomic analysis in temephos resistance condition revealed a wide variety of P450s, GSTs and CCEs overexpressed; some overexpressed CCEs are common in both *Aedes albopictus* and *Ae. aegypti* mosquitoes resistant to OPs and carbamates [102].

▪ Reduced Penetration

Reduced penetration of insecticides into the insect body occurs either by modifying the composition or enhancing the thickness of the cuticle, through increased deposition of its structural components. This alteration creates a barrier that slows down the rate of insecticide penetration into the mosquito body; therefore, the insecticide molecules delay to reach their target and detoxification enzymes have more time to act, resulting in a stronger resistance phenotype [48].

The mosquito cuticle is the exoskeleton of the body, constituting the first barrier to the insecticide penetration. Epicuticle, the outer layer, comprises of hydrocarbons, proteins and lipids, mostly fatty acids. Hydrocarbons are specifically produced by oenocytes. In a deltamethrin resistant strain of the major malaria vector, *An. gambiae*, cuticular analysis revealed both thicker epicuticular layer and increased content of hydrocarbons. *cyp4g16* and *cyp4g17* genes, involved in cuticular hydrocarbon biosynthesis, are overexpressesed in oenocytes of resistant *An. gambiae* and *An. arabiensis* populations from African regions [49].

The modification of the cuticle occurs also in legs, the most relevant tissue for insecticide uptake. Mosquitoes remodel their leg cuticles by increased deposition of cuticular proteins and chitin, producing a thickened leg that contributes to a resistant phenotype [50].

▪ Behavioral Resistance

This mechanism refers to any change in mosquito behavior that permits the avoidance of the lethal effects of an insecticide. It has been reported against several classes of insecticides, including OCs, OPs, carbamates and pyrethroids (IRAC; [16]). However, not much research has been conducted on it, as the investigation requires the examination of field populations and the changes in behavior are not always easy to observe or quantify. The contribution of this mechanism to the decreased efficacy of vector chemical control is underestimated, in comparison to the other resistance mechanisms [126].

The aversion behavior can be either learned or based on simple repellency. It may be associated with the mobility or immobility of the insect in order to avoid the toxic compound or limit the duration of being in contact to it. For example, mosquitoes may stop feeding on a plant if they come across an insecticide or move to the underside of a sprayed leaf or fly away. Other observed behavior adaptations in the field may concern changes in the biting time and/or site, the resting time and/or site, the preferred host species, etc. In many African mosquito species, behavioral

modifications have been observed after the implementation of IRS and ITNs; there are some typical examples following: *Anopheles gambiae s.l.* in Bioko Island and *An. funestus* in Tanzania have displayed an increased outdoor host-seeking behavior, while in areas with high ITNs coverage in Kenya, *Culex quinquefasciatus*, *An. funestus* and *An. gambiae s.s.* increased their zoophilic behavior [59].

6.2. Insecticide Resistance Status of *Aedes* Vectors Worldwide

Both *Aedes aegypti* and *Aedes albopictus* are major arbovirus vectors; controlling disease transmission by these species largely relies on the use of insecticides. Resistance to all 4 main insecticide classes, associated with target-site mutations and metabolic detoxification, has emerged globally. Most studies have been conducted in Asia and America, while there are important data gaps for Africa and Europe. Moreover, the knowledge concerning the resistance status of *Aedes albopictus* is fragmented and more resistance data refers to *Aedes aegypti* [46].

Knock-down resistance (*kdr*) mutations in the *vssc* gene are very commonly detected in *Ae. aegypti* populations from Africa, Asia and America; 10 *kdr* mutations (in 15 haplotypes) have been reported to confer resistance to pyrethroids, individually or in combination to another one, located at 8 codon positions in VSSC domains II, III, and IV. The geographical distribution of these mutations across the 3 continents is presented in **Image 22** [69, 102].

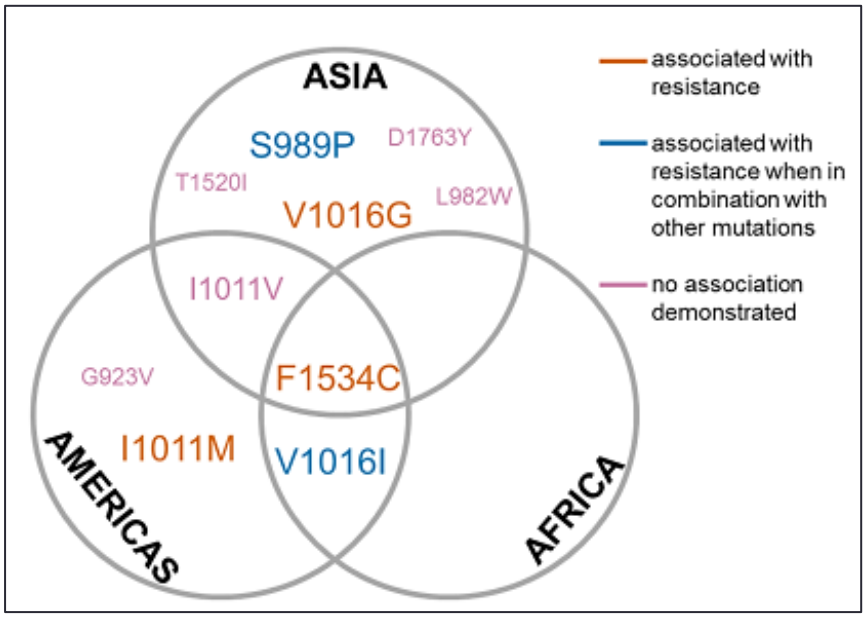


Image 22: The geographical distribution of the 10 known *vssc* mutations in *Aedes aegypti* across the 3 continents in which they have been detected. Association with pyrethroid resistance is shown in the key. Font size gives an indication of relative frequency [102].

In *Ae. albopictus*, 5 mutations in 3 loci (1016, 1532 and 1534) of *vssc* gene domains II and III associated with pyrethroid resistance have been reported, to date. Substitution V1016G leads to both type I and II pyrethroids resistance, while *kdr* F1534C confers mainly resistance to permethrin and *kdr* F1534S/L to deltamethrin; however, mutation at locus 1016 gives much higher resistance levels than those at locus 1534. It remains ambiguous based on literature whether mutation I1532T plays a role in pyrethroid resistance [88].

In European *Ae. Albopictus* populations, mutation I1532T has been detected in Vlore, Albania at a frequency of 11% [114], while the mutations V1016G [88] and I1532T [114] have been reported across Italy at various frequencies, the highest being 36% and 32% respectively, in the Emilia-Romagna region. Mutation F1534L has been recorded once in Acro, Italy at an allele frequency of only 1%. Recently, a high F1534C allele frequency of 66% was detected in Athens, Greece [114]. Regarding other continents, mutation V1016G has been found in Vietnam [88] and I1532T in China [139]. Substitutions at position F1534 have been found in populations from Asia and America; F1534C in Brazil, China and Singapore, F1534L in Florida (USA), China and Malaysia and F1534S in Florida (USA) and China [46, 88, 102, 114 125, 139].

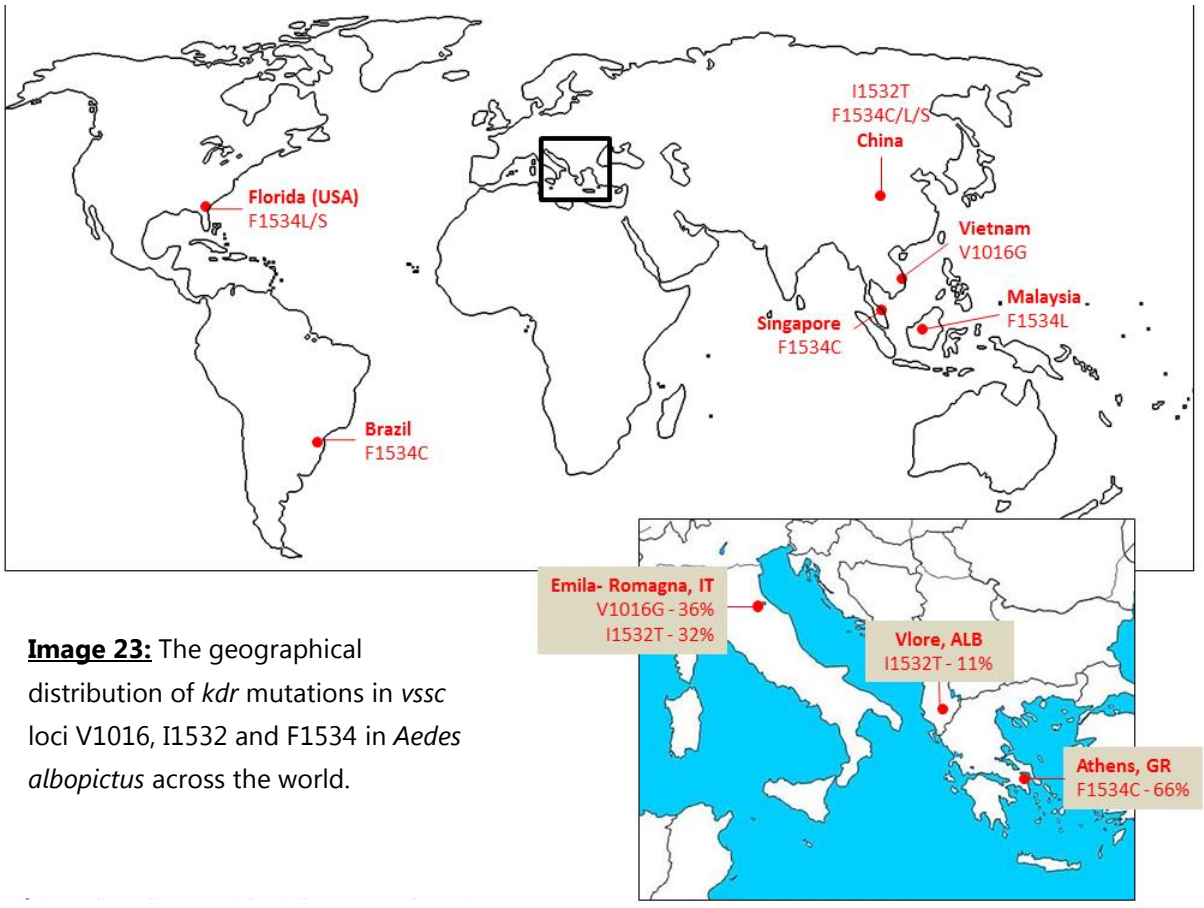


Image 23: The geographical distribution of *kdr* mutations in *vssc* loci V1016, I1532 and F1534 in *Aedes albopictus* across the world.

In *Aedes* vectors, multiple P450 genes are implicated in metabolic resistance to pyrethroids, but this has been linked especially to overexpression of *CYP6* and *CYP9* subfamilies, the most commonly duplicated P450s; some validated members are *CYP9J10, CYP6BB2, CYP9J26* and *CYP6J28* [102]. Through transgenic expression in *Drosophila melanogaster*, *CYP6P12* has been proven to confer resistance to pyrethroids in *Ae. albopictus* [85].

Resistance to OPs and carbamates is also common in *Aedes* vectors; data from bioassays until 2015 indicate high levels of temephos (OP) resistance in *Ae. aegypti* from Central and Latin America and in *Ae. albopictus* from SE Asia, while carbamate resistance has been recorded in SE Asia for both vectors. However, target-site mutations in *ace-1* gene do not occur widely; substitution G119S associated with OP resistance has only been detected in *Ae. aegypti* populations from India so far [103], but studies monitoring such mutations are scarce.

As mentioned previously, metabolic resistance to OPs involves a large variety of P450s, CCEs and GSTs identified as overexpressed, especially in *Ae. aegypti*, but also in *Ae. albopictus*. There are some common genes overexpressed in resistant populations of both species, like the alpha-esterases CCEae3a and CCEae6a, the most possible candidates mediating temephos resistance when upregulated, due to gene amplification. Upregulation of *CCEae3a* and *CCEae6a* has been correlated with temephos resistance in *Ae. aegypti* populations from Thailand [116]. A similar result has also been shown for *Ae. albopictus* populations from Greece and Florida, USA [78].

CCEae3a and *CCEae6a* are very closely located on the genome; this genomic region probably consists a "hot spot" for recombination and amplification, but the mechanism has not been thoroughly investigated yet. CCEae3a is located in the malpighian tubules and nerve tissues of *Ae. albopictus* larvae, where it metabolizes temephos oxon, the activated form of temephos **(Image 24)** [77].

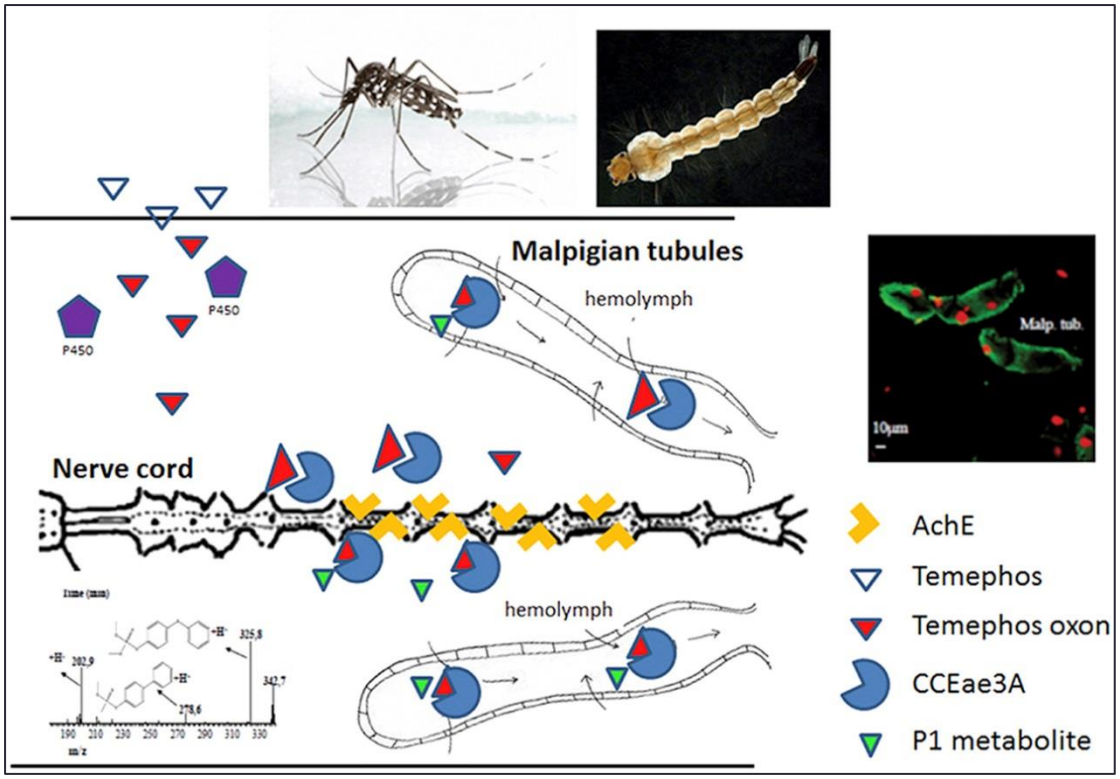


Image 24: CCEae3a is localized in the Malpighian tubules and nerve tissues in *Ae. albopictus* and is associated with temephos resistance, when upregulated due to gene amplification, through metabolizing temephos oxon [77].

As far as resistance to alternative insecticides currently being used, like IGRs, there is a little evidence on field populations of *Aedes* mosquitoes; bioassay data from *Ae. aegypti* population of Martinique and *Ae. albopictus* of Malaysia demonstrated low levels of resistance to diflubenzuron and other IGRs [93, 97]. It should be noted, that to date there has been no mutation correlated with resistance to diflubenzuron in *Aedes* mosquitoes.

PART A:

Molecular analysis of the insecticide resistance status of *Aedes albopictus* populations from Greece

The aim of the study

Public health preparedness against vector borne diseases (VBDs) requires continuous surveillance of the vector species composition, distribution and dynamics and systematic analyses of their insecticide resistance status, in order to design and implement efficient control programs that will reduce and may prevent VBD transmission. The alarming re-appearance of VBDs in Europe, the widespread distribution of *Ae. albopictus* in Southern Europe and the limited selection of available insecticides make the continuous monitoring and analysis of *Ae. albopictus* insecticide resistance a necessity.

The Asian tiger mosquito was first recorded in Greece 15 years ago [47] and, to date, has been established in many Regional Units. Taking into account that our country suffers from consecutive WNV epidemics since 2010 (mainly attributed to *Culex pipiens* mosquitoes) and that *Ae. albopictus* is a secondary vector of WNV, we cannot exclude its possible implication in disease circulation, over the years; hence, it poses a threat for public health.

There is a limited number of recent studies concerning *Ae. albopictus* surveillance in Greece [47]. Here, in the framework of organized evidence-based mosquito control programs, we conducted an analysis of its distribution in the country and monitored the presence of previously characterized alleles conferring insecticide resistance, in populations of different regions of the country. Insecticides, to which resistance was examined, were: **(a)** diflubenzuron (IGR), used for *Cx. pipiens* mosquitoes and agricultural pests control in Greece, **(b)** pyrethroids, widely implemented both in organized mosquito control programs and at household level, and **(c)** temephos (OP) which is currently suspended in the EU for mosquito control, but still used against agricultural pests. Utilizing molecular diagnostic tools we investigated the presence, frequency and distribution of *vssc* mutations (*kdr* V1016G, I1532T and F1534C/L/S), *CHS1* mutations (I1043M/L) and *CCEae3a* and *CCEae6a* gene amplification associated with pyrethroid, diflubenzuron and temephos resistance, respectively.

MATERIALS & METHODS

1. Study sites, sample collections and mosquito handling

Adult and immature stage *Ae. albopictus* collections were conducted during the summer of 2017 and 2018 in a total of 29 urban and peri-urban localities in Greece, located in the regions of Thessaloniki and Rodopi (Northern Greece), Attica (Agios Stefanos, Aghios Eleftherios, Aigaleo and Filothei) and Argolida (Central Greece), Chios Island (North Eastern Aegean Island complex), Kefalonia (Ionian Island complex) and Crete (Chania, Rethymnon and Lasithi / Southern Greece) **(Table A2)**.

Adult specimens were collected with aspiration catches and CDC-light traps baited with dry ice. Larvae were sampled with dipping collections and eggs were collected with oviposition traps (black plastic cup with 2 wooden tongue depressors) **(Image 25)**. Both larvae and eggs were collected from at least five different sites within each locality in order to minimize the probability of including isofemale mosquitoes in the molecular analyses.

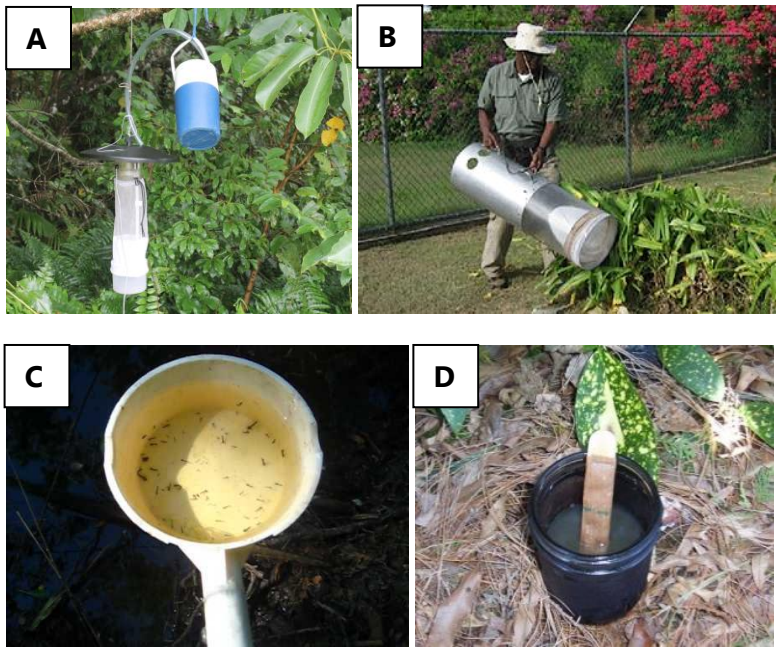


Image 25: Sample collection methods: **(A)** CDC-light trap, **(B)** Aspirator, **(C)** Larvae dipper, **(D)** Oviposition trap

A subgroup of eggs were reared to adults in the lab and all 3rd-4th instar larvae. Adult mosquitoes were identified morphologically to species ("Mosquitoes and Their

Control", N. Becker, Springer, 2nd edition). All specimens were stored in ethanol at -4°C.

2. Genomic DNA extraction (DNazol reagent)

Genomic DNA was extracted from individual larvae or adult mosquitoes and pools of eggs (10 eggs per pool/ per collection site), using DNazol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA):

- Individual larvae or adult mosquitoes were placed in 1.5 ml sterilized tubes. 50 ul DNAzol were added and the mosquito was homogenized using a grinder and a pestle. 150 ul DNAzol were added, after the homogenization.

- Samples were centrifuged at 10.000 rpm for 10 minutes at room temperature (RT). The supernatant was transferred into a new 1.5 ml tube

- 100 ul 100% ethanol were added, mixed and the samples were incubated at RT for 1-3 minutes

- Samples were centrifuged at 13.000 rpm for 20 minutes at RT. The supernatant was discarded and 1 ml 75% ethanol was added.

- Samples were centrifuged at 13.000 rpm for 5 minutes at RT. The supernatant was discarded and the pellet was airdried at 30°C for 30 minutes.

- The pellet was resuspended in 30 ul of sterilized ddH_2O

- DNA is preserved at -20°C

3. Molecular identification of mosquito species

Species identification was based on amplification of the nuclear ribosomal spacer gene ITS2 (internal transcribed spacer 2) by polymerase chain reaction (PCR) (KAPA Taq PCR Kit) (*Patsoula* et al.. 2006; [110]) (primers 5.8S, 28S; **Table A1**):

PCR Protocol	
10x Taq A Buffer	2.5 ul
25 mM $MgCl_2$	1 ul
dNTPs 10mM (Invitrogen)	1 ul
Primer 28S 10uM	1 ul
Primer 5.8S 10uM	1 ul
5 U/ ul KAPA Taq poymerase	0.3 ul
Genomic DNA	1 ul`
ddH_2O	Up to 25 ul

This assay discriminates between *Aedes albopictus, Aedes cretinus* and *Aedes aegypti*, (which are hard to distinguish morphologically) giving a PCR product of 509 bp (according to GenBank published sequence M95127), 385 bp and 324 bp in length, respectively. The applied thermal protocol was the following: initial denaturation at 94°C for 10 min, 40 cycles x [denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min, primer extension at 72°C for 1 min] and final extension at 72°C for 10 min. The PCR products were electrophoresed in 1.5% w/v TAE agarose gel containing ethidium bromide.

4. Genotyping of target- site resistance mutations

a) Monitoring of the *CHS* mutation I1043L/M

The monitoring of the *CHS* mutation I to L/M at position 1043 leading to diflubenzuron target-site resistance in *Culex pipiens* mosquitoes was performed in pools of mixed DNA extracted of 5-8 *A. albopictus* individuals of the same population. Each DNA pool contains 1 ul of each individual gDNA and ddH_2O added up to 10 ul.

A fragment of 350 bp of *A. albopictus* chitin synthase gene, including locus 1043 (numbering based on *Musca domestica* genomic sequence) was amplified in a 25 ul PCR (KAPA Taq PCR Kit) containing:

PCR Protocol	
10x Taq A Buffer	2.5 ul
dNTPs 10mM (Invitrogen)	1 ul
Primer kkv F3 10uM	1 ul
Primer kkv R3 10uM	1 ul
5 U/ ul KAPA Taq poymerase	0.3 ul
Genomic DNA (pool)	1.5 ul`
ddH_2O	Up to 25 ul

The thermal conditions were: initial denaturation at 95°C for 5 min, 40 cycles x [denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, primer extension at 72°C for 1 min] and final extension at 72 °C for 10 min. A small amount of the PCR products were electrophoresed in 1.5% w/v TAE agarose gel containing ethidium bromide. The remaining amount of the PCR products were purified using the Nucleospin PCR & Gel Clean-Up Kit (Macherey Nagel, Dueren, Germany) and sequenced using the kkv F3 primer **(Table A1)**.

b) Detection *of knock-down resistance (kdr)* mutations in the *vssc* gene

The *vssc* gene domains II and III were analysed for the presence of *kdr* mutations associated with pyrethroid resistance at positions 1016 (mutation V to G) in domain II and 1532 (mutation I to T) and 1534 (mutation F to L/C/S) in domain III. The PCR (KAPA Taq PCR Kit) reactions were carried out in 25 ul containing:

PCR Protocol for *vssc* domain II	
10x Taq A Buffer	2.5 ul
dNTPs 10mM (Invitrogen)	1 ul
Primer kdr2R 10uM	1 ul
Primer kdr2F 10uM	1 ul
5 U/ ul KAPA Taq poymerase	0.3 ul
Genomic DNA (pool)	1.5 ul`
ddH_2O	Up to 25 ul

PCR Protocol for *vssc* domain III	
10x Taq A Buffer	2.5 ul
25 mM $MgCl_2$	1 ul
dNTPs 10mM (Invitrogen)	1 ul
Primer kdr3R 10uM	0.75 ul
Primer kdr3F 10uM	0.75 ul
5 U/ ul KAPA Taq poymerase	0.3 ul
Genomic DNA (individual)	1.5 ul`
ddH_2O	Up to 25 ul

The thermal conditions for the PCR amplification of the *vssc* domain II were: initial denaturation at 95^{o}C for 3 min, 40 cycles x [denaturation at 94^{o}C for 30 sec, primer annealing at 55^{o}C for 30 sec, primer extension at 72^{o}C for 1 min] and final extension at 72^{o}C for 5 min. A small amount of the PCR products was electrophoresed in a 1% w/v TAE agarose gel expecting a band of 500 bp in length. For the samples carrying the specific band, the remaining amount of PCR product was purified using the Nucleospin PCR & Gel Clean-Up Kit and sequenced using primer kdr2F **(Table A1)**.

The thermal conditions for the PCR amplification of *vssc* domain III were: initial denaturation at 95^{o}C for 3 min, 40 cycles x [denaturation at 94^{o}C for 30 sec, primer annealing at 57 ^{o}C for 30 sec, primer extension at 72^{o}C for 1 min] and final extension at 72 ^{o}C for 5 min. The products were electrophoresed in a 1% w/v TAE agarose gel and the specific 740 bp band was extracted from the gel, purified using the Nucleospin PCR & Gel Clean-Up Kit and sequenced with primer kdr3Rin **(Table A1)**.

Following the initial PCR product sequencing, the presence of mutations in the *vssc* domain III detected for the first time in Greece was verified with a plasmid- *E. coli* cloning vector system. The *vssc* domain III of the specimens carrying the mutations of

interest was amplified by PCR, as described above, and the PCR products were gel extracted and ligated into the pGEM-T-Easy vector (PROMEGA), using the T4 DNA ligase. *E.coli* competent cells were transformed with the plasmid vector and the recombinant clones were identified by color screening on LB/ X-Gal/ IPTG/ ampicillin plates; white colonies were selected and screened by PCR for the presence of the insert (primers kdr3F, kdr3R; **Table 1**). Positive clones were grown overnight in liquid LB/ampicillin, followed by plasmid extraction (NucleoSpin Plasmid Kit, Macherey-Nagel) and diagnostic *ECoR*I digestion to confirm the presence of the insert. Plasmids carrying the *vssc* domain III insert were finally sequenced using the kdr3Rin primer **(Table A1)**.

5. Metabolic Resistance: Detection of CCEs gene amplification

CCEae3a and *CCEae6a* gene copy number was determined using quantitative real-time PCR on individual *A. albopictus* specimens. Amplification reactions at 10ul final volume were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) containing:

qPCR Protocol	
2x SYBR Select Master Mix (*)	5 ul
Primer CCEae3aF/ CCEEae6aF 10uM	0.2 ul
Primer CCEae3aR/ CCEEae6aR 10uM	0.2 ul
Genomic DNA	0.5 ul`
ddH_2O	Up to 10 ul

*Applied Biosystems, Thermo Fischer Scientific

Histone 3 (NCBI: XM_019696438.1) was used as a reference gene for normalization (primers His3 TaqF, His3 TaqR; **Table 1**). The thermal parameters were: 50°C for 2 min, 95°C for 2 min and 40 cycles x [95°C for 3 sec , 60°C for 30 sec]. Melting curves were performed for reference and target genes to verify the presence of a unique specific PCR product, which was also checked visualized in an 1% w/v TAE agarose gel. A no-template control was included to detect any possible contamination. Samples were amplified in duplicates. *CCEae3a* and *CCEae6a* gene copy numbers were estimated relatively to a temephos susceptible laboratory strain.

Table A1: Primers used in this study for regular and real-time (*) PCR.

Primer Name	Primer Sequence
5.8S	5′ TGTGAACTGCAGGACACATG 3′
28S	5′ ATGCTTAAATTTAGGGGGTA 3′
kkv F3	5' TCGGAAGTCCTTCGGCTTATTC 3'
kkv R3	5' TGGATACTTCAATGGAACCTTCC 3'
kdr2 F	5′ TTCACCGACTTCATGCACTC 3′
kdr2 R	5′ CGCAATCTGGCTTGTTAACTT 3′
Kdr3 F	5′GAGAACTCGCCGATGAACTT 3′
Kdr3 R	5′GACGACGAAATCGAACAGGT 3′
Kdr3 R in	5′ AGCTTTCAGCGGCTTCTTC 3′
His3 Taq F*	5′ CCCAAGATTTCAAGACCGATCT 3′
His3 Taq R*	5′ GGTAGGCTTCACTGGCTTCCT 3′
CCEae3a F*	5′ AGAGTGCGTTACGGATCAAG 3′
CCEae3a R*	5′ TAGCCTCATTGCTGGTTAGC 3′
CCEae6a F*	5′ CAGCATGTCCTCGTTAAAGC 3'
CCEae6a R*	5′ GACAACACACTTCCCTACCG 3'

RESULTS

1. Molecular identification of mosquito species

A total of 369 individual larvae/adult mosquitoes and 30 eggs in pools (grouped in pools of 10 eggs) were identified to species by PCR discrimination of ITS2 genomic sequence length: 96,7% of all samples were identified as *Ae. albopictus*, while only 12 specimens all originated from Chania, (consisting 17,4% of this population) were identified as *Ae. cretinus*, **(Table A2)**.

Table A2: Study sites in Greece, year of sample collections and molecular identification of mosquito species

				Species ID	
Region	**Study sites**	**Year of Collection**	**N**	***Ae. albopictus***	***Ae. cretinus***
Rodopi	Komotini, Iasmos	2018	14	14	0
Thessaloniki	Thermi- Litsa, Ref. Camp Diavata, Ref. Camp Lagkadikia	2017, 2018	58**	58**	0
Chios	Town, Ref. Camp Souda	2017, 2018	23*	23*	0
Athens	Aigaleo, Filothei, Aghios Stefanos, Aghios Eleftherios	2018	98	98	0
Kefalonia	Town	2018	8	8	0
Argolida	Myloi, Kallithea, Laloukas, Aghia Triada, Ira, Koronida	2018	65	65	0
Kalamata	Town	2018	7	7	0
Chania	Town, Souda, Platanias, Pithari, Makrys Toixos, Georgioupoli	2018	69	57	12
Rethymno	Town, Panormos, Violi Xaraki	2018	55	55	0
Lasithi	Ierapetra, Siteia	2018	2	2	0

N: total number of specimens analysed per sampling region for species identification

/*: 10 or 20 eggs analysed in pools (10 eggs per pool) are included

3. Genotyping of target- site resistance mutations

a) *CHS* genomic sequence: mutation I1043L/M

The *CHS1* genomic sequence of N=213 larvae/adult *Ae. albopictus* mosquitoes and 20 eggs was analysed in pools (genomic DNA of 5-7 individuals or 10 eggs of the

same population per pool) for the presence of mutation I to L/M at position 1043. No mutation was detected in any of the genotyped pools; the codon at position 1043 was ATT (isoleucine, I) **(Table A3)**.

Table A3: Genotype and allele frequencies (%) of C*HS-1* locus 1043.

Region	N	CHS1 I1043M/L		
		Genotype	Allele freq (%)	
		II	I	M or L
Rodopi	13	13	100	0
Thessaloniki	68**	68**	100	0
Chios	12	12	100	0
Athens	52	52	100	0
Kefalonia	8	8	100	0
Argolida	22	22	100	0
Kalamata	7	7	100	0
Chania	25	25	100	0
Rethymno	24	24	100	0
Lasithi	2	2	100	0

N: total number of specimens genotyped in pools per sampling region

I: 1043I susceptible allele, **L/M:** 1043L/M mutant alleles

****:** 20 eggs analysed in pools (10 eggs per pool) are included

b) *VSSC* genomic sequence: *kdr* mutations

Genotyping of *vssc* gene was performed on N=211 larvae/adult mosquitoes and 20 eggs for domain II (*kdr* mutation V1016G) in pools (genomic DNA of 5-7 individuals or 10 eggs of the same population per pool), and on N=207 individuals and 2 pools of 10 eggs for domain III (*kdr* mutations I1532T and F1534C/L/S).

No mutation was detected at locus 1016. The wild type allele V1016 (codon GTA: valine, V) was detected in all cases **(Table A4)**.

Mutation I1532T (ATC → ACC: isoleucine → threonine) in *vssc* domain III was detected, for the first time in Greece, in 10 genotyped specimens, all in heterozygosis, with a total 1532T allele frequency of 2,4%. The mutation was detected in 5 out of the 10 surveyed regions, particularly in Thessaloniki, Rodopi, Argolida, Rethymno and Chania, with the mutant allele frequency varying from 1.7% to 6.5% in these sites **(Table A4)**.

Mutation F1534C (TTC → TGC : phenylalanine → cysteine) in *vssc* domain III, to date the only *kdr* mutation reported in Greece, was observed in homo- or hetero-zygosis in all regions **(Table A4)**, with an overall mutant allele 1534C frequency of 40.1%. The regions with the highest frequency were Athens (68,3%), Argolida (45,2%), Rethymno (48,3%), Chania (29%), Lasithi and Kefalonia; however the number of specimens of the two latter regions was not sufficient (N=2 and N=4, respectively - further analyses are required). Northern Greece regions, Thessaloniki and Rodopi, displayed a lower mutant allele frequency of 6,6% and 16,7%, respectively, and Chios Island a frequency of 27,3%. In the majority of the surveyed sites, the mutant allele 1534C appeared mainly in heterozygosis, with the exception of Athens, where 31 out of 52 specimens (51,6%) were homozygous 1543C/1534C **(Table 4)**.

There was only one individual harbouring both mutations, I1532T and F1534C, in heterozygosis (genotype: I1532/T1532, F1534/C1534), sampled from Argolida (Koronida).

Table A4: Genotype and allele frequencies (%) of *vssc* domain II locus V1016 and domain III loci I1532 and F1534.

	***vssc* domain II**				***vssc* domain III**									
		V1016G				**I1532T**				**F1534C**				
		Genotype	**Allele freq (%)**			**Genotype**		**Allele freq (%)**		**Genotype**			**Allele freq (%)**	
Region	**N**	**VV**	**V**	**G**	**N**	**II**	**IT**	**I**	**T**	**FF**	**FC**	**CC**	**F**	**C**
Rodopi	13	13	100	0	12	11	1	95.8	4.2	8	4	0	83.3	16.7
Thessaloniki	48*	48*	100	0	38	36	2	97.4	2.6	33	5	0	93.4	6.6
Chios	22*	22*	100	0	11	11	0	100	0	6	4	1	72.7	27.3
Athens	59	59	100	0	52	52	0	100	0	12	9	31	31.7	68.3
Kefalonia	8	8	100	0	4	4	0	100	0	0	1	3	12.5	87.5
Argolida	21	21	100	0	21	19	2	95.2	4.8	10	3	8	54.8	45.2
Kalamata	7	7	100	0	6	6	0	100	0	2	3	1	58.3	41.7
Rethymno	20	20	100	0	30	29	1	98.3	1.7	10	11	9	51.7	48.3
Chania	31	31	100	0	31	27	4	93.5	6.5	15	14	2	71	29
Lasithi	2	2	100	0	2	2	0	100	0	0	1	1	25	75

N: total number of genotyped specimens for each domain **in pools (domain II)** or **individually (domain III)** per sampling region.

***:** 10 eggs analysed in pools (10 eggs per pool) are included.

V: 1016V susceptible allele**, G:** 1016G mutant resistant allele, **I:** 1532I susceptible allele, **T:** 1532T mutant allele, **F:** 1534F susceptible allele, **C:** 1534C mutant allele; **VV:** 1016V/1016V homozygous susceptible, **II:** 1532I/1532I homozygous susceptible, **IT:** 1532I/1532T heterozygote, **FF:** 1534F/1534F homozygous susceptible, **FC:** 1534F/1534C heterozygote, **CC:** 1534C/1534C homozygous mutant.

5. Detection of CCEs gene amplification

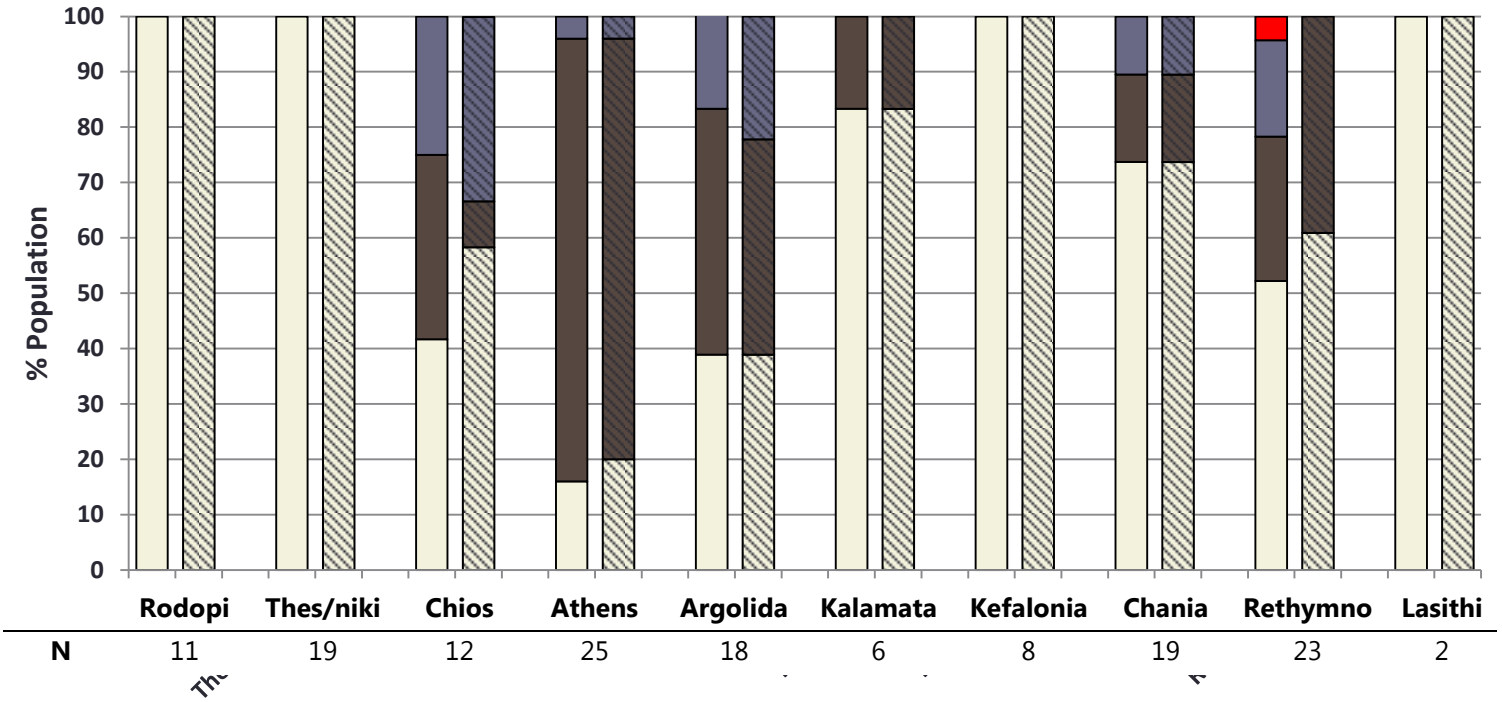
CCEae3a and *CCEae6a* amplification associated with temephos resistance was recorded in 6 out of the 10 surveyed regions in Greece. Two types of amplification were found: *CCEae3a* - *CCEae6a* co-amplicon and *CCEae3a* amplicon.

Amplified esterases were detected in specimens from Chios, Athens, Argolida, Kalamata, Chania and Rethymno. The highest percentages of temephos resistant samples (≥2 copies of CCEs genes) were detected in Athens and Argolida: 84% and 50% of the population samples, respectively. In Chios, Kalamata, Chania and Rethymno, the frequencies of samples with more than 1 copies of *CCEae3a* and *CCEae6a* ranged from 16,7 - 58,3% and 16,7 – 41,6%, respectively. The majority of temephos resistant samples (CCE amplification) had between 2-10 gene copies. The highest frequency of samples with 11-20 gene copies of *CCEae3a* and *CCEae6a* was recorded in Chios (25% and 33,3%), Argolida (8,3% and 16,7%) and Chania (10,5% for both) **(Figure A1)**. There was only one specimen from Rethymno (4,3%) with 24 *CCEae3a* copies.

No carboxylesterase gene amplification was detected in Rodopi, Thessaloniki, Kefalonia and Lasithi; however, only a small number of samples was analysed from the latter two regions.

Figure A1:

Distribution of relative *CCEae3a* and *CCEae6a* gene copy number per population.



N: total number of specimens analysed per population.

qPCR for each sample was performed in duplicates. *Histone 3* was used as a reference gene. Gene copy number was estimated relative to a control lab strain susceptible to temephos.

DISCUSSION

This study verified the presence of the invasive mosquito species *Ae. albopictus* in Greece and examined the vector's insecticide resistance status against the major insecticides used for its control, in 10 urban or semi-urban areas throughout the country.

Identification of mosquito species based on both morphology and molecular markers, showed the dominant presence of *Ae. albopictus* over *Ae. cretinus* and *Ae. aegypti* (no specimens), in all surveyed regions across Greece. *Ae. cretinus* was found only in Chania, Crete in a frequency of 17,4%, but this result does not imply the absence of this species from other regions of the country. It was important to discriminate *Ae. albopictus* from the other two *Stegomyia* species, since *Ae. cretinus* has never been reported to transmit pathogens, but is a native species in Greece (Edwards et al., 1921; Samanidou-Voyadjoglou and Koliopoulos 1998), while *Ae. aegypti* is the primary vector of CHIKV, ZIKV, DENV, etc. The latter was well-established in southern Europe until the mid- 20th century, but now appears to be absent in the region (ECDC- July 2019; https://www.ecdc.europa.eu/en/publications-data/aedes-aegypti-current-known distribution-july-2019). As *Ae. albopictus* is an antagonistic invasive species displaying strong adaptability to new and diverse environmental niches, it may have gradually displaced *Ae. cretinus* and *Ae. aegypti* populations. However this hypothesis requires further monitoring and investigation of available literature regarding the presence/dynamics of the three species in previous years.

Aedes albopictus has a dominant vector role in many recent mosquito-borne disease outbreaks in Europe (e.g. CHIKV, DENV) over the last 2 decades; this, in combination with the increasing insecticide resistance worldwide, highlights the significance of evidence-based vector control strategies. Here, we investigated the presence, geographical distribution and frequency of mutations associated with target-site or metabolic resistance to diflubenzuron (IGR), pyrethroids and temephos (OPs).

No mutations in locus 1043 of the *CHS1* gene conferring diflubenzuron resistance were detected in the genotyped *Ae. albopictus* samples from Greece. To our knowledge, to date no genotypic resistance to diflubenzuron has been found in any *Aedes* mosquitoes worldwide. Diflubenzuron is one of the most effective larvicides used globally. Substitutions I1043L/M in the Chitin Synthase 1 (*CHS1*) gene are the only mutations identified to confer to diflubenzuron resistance [79], reported in *Culex pipiens* mosquito populations across Italy [79, 142] and associated with intense mosquito control and agricultural diflubenzuron based applications. The absence of

the respective mutations in *Ae. albopictus* may be attributed to the limited selection pressure imposed on the populations' hard-to-reach- breeding sites, as *Ae. albopictus* is a known container breeder and small artificial and natural water-bodies are seldom treated with the larvicide. In any case, the absence of DFB resistance in the *Ae. albopictus* populations indicates the suitability of DFB for their control.

Mutation V1016G in *vssc* domain II was not detected in any of the *Ae. albopictus* specimens from Greece. This substitution has been recorded only in populations from Vietnam and across Italy until now [88, 114], contributing to high levels of pyrethroid resistance.

However, genotyping of *vssc* domain III revealed the presence of 2 distinct mutations. I1532T was reported for the first time in Greece, particularly, in 5 out of 10 surveyed regions with an overall frequency varying from 1.7 to 6.5%. This mutation has been previously detected in *Ae. albopictus* field populations across Italy with an allele frequency of up to 32% [114] and in Vlore, Albania [114], but according to Kasai et al., 2019 [88], based on bioassays data, there is no association between 1532T allele and pyrethroid resistance. Additional evidence from functional characterization of this mutation (e.g. heterologous expression of *vssc* 1532T in *D. melanogaster* or in *Xenopus* oocytes using electrophysiology techniques) are necessary to justify its selection and spread across Italy. Additionally, *kdr* mutation F1534C, correlated with permethrin resistance, was recorded in all sampling regions, with the highest 1534C mutant allele frequency (in a representative number of samples) detected in Athens (68.3%), Argolida (45.2%), Chania (29%) and Rethymno (48.3%). The high mutation 1534C frequency in Athens is in line with the study conducted by Pichler et al., 2019 [114]. This mutation has not been reported in neighboring countries of Greece or elsewhere in Europe, but in Brazil, Singapore and China, while other substitutions in the same genomic locus, F1534L/S, occur in Florida (USA), China and Malaysia [46, 125, 139]. Whether resistance was initially selected for in Greeece (one or multiple selection procedures in the different regions) or individuals/populations with high mutation frequencies (selected for elsewhere) entered Greece remains unknown and requires further investigation. In either scenario the high mutation frequencies recorded indicate the ongoing presence of pyrethroid selection pressures in the sampling regions. Although the operational significance and the possible effect of the *kdr* mutation frequencies recorded, in terms of pyrethroid suitability/efficiency for their control, has to be studied, the high mutation frequencies indicate the need for expanding the currently available methods to include novel strategies as well as novel insecticidal groups.

The mutant allele 1534C frequencies in the surveyed areas of northern Greece, Thessaloniki and Rodopi (6.6% and 16.7% respectively), were lower than in Central and Southern Greece which may be associated with different intensity of selection

pressures imposed on the populations. For example, the specimens from Thessaloniki were collected from a biological farm where no insecticides have been applied over the last 10 years.

Notably, substitutions at *vssc* locus F1534 conferring pyrethroid resistance have been found in *Ae. albopictus* populations from countries of Asia, America and Europe, while another much stronger *kdr* mutant allele 1016G (Kasai et al, 2019; [88]) has been detected in Vietnam and Italy; these data imply that occurrence and geographical spread of resistance to the most effective insecticides become wider.

The amplified carboxylesterase genes, *CCEae3a* and *CCEae6a*, associated with temephos resistance in *Ae. albopictus* [77, 78] were detected in samples from Chios, Athens, Argolida, Chania and Rethymno, but not in Thessaloniki and Rodopi. Two amplification patterns were recorded, *CCEae3a* amplicon and *CCEae3a-CCEae6a* co-amplicon. Furthermore, a significant portion of the resistant positive samples had 10 or more copies of *CCEae3a* or both genes indicating elevated detoxification activity. It should be noted, that temephos has been officially banned in Europe for mosquito control since 2007, but the occurrence of carboxylesterases gene amplification is persistent. This could be attributed to selection pressure from agricultural use of organophosphates and/or to the lack of any fitness cost associated with these gene duplications (requires further investigation). A previous study by Grigoraki et al., 2017 [78] recorded both amplification types in samples from Athens and Florida, USA. Both populations shared a common *CCEae3a-CCEae6a* co-amplicon haplotype, providing evidence of a single amplification event either in Athens or in Florida and transportation of resistant specimens from one site to the other.

The simultaneous presence of the same insecticide resistance mutations in geographically distinct *Ae. albopictus* populations raises concern regarding passive transportation of resistant vectors via global trade, shipping, travelling, etc. The development and increasing occurrence of insecticide resistance in *Ae. albopictus* worldwide, in the current context of a limited number of available insecticides, poses a major threat to the vector control efforts and constitutes an important risk factor facilitating VBD transmission in Europe. Our study highlights the need for integrated vector control strategies incorporating biological methods, new insecticide formulations, larval source management, and insecticide resistance management programs, as well as continuous monitoring of the insecticide resistance traits in order to effectively control the major invasive species *Ae. albopictus* and the diseases it transmits. It should, finally, be noted, that added to genotypic resistance, phenotypic data from bioassay surveillance are also required to provide a more comprehensive assessment of *Ae. albopictus* insecticide resistance status in Greece, something that is included in our future plans, as well as further sampling and

monitoring (molecular diagnostic tools and bioassays) in more localities (e.g. Patra, Kefalonia, Heraklion).

PART B:

Nanoparticle-mediated targeted delivery of dsRNA for blocking *Plasmodium* parasite transmission into *Anopheles* mosquitoes

The aim of the study

This study is part of the project "Development of nanovectors for the targeted delivery in *Anopheles* mosquitoes of agents blocking transmission of *Plasmodium* parasites (NANOpheles)" of the European Innovative Research & Technological Development Project in Nanomedicine. The objective behind is to design polymeric nanovectors for the delivery of antimalarial agents (e.g. drugs, antimicrobial peptides, antibodies, dsRNA) to *Plasmodium* stages inside *Anopheles* mosquito that will block the parasite transmission by the mosquito. These nanovectors are proposed to enter the mosquito via sugar-feeding in field application (e.g. incorporated into sugar bait stations).

The antimalarial agent used, here, is dsRNA encapsulated in polymers. The implementation of RNA interference (RNAi) approach targets mosquito genes that are necessary for the parasite development; in particular, they are important for the parasite invasion of the mosquito midgut for ookinete-to-oocyst transition **(Image 26)**. Ookinete is the most vulnerable parasite stage to mosquito immune response [123].

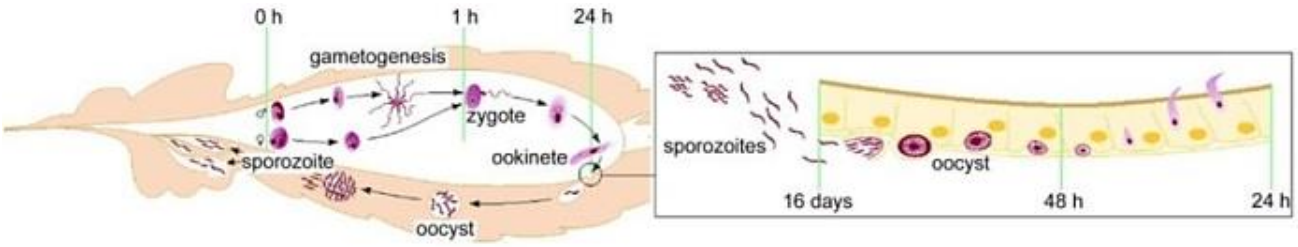


Image 26: *Plasmodium* stages in the *Anopheles* mosquito [19].

Many loss-of-function experiments have been performed, revealing *Anopheles* genes that promote parasite development into the invertebrate host, e.g. *FREP1* [122], *ESP* [118], *gelsolin* [133], *lipophorin* [67], *SOCS* [81], *Caspar* [141], *CACTUS* [73] (see introduction- paragraph 3.B). Here, the mosquito genes selected as dsRNA targets are Epithelial Serine Protease (*ESP*) and *CACTUS*. The cationic polymeric carriers used are poly-amido-amines (PAAs) with zero (PAA_{0m}) or four (PAA_{4m}) methyl-group substitutions in the disulfide bond **(Image 27)**. These nanoparticles are non-toxic, stable in the extracellular environment protecting the genetic cargo, but rapidly degradable after uptake in reductive intracellular conditions, releasing efficiently the dsRNA payload.

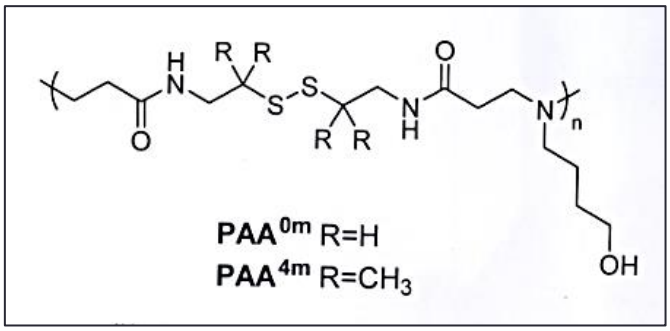


Image 27: Chemical structure of biodegradable polyamidoamines used in this study, with varying degrees of steric hindrance (0- or 4- methyl-groups) adjacent to the disulfide bonds.

In this part of NANOpheles project, we investigated whether PAAs can be used as valuable polymeric vectors of dsRNA in *Anopheles gambiae* midgut, in terms of both protecting the cargo from extracellular degradation and efficiently releasing it intracellularly, in response to the different reduction potential. Subsequently, the ability of PAAs-dsRNA nanoparticles to block *Plasmodium* parasite development into the mosquito was evaluated.

MATERIALS & METHODS

1. Mosquito strain and rearing

The *Anopheles gambiae* N'Gousso (NG) strain from Cameroon was reared under standard insectary conditions at 27°C and 70–80% humidity under a 12 h : 12 h photoperiod and fed on 10% sucrose solution (in tap water).

2. RNA extraction (Trizol Reagent)

RNA was extracted from female NG whole mosquitoes or isolated midguts, using Trizol Reagent protocol:

-Tissues were homogenized in Trizol (grinder- pestle) at room temperature (RT). For 50-100 mg of tissue, 1 ml Trizol is required.

-Samples were centrifuged at 12,000xg for 10 minutes, at 4^{o}C. The supernatant (containing RNA) was transferred into a new 1.5 ml tube and incubated for 5 minutes, at RT.

-200 ul chloroform were added (hood) per 1 ml Trizol, the tubes were closed securely and the samples were shaken vigorously by hand per 15 seconds. An incubation for 2-3 minutes at RT followed and, then, the samples were centrifuged at 12,000xg for 15 minutes, at 4^{o}C. The upper colorless aqueous phase, that contains the RNA, was placed into a new tube, without disturbing the other two phases.

-500 ul 100% isopropanol were added per 1 ml Trizol and the samples were incubated for 10 minutes at RT. Subsequently, they were centrifuged at 12,000xg for 10 minutes, at 4^{o}C; the supernatant was carefully removed.

-The pellet was washed with 1 ml 75% ethanol per 1 ml Trizol. The samples were mixed briefly (vortex) and centrifuged at 7,500xg for 5 minutes, at 4^{o}C.

-Tubes were left open and the RNA pellet was air-dried for 10 minutes, at RT. The remaining ethanol was carefully removed.

-The RNA pellet was resuspended in 20-50 ul DEPC-treated water and incubated for 10-15 minutes, at 60^{o}C.

-RNA quantity (ng/ul) and quality (ratios A_{260}/A_{280}, A_{260}/A_{230}) of each sample were estimated using Nanodrop

DNase Treatment

The RNA samples were further treated for the removal of any remaining genomic DNA (Thermo scientific, DNAse I, RNase-free). In an RNase- free tube, the following reagents were added:

DNase- treatment mixture	
RNA	1 ug
10x Reaction Buffer with $MgCl_2$	1 ul
RNase-free DNase I	1 ul (1 U)
DEPC- treated water	Up to 10 ul

Samples were incubated for 30 minutes at 37°C. 1 ul 50 mM EDTA was added and the samples were incubated for 10 minutes at 65°C.

After the DNase treatment, RNA quantity and quality were estimated again using Nanodrop.

RNA samples are stored at -80°C.

3. Reverse transcription

DNase- treated RNA samples were used as template for reverse transcriptase. cDNA synthesis was performed using MINOTECH RT reagents, as described below:

cDNA synthesis mixture	
5 ug total RNA	maximum 11 ul
50 uM oligo(dT)-20 primers	1 ul
10 mM dNTPs mix	1ul
Sterile DEPC- water	Up to 13 ul

The mixture was heated for 5 minutes at 65°C and incubated on ice for at least 1 minute. Then, the following reagents were added:

5x MINOTECH RT assay buffer	4 ul
0.1 M DTT	1 ul
40 U/ ul RNase inhibitor	1 ul
200 U/ ul MINOTECH RT	1 ul

The samples was mixed gently and incubated for 60 minutes at 42°C, and subsequently, for 15 minutes and 70°C (inactivation step).

In the reverse transcription reaction, a negative control is included; usually, this is the sample with the maximum quantity of RNA without adding reverse transcriptase.

4. Construction of dsRNA against specific *Anopheles* genes

RNA from adult mosquito midgut was reverse transcribed. cDNA was, subsequently, used as template for the amplification of our target genes, *ESP* and *Cactus*, as described below:

PCR for *ESP* amplification	
Midgut cDNA	0,5 ul
dNTPs (10mM)	0,5 ul
Primer ds1ESP F (10µM)	0,5 ul
Primer ds1ESP R (10µM)	0,5 ul
5X Green GoTaq Buffer	5 ul
GoTaq DNA polymerase	0,125 ul
ddH_2O	Up to 25 ul

The applied thermal protocol was the following: initial denaturation at 95°C for 2 minutes, 40 cycles x [denaturation at 95°C for 1 min, primer annealing at 54°C for 1 minute, primer extension at 72°C for 50 seconds] and final extension at 72°C for 5 minutes.

PCR for *Cactus* amplification	
Midgut cDNA	0,5 ul
dNTPs (10mM)	0,5 ul
Primer ds2Cactus F (10µM)	0,5 ul
Primer ds2Cactus R (10µM)	0,5 ul
5X Green GoTaq Buffer	5 ul
GoTaq DNA polymerase	0,125 ul
ddH_2O	Up to 25 ul

The applied thermal protocol was the following: initial denaturation at 95°C for 2 minutes, 40 cycles x [denaturation at 95°C for 1 min, primer annealing 59°C for 1 minute, primer extension at 72°C for 50 seconds] and final extension at 72 °C for 5 minutes.

PCR for *GFP* amplification	
Plasmid (*) 9 ng/ul	1,75 ul
dNTPs (10mM)	0,5 ul
Primer dsGFP F (10µM)	0,5 ul
Primer dsGFP R (10µM)	0,5 ul
5X Green GoTaq Buffer	5 ul
GoTaq DNA polymerase	0,125 ul
ddH_2O	Up to 25 ul

The applied thermal protocol was the following: initial denaturation at 95°C for 2 minutes, 40 cycles x [denaturation at 95°C for 1 min, primer annealing at 60°C for 1 minute, primer extension at 72°C for 50 seconds] and final extension at 72°C for 5 minutes.

*: *gfp* cDNA cloned in a plasmid vector

Four replicates were prepared for each gene. All primer pairs used are described in **Table B1.**

A small amount of the PCR products were run on a 1,5% agarose gel, prepared in TAE and containing ethidium bromide. The expected bands are 569 bp for *ESP*, 556 bp for *CACTUS* and 550 bp for *GFP*. The remaining amount of the PCR products (all replicates merged in one) was purified using the Nucleospin PCR & Gel Clean-Up Kit (Macherey Nagel, Dueren, Germany).

cDNA of the three genes was used as template for dsRNA construction, using the HiScribe T7 Kit:

dsRNA synthesis reaction	
cDNA (template)	maximum 1 ug
ATP	2 ul
GTP	2 ul
UTP	2 ul
CTP	2 ul
T7 Buffer	2 ul
T7 enzyme	2 ul
DEPC- water	Up to 20 ul

*reaction prepared in duplicates for each transcript.

dsRNA synthesis was performed at 37^{o}C for 2 hours.

The dsRNA products were purified using the MegaClear Kit, as described below:

-Each RNA sample were brought to 100 ul using elution buffer.

-Each reaction was placed in a new tube, 350 ul binding solution were added and mixed gently.

-250 ul 100% ethanol were added and mixed gently.

-Samples were transferred to a filter column and centrifuged at 15,000xg for 15 seconds. The flow-through was discarded. The step is repeated for the other replicate.

-Samples were washed 2 times using 500 ul wash solution and centrifuged at 15,000xg for 30 seconds.

-The flow-through was discarded and the samples were centrifuged at 15,000xg for 1 minute to remove any remaining ethanol.

-The column was placed in a new tube and the RNA was eluted in 50 ul DEPC-treated water. The tube was closed, heated for 10 minutes at 70°C and centrifuged for 1 minute at 10,000 rpm.

dsRNA quantity and quality were estimated in Nanodrop. SpeedVac was used to bring the quantity at 3 ug/ul.

dsRNA is preserved at -20°C.

5. Mosquito dsRNA injections and post-injection handling

Mosquito dsRNA injections were performed as described in Garver L., Dimopoulos G., 2007 [74]. 3 to 5-day old female *An. gambiae* N'Gousso (NG) mosquitoes were CO_2 anaesthetized and 69 nl of 3 ug/ ul dsRNA (dsESP, dsCactus) were injected intrathoracically into the thin part of the cuticle (at the side point where the wing is attached to the thorax) using a Nanoinjector (Drummond Nanoinject II). The control group was injected with dsGFP. Subsequent handling of the mosquitoes depends on the purpose of the experiment.

Silencing efficiency of dsRNA constructs

In order to validate the silencing efficiency of ds*ESP* and ds*CACTUS* constructs against the endogenous genes in the midgut, mosquitoes were allowed to recover at insectary conditions (28°C, 70-80% humidity) for 3 days post-injection. Then, midguts were dissected and gene silencing was determined using real-time PCR, after RNA extraction and cDNA synthesis.

Oocysts counting

All injected mosquitoes were collected in a covered cup and allowed to recover for 48 hours at 19°C, under appropriate humidity conditions, feeding on 10% sucrose solution. Then, a blood-meal on a *Plasmodium berghei*-infected anaesthetized mouse for 30 minutes was carried out. The parasite was tagged with a GFP-expression cassette under control of the eef1aa-promoter [86]. Mouse parasitemia levels were determined using light microscopy by methanol fixation of air-dried blood smears and staining with 10% Giemsa. Injected mosquitoes were sucrose-deprived for 2 hours before the blood-meal (starvation), in order to ensure that they will feed on the mouse adequately. One day post-blood feeding, 10% sucrose solution was provided to the mosquitoes. 8-10 days post-blood feeding, mosquito midguts were dissected

to determine the level of parasite infection by counting oocysts in a fluorescent microscope.

6. Mosquito midgut dissections

Treated female NG mosquitoes were collected using an aspirator and were ice-anaesthetized, in a Petri dish.

Each mosquito was placed under the stereoscope, in a PBS drop; the gut was removed from the abdomen while pulling with a forceps the last abdominal segment. The gut was carefully separated from the ovaries, the Malpighian tubules and the salivary gland **(Image 28)**; it is important to complete the dissection in the PBS drop and not let the midgut dry.

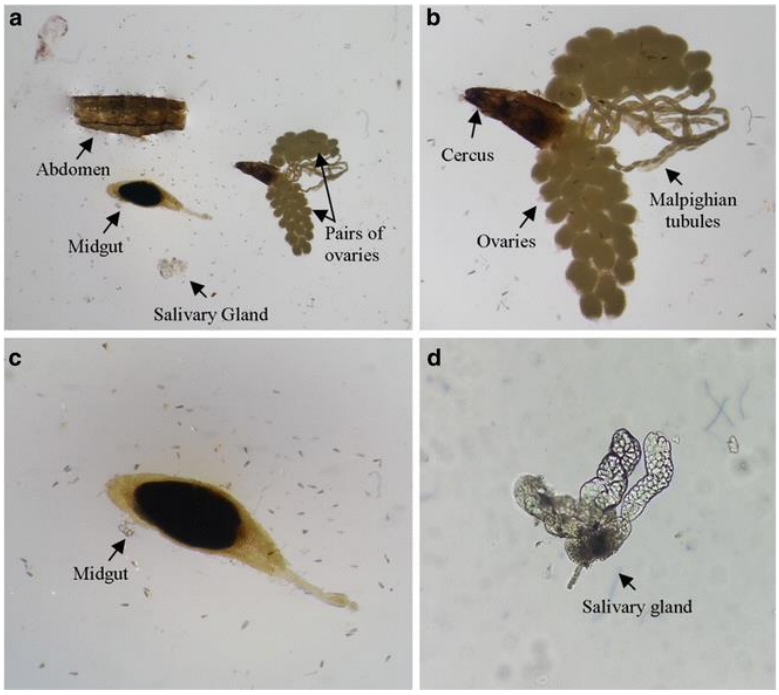


Image 28: Overview of individual mosquito organs dissected: salivary glands, pair of ovaries with developed eggs, Malpighian tubules and midgut with undigested blood [137].

Guts isolated from mosquitoes that were same-treated were placed together in 1 ml Fixation Buffer under agitation for 10 minutes.

Fixation Buffer (for 1 ml)	
Paraformaldehyde (PFA) 10%	400 ul
PBS 1x	600 ul

After carefully removing the Fixation Buffer, guts were washed in 1 ml PBS under agitation for 5 minutes.

PBS was discarded and, subsequently, 1 ml Hoechst dye 1: 2000 in PBS was added and the guts were incubated for 5 minutes under agitation. Hoechst is a blue fluorescent dye which stains DNA.

The dye was removed and after one more PBS wash, guts were mounted in VectaShield for observation in a fluorescent microscope.

7. Estimation of dsRNA silencing efficiency with real-time PCR

After mosquito midgut dissections, 7-8 midguts were grouped, followed by RNA extraction (Trizol) and cDNA synthesis, as described above.

Silencing efficiency of ds*ESP* and ds*CACTUS* constructs was determined using quantitative real-time PCR. Reactions were of final volume 20 ul, containing 2X SYBR Master Mix, 0,3 ul of each 10 uM primer and 1 ul of cDNA (diluted) and performed in duplicates. Gene silencing was measured relative to control ds*GFP*- injected mosquitoes. The reference gene was *S7* encoding a ribosomal. The thermal conditions were: 95^{o}C for 3 minutes and 39 cycles x [95^{o}C for 10 seconds, 60^{o}C for 45 seconds]

All primer pairs used for real time-PCR are described in **Table B1.**

Table B1: Primers used in this study for regular and real-time (*) PCR.

Primer Name	Primer Sequence
ds1ESP F	5′ CAACTTCCTGGAGGGGTCAG 3′
ds1ESP R	5′ TCACTGGTACGTCCGAATCC 3′
ds2Cactus F	5' AAAATCTTGACGCCCCAACG 3'
ds2Cactus F	5' GCCAGGTGCAGTTGAGTTTC 3'
dsGFP F	5′TAATACGACTCACTATAGGGAGAACGTAAACGGCCACAAGTTC 3′
dsGFP R	5′ TAATACGACTCACTATAGGGAGACTTGTACAGCTCGTCCATGCC 3′
qESP F (*)	5′ TGGTGTGGTGTCCTTCGTTG 3′
qESP R (*)	5′ TTACGGAAATGGGTGGTGCG 3′
qCactus F (*)	5′ GGCAGCATGGAAACACTTCG 3′
qCactus R (*)	5′ GCTGATGTGTAGAGCGGACA 3′
qS7 F (*)	5′ AGAACCAGCAGACCACCATC 3′
qS7 R (*)	5′ GCTGCAAACTTCGGCTATTC 3′

8. Preparation of PAA-dsRNA nanoparticles and stability evaluation

Procedure of polyplex formation was based on Elzes et al., 2016 [70]. PAA_{0m} and PAA_{4m} polymer solutions of initial concentration 6 mg/ml in MilliQ water were prepared, followed by serial dilutions of 3 mg/ml, 1.5 mg/ml, 0.75 mg/ml and 0.375 mg/ml. 36,3 ul of each dilution were used to achieve various w/w ratios of polymer/ dsRNA, when mixed with 2,2 ul of 1 ug/ul dsRNA (ds*GFP*, ds*ESP*, ds*Cactus*) and 34,1 ul of MilliQ water, as described in the table below. Polyplex solutions were mixed briefly (vortex).

PAA Concentration (mg/ml)	PAA volume (ul)	1 ug/ul dsRNA volume (ul)	MilliQ water volume (ul)	Polymer/ dsRNA ratio (w/w)
6	36,3	2,2	34,1	100
3	36,3	2,2	34,1	50
1,5	36,3	2,2	34,1	25
0,75	36,3	2,2	34,1	12.5
0,375	36,3	2,2	34,1	6.25

12 ul of each solution were mixed with 3 ul of loading dye. A positive control containing 0,5 ul of 1 ug/ul dsRNA and 11,5 ul MilliQ water was also used. Samples were run on a 1,5% agarose gel, prepared in TAE and containing ethidium bromide, at 75-80 mA for approximately 30 minutes. Visualization of the gel revealed the weight ratio that provides maximum dsRNA encapsulation in PAA_{0m} and PAA_{4m}.

Polyplex degradation

Polyplex stability was evaluated in presence of the following reducing agents, diluted in MilliQ water to obtain various concentrations:

- **Heparin sodium salt:** 8.5 mg/ml, 4.25 mg/ml, 0.85 mg/ml, 0.425 mg/ml, 0.085 mg/ml, 0.0425 mg/ml
- **DTT:** 8.5 M, 850 mM, 85 mM, 8.5 mM, 0.85 mM, 0.085 mM
- **Hydrazine monohydrate**: 2 M

7,5 ul of the PAA/dsRNA ratio, that demonstrated the maximum dsRNA binding in gel electrophoresis, were mixed with 1 ul of each Heparin, DTT, Hydrazine concentration or MilliQ water (control) and incubated for 30 minutes at RT. 1,5 ul of loading dye was added and samples were run on a 1,5% agarose gel, prepared in TAE and containing ethidium bromide, at 75-80 mA for approximately 30 minutes. Visualization of the gel showed the decomplexation behavior of the polyplexes in presence of increasing concentrations of the reducing agents used.

9. Administration of PAAs-dsRNA nanoparticles to mosquitoes

PAA- dsRNA polyplexes were administered to 3- to 5-days old adult female *An. gambiae* NG mosquitoes either by injecting or feeding them.

Polyplex injections

Mosquito injections (69 nl) were performed using:

- PAA_{0m}- ds*GFP* 25 w/w
- PAA_{0m}- ds*CACTUS* 25 w/w
- 30 ng/ul ds*GFP* (control)
- 30 ng/ul ds*CACTUS* (control)

Injected mosquitoes were, then, fed on 10% sucrose solution and 3 days post-injection, they were cold anaesthetized, midguts were dissected and pooled (7-8 midguts per pool x 3). Subsequently, RNA was extracted from midgut pools and reverse transcribed, as described above. cDNA was used for the evaluation of polyplex silencing efficiency against *CACTUS,* using real-time PCR, using the same conditions previously described.

Polyplex feeding

Alternatively, polyplexes were given to mosquitoes through their sugar-meal. At first, it was necessary to estimate the effect of sucrose solution on the conjugation of dsRNAs to PAAs polymers. This was done by mixing 34,1 ul of 10% sucrose solution (MilliQ water as control) with 36,3 ul of PAA_{0m} or PAA_{4m} and 2,2 ul of 1 ug/ul dsRNA (ds*GFP* and ds*CACTUS*) in 6.25, 12.5, 25, 50 w/w ratios. Samples were incubated for 30 minutes at RT and results were visualized in a 1,5% agarose gel, prepared in TAE and containing ethidium bromide.

Mosquitoes were fed on the following polyplexes:

- PAA_{0m}- ds*GFP* 6.25 w/w
- PAA_{0m}- ds*CACTUS* 6.25 w/w
- PAA_{4m}- ds*GFP* 50 w/w
- PAA_{4m}- ds *CACTUS* 50 w/w

prepared in sucrose solution, as described in the table below:

Polyplex feeding mixture	
1 ug/ul dsRNA	90,9 ul
PAA polymer	1,5 ml
21,3% sucrose solution	1,41 ml
	Volume: 3 ml

Female *An. gambiae* mosquitoes were transferred to a paper cup and 3 ml mixture (final sucrose concentration: 10%) was provided for 4 days through a syringe-filter system adjusted to the side of the cup. Then, 10% sucrose solution (prepared in tap water) replaced the previous meal for 1 day. Mosquitoes were subsequently cold anaesthetized, midguts were dissected and pooled (7-8 midguts per pool x 3). RNA was extracted from midgut pools, reverse transcribed and silencing efficiency of polyplex against *Cactus* was evaluated using real-time PCR, as described above.

RESULTS

1. Gene silencing efficiency of ds*ESP* and ds*CACTUS* constructs

CACTUS and *ESP* endogenous genes were dsRNA- silenced in adult female NG *An. gambiae* mosquitoes. ds*CACTUS* and ds*ESP* injections reduced the expression of *CACTUS* by ~62% **(Figure B1a)** and of *ESP* by ~74% **(Figure B1b)**, respectively, in the midgut, 3 days post-injection, both in comparison to ds*GFP*- injected mosquitoes. Differences in *CACTUS* and *ESP* mRNA levels are statistically significant (*, $p<0,05$) compared to the control.

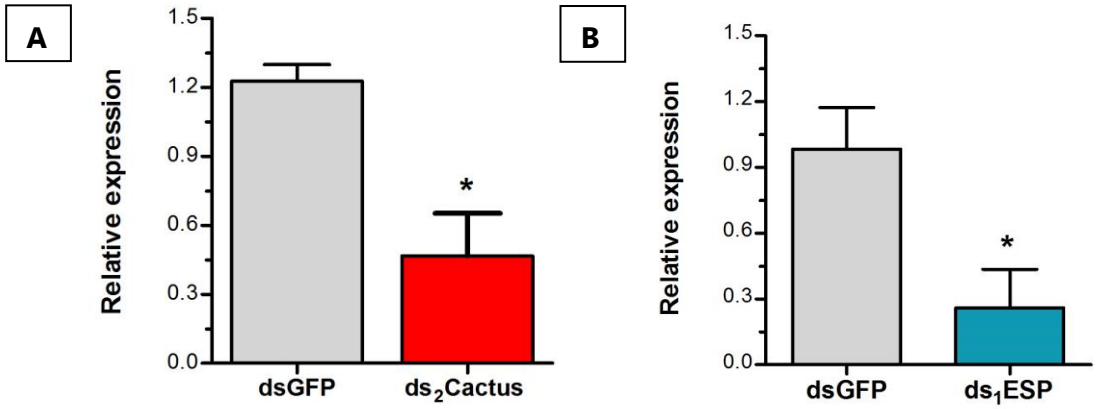


Figure B1: (A) *CACTUS* and **(B)** *ESP* mRNA silencing in the midgut 3 days post- ds*CACTUS* and ds*ESP*- injections, respectively, in adult female NG *An. gambiae* mosquitoes. The unpaired two-tailed t-test was used to compare the differences in mRNA levels (*, $p<0,05$). Expression analysis was performed using real-time PCR, in cDNA from pools of 7-8 same dsRNA-injected mosquitoes, in 3 biological replicates. Results were analyzed in GraphPad Prism v.5.

2. Effect of ds*ESP*- and ds*CACTUS*- injections on the number of oocycts in mosquito midgut

After validating the efficient silencing of *ESP* and *CACTUS* expression in the midgut by these dsRNAs, the effect of intrathoracic injections on *P. berghei* ookinete invasion of the midgut was investigated. Oocysts were counted 8-10 days post infective blood-meal. The results showed a decrease in the number of emerging oocysts in the midgut from individual ds*ESP*- or ds*CACTUS*- injected mosquitoes, in comparison to

ds*GFP*- injected. In detail, total absence of *P. berghei* oocysts was observed in a small number of midguts, n=12 and n=7, from ds*CACTUS*- and ds*ESP*- injected mosquitoes, respectively **(Figure B2, a&b)**. In contrast, 20-230 oocysts were present in half of control-group midguts (ds*GFP*- injected mosquitoes) **(Figure B2, c&d;** representative images**)**; however, in ~28% of ds*GFP*- injected mosquitoes no oocysts developed.

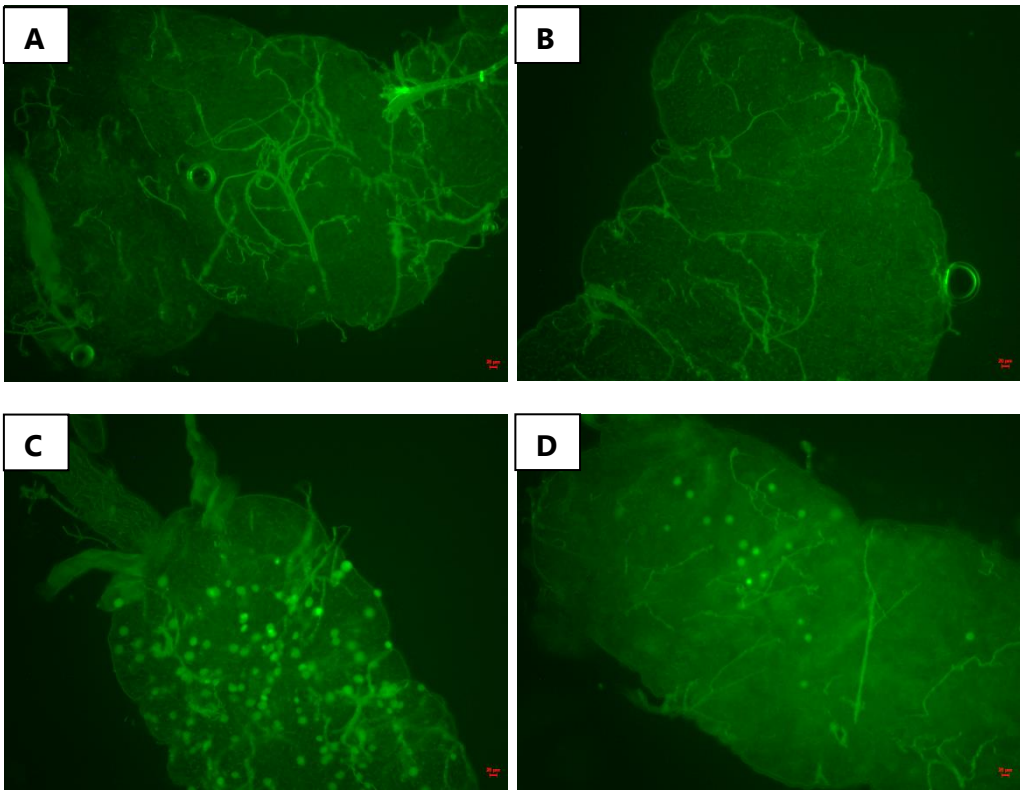


Figure B2: Microscopic analysis of midgut parts from female *An. gambiae* mosquitoes injected with: **(A)** ds*CACTUS*, **(B)** ds*ESP*, and **(C,D)** ds*GFP*. Green fluorescent dots in C,D indicate the presence of *P. berghei* oocysts, 8-10 days post infective blood-meal.

Finally, the decrease in parasite development upon ds*CACTUS*- and ds*ESP*- injections in comparison to the control is statistically significant (*, $p<0,05$) **(Figure B3)**.

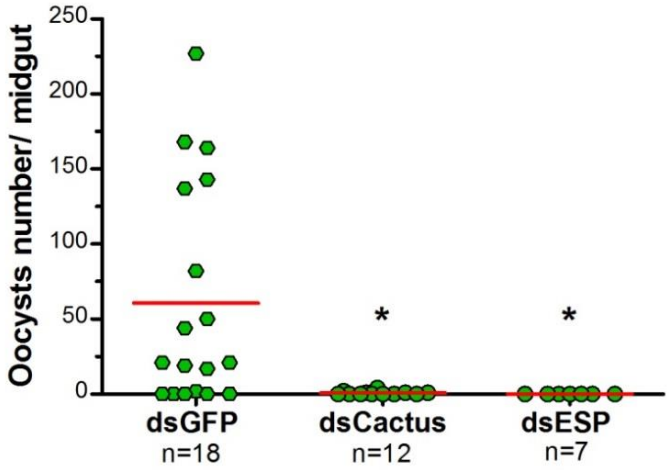


Figure B3: Effect of ds*CACTUS* and ds*ESP* injections on the number of *P. berghei* oocysts in the midgut of adult female NG *An. gambiae* mosquitoes, 8-10 days post blood-feeding. Each green dot represents the number of oocysts in an individual midgut and the red line indicates the median number. The unpaired two-tailed t-test was used to compare the different experimental groups with the control (*, $p<0,05$). Results were analyzed in GraphPad Prism v.5.

3. Synthesis of polyamidoamine- dsRNA nanoparticles

Varying PAA_{0m} and PAA_{4m} polymer concentrations were mixed with standard concentration of ds*GFP*, ds*ESP* and ds*CACTUS* to obtain several polymer/ dsRNA weight ratios from 6.25 to 100. Agarose gel electrophoresis revealed increased encapsulation of dsRNA as the polymer weight increases, for all polyplexes. In particular, dsRNAs totally bind to PAA_{0m} either from 6,25 **(Figure 4)** or 12,5 w/w ratio and higher; there was a variation when this procedure was repeated (total binding at 6.25 w/w ratio was a more reproducible result). It was, additionally, shown that PAA_{4m} nanoparticles allow all three dsRNAs to efficiently bind from a ratio of 50 w/w and on **(Figure B4)**.

10% sucrose solution was also mixed with the polyplexes and it was verified through gel electrophoresis that it does not modify dsRNAs binding to the polymers (results not shown).

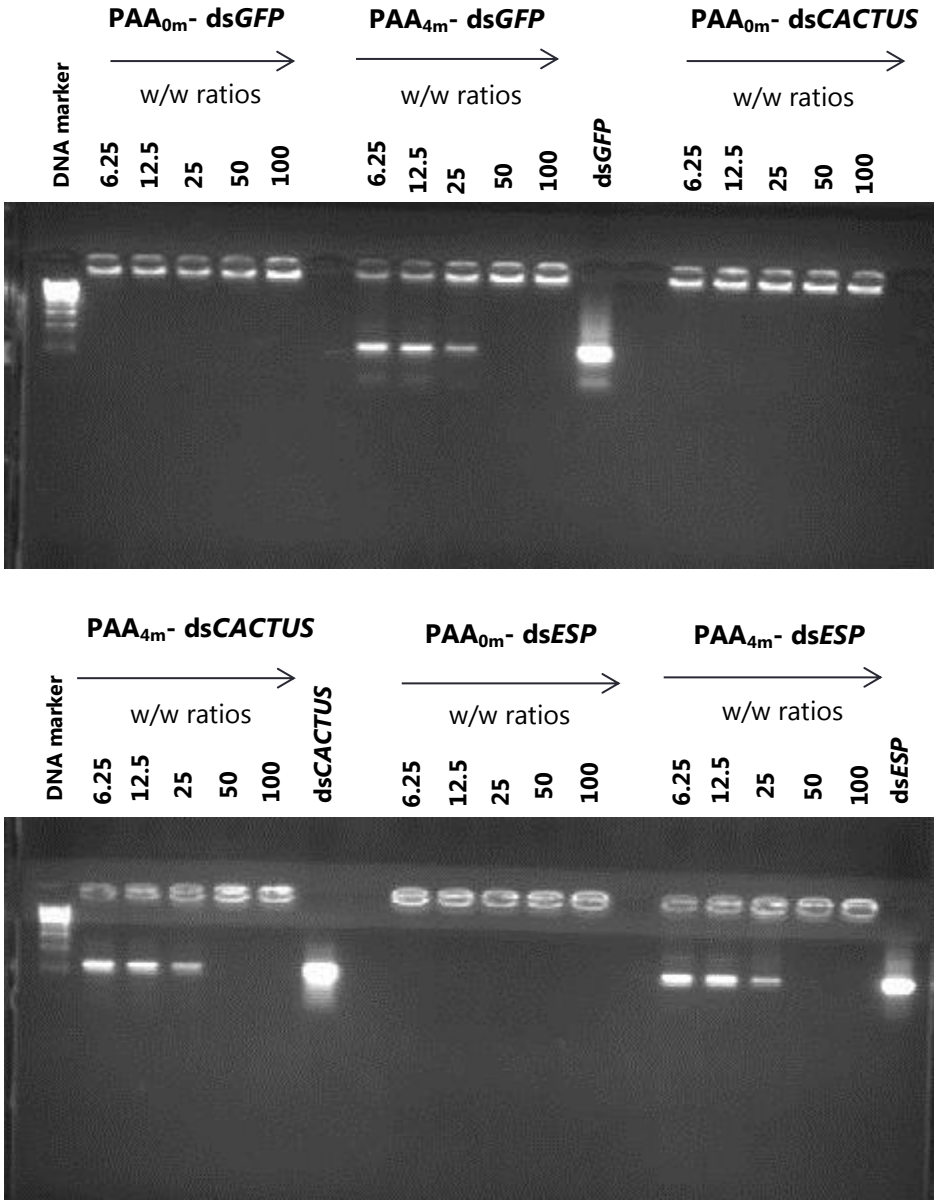


Figure B4: Agarose gel (1,5%) electrophoresis results of PAA_{0m}- dsRNA and PAA_{4m}- dsRNA polyplexes synthesis in increasing weight ratios of polymer/dsRNA. A free dsRNA was used as a positive control.

4. Polyplex degradation behavior

Polyplex stability was evaluated in presence of reducing agents. To investigate decomplexation due to polymer degradation, polyplexes were incubated in various DTT and Hydrazine concentrations. Dissociation of the polyplexes upon polyanion exchange was tested under the influence of increasing Heparin concentrations.

PAA_{0m}- dsRNA and PAA_{4m}- dsRNA polyplexes at the weight ratio that demonstrated the maximum conjugation (Fig.4) were exposed to the reducing agent for 30 minutes at RT; the release of dsRNA cargo was monitored in gel electrophoresis **(Figure B5)**.

PAA_{0m}- dsRNA polyplexes demonstrated an early degradation from the lowest concentrations of reducing agents, and more intensely at higher concentrations. Particularly, a partial release of ds*GFP* was observed already from 8.5 mM DTT, from 0.009 mg/ml Heparin and in 0.2 M Hydrazine; PAA_{0m}- ds*CACTUS* and PAA_{0m}- ds*ESP* were dissociated from 8.5 mM DTT and on and from 0.04 mg/ml Heparin and on, while 0.2 M Hydrazine degraded both polyplexes **(Figure B5a)**.

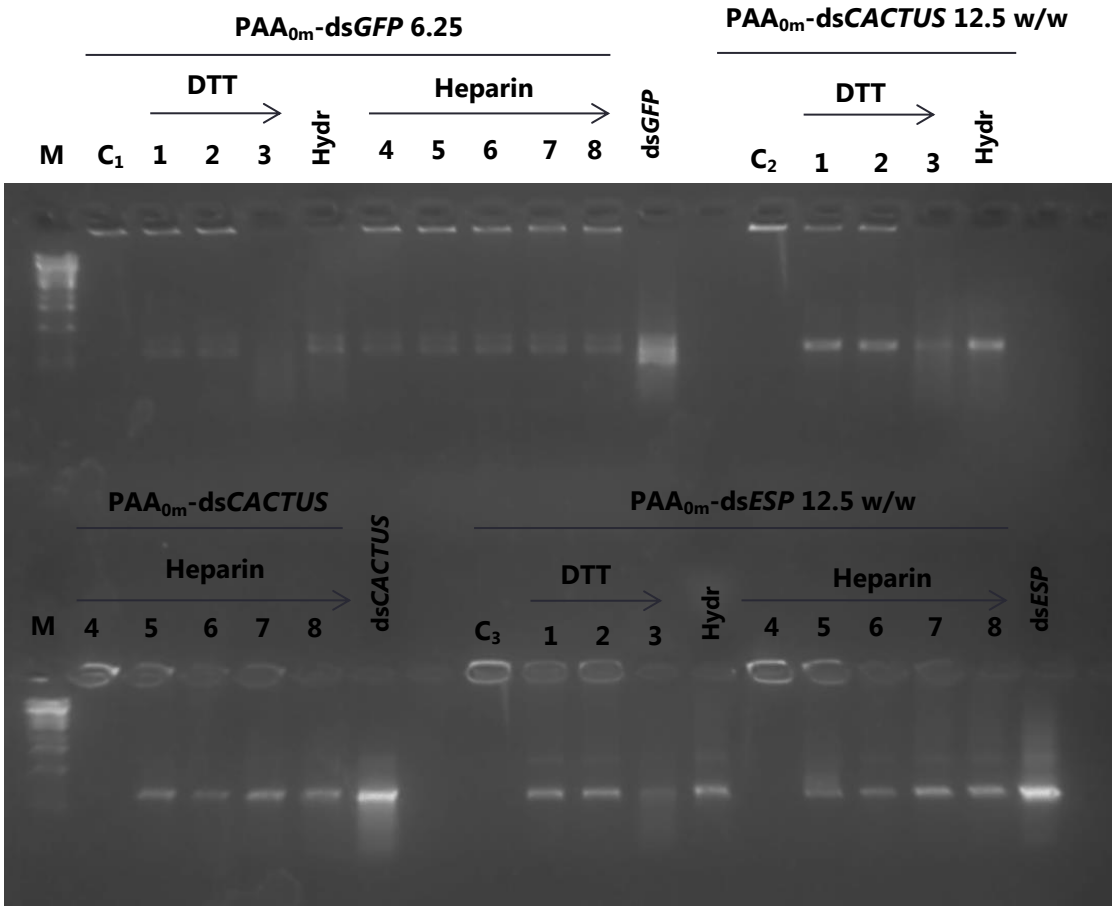


Figure B5a: Agarose gel (1,5%) electrophoresis results of PAA_{0m}- dsRNA polyplexes exposed to increasing concentrations of DTT, Hydrazine and Heparin. M: DNA marker; C1: PAA_{0m}- ds*GFP* 6.25 w/w untreated, C2: PAA_{0m}- ds*CACTUS* 12.5 w/w untreated, C3: PAA_{0m}- ds*ESP* 12.5 w/w untreated; DTT concentrations: 1) 8.5 mM, 2) 85 mM, 3) 850 mM; Hydrazine (Hydr) concentration: 0.2 M; Heparin concentrations: 4) 0.009 mg/ml, 5) 0.04 mg/ml, 6) 0.09 mg/ml, 7) 0.4 mg/ml, 8) 0.9 mg/ml. Concentrations shown are concentrations mentioned in Materials & Methods- paragraph 8 diluted 10 times, after being mixed with the nanoparticles and loading dye. A free dsRNA was used as a control.

PAA_{4m}- dsRNA 50 w/w polyplexes remained intact in presence of the highest DTT concentrations (85 mM and 850 mM) and dsRNAs were only released when exposed to high concentrations (0.2 M and 1 M) of Hydrazine, a stronger reducing agent than DTT **(Figure B5b)**. As far as polyplex behavior in presence of Heparin is concerned, PAA_{4m}- ds*GFP* was partially degraded in 0.4 mg/ml and on, but the other two polyplexes released their genetic cargo, ds*CACTUS* and ds*ESP*, already from the lowest Heparin concentration of 0.009 mg/ml **(Figure B5b)**.

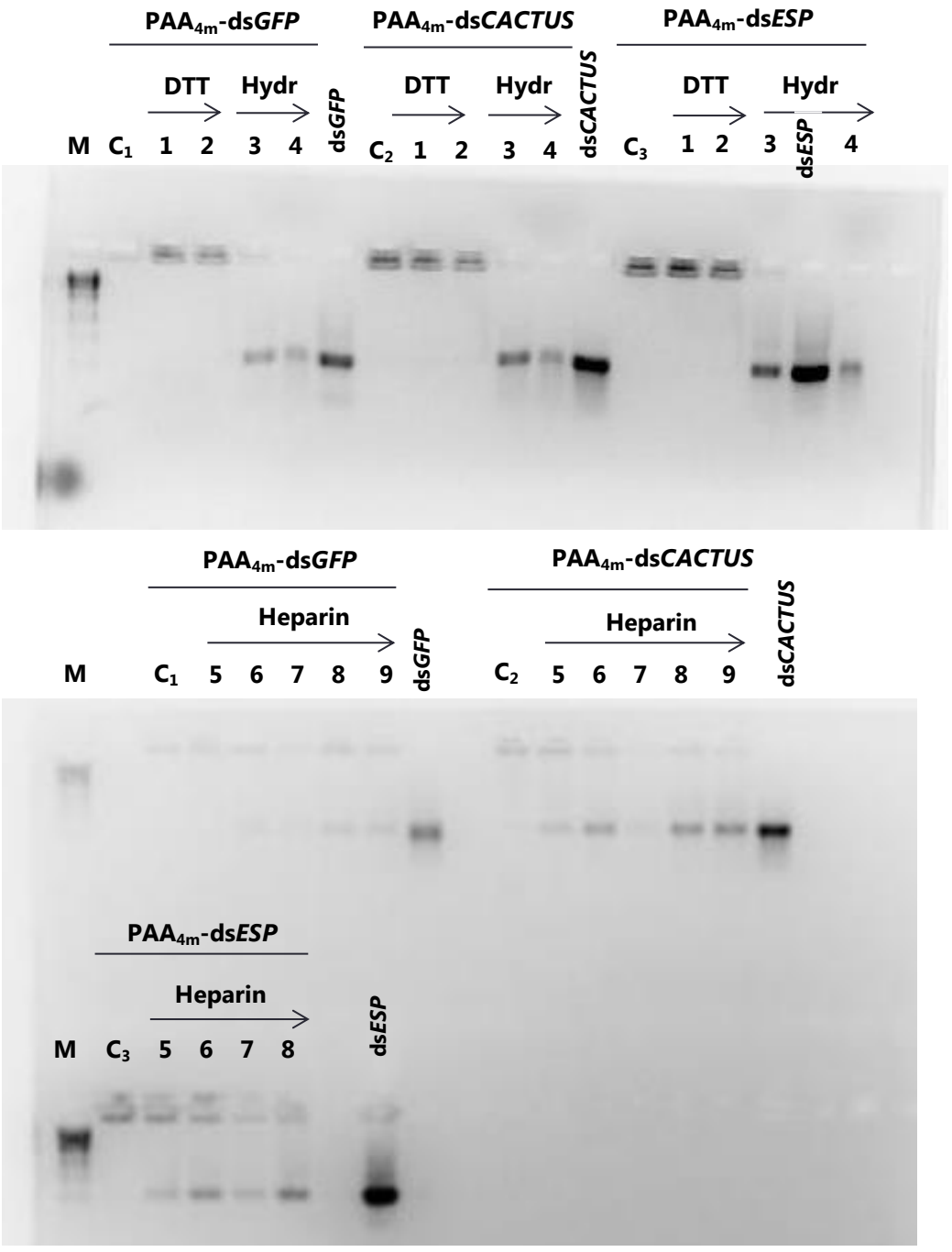


Figure B5b: Agarose gel (1,5%) electrophoresis results of PAA_{4m}- dsRNA 50 w/w polyplexes treated with increasing concentrations of DTT, Hydrazine and Heparin. M: DNA marker; C1: PAA_{4m}- ds*GFP* 50 w/w untreated, C2: PAA_{4m}- ds*CACTUS* 50w/w untreated, C3: PAA_{4m}- ds*ESP* 50w/w untreated; DTT concentrations: 1) 85 mM, 2) 850 mM; Hydrazine (Hydr) concentration: 3) 0.2 M, 4) 1 M; Heparin concentrations: 5) 0.009 mg/ml, 6) 0.04 mg/ml, 7) 0.09 mg/ml, 8) 0.4 mg/ml, 9) 0.9 mg/ml. Concentrations shown are concentrations mentioned in Materials & Methods- paragraph 8 diluted 10 times, after being mixed with the nanoparticles and loading dye. A free dsRNA was used as a control.

5. Gene knock-down efficiency of PAA_{0m}- ds*CACTUS* and PAA_{4m}- ds*CACTUS* polyplexes

To assess the potential of these polymers for dsRNA delivery to *An. gambiae*, repression of endogenous *CACTUS* gene expression (RNAi) in the midgut was investigated after PAA_{0m}- and PAA_{4m}- ds*CACTUS* administration either by feeding or injecting adult female mosquitoes.

- **Polyplex injections**

Injecting (69 nl) PAA_{0m}- ds*CACTUS* 25 w/w to 5-days old female mosquitoes caused 17% reduction in *Cactus* expression in the midgut compared to PAA_{0m}- ds*GFP* 25 w/w injection **(Figure B6)**. This difference is consistent to the one observed between ds*GFP*- and ds*CACTUS*- injected mosquitoes, meaning that polyamidoamines have the potential to be efficient carriers of dsRNA. However, due to low number of replicates (3 per injection group), the mentioned differences are not statistically significant (unpaired two-tailed t-test; $p>0,05$).

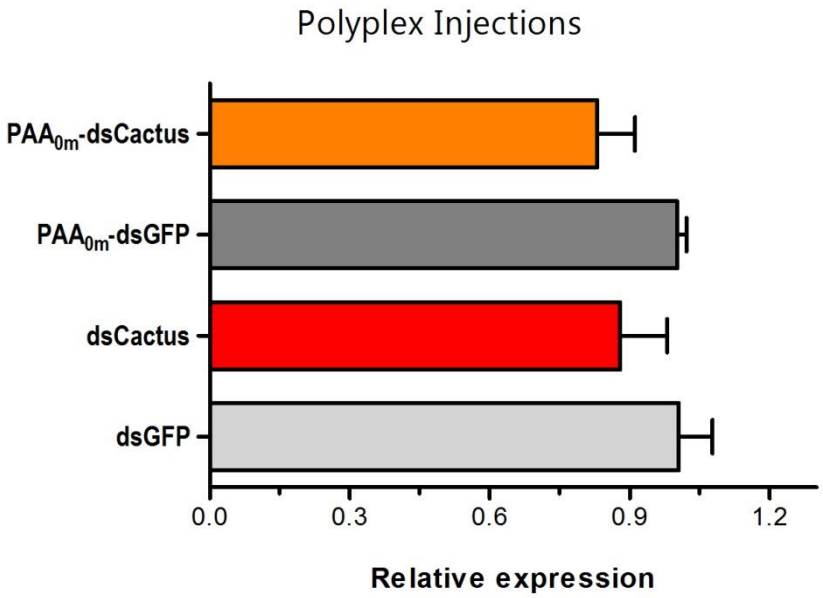


Figure B6: *CACTUS* mRNA silencing in female *An. gambiae* midguts 3 days post-injection. The unpaired two-tailed t-test was used to compare the differences in mRNA (not statistically significant). Expression analysis was determined by real-time qPCR, in cDNA pools of 7-8 same-injected mosquitoes, in 3 biological replicates. Results were analyzed in GraphPad Prism v.5

It is important to clarify why ds*CACTUS*- injections in this experiment led to less than 20% knock-down of *Cactus* expression in the midgut, while silencing efficiency of this dsRNA construct is more than 60% (Fig.1). The standard volume of an injection used for adult *Anopheles* mosquito accepts is 69 nl [74]. The concentration of ds*CACTUS* injected to mosquitoes for evaluation of the silencing efficiency of the construct was 3 ug/ ul; finally, 0.207 ug of ds*CACTUS* are injected to the mosquito. However, polyplex formation at a specific weight ratio requires a standard dsRNA concentration encapsulated; here, in PAA- ds*CACTUS* nanoparticles, ds*CACTUS* concentration is 0.03 ug/ul, meaning that finally 0.002 ug of ds*CACTUS* are injected to the mosquito. This 100 times less ds*CACTUS* mass entering the mosquito explains the lower silencing efficiency observed in this polyplex-injection experiment. A single dsRNA molecule may produce several siRNA molecules, each of which may activate a separate RISC. Thus, the number of mRNA molecules degraded is far in excess of the number of dsRNA molecules introduced into a cell. Typical amounts of injected dsRNAs range from one to hundreds of nanograms.

- **Polyplex feeding**

The silencing of *CACTUS* gene in mosquito midguts after 4-days of feeding on PAA-dsRNA nanoparticles in sucrose solution was analysed by real-time PCR. PAA_{0m}-ds*CACTUS* nanoparticle delivery resulted in a 32,3% repression of the endogenous gene expression in the midgut, compared to the PAA_{0m}- ds*GFP* control group, a difference that is marginally statistically significant (unpaired two-tailed t-test; $p=0.05$) **(Figure B7)**. This difference was not observed between PAA_{4m}- ds*GFP* and PAA_{4m}- ds*CACTUS*; in particular, mosquitoes fed on PAA_{4m}- ds*GFP* demonstrated a lower *CACTUS* expression in the midgut than the PAA_{4m}- ds*CACTUS* fed, but the difference is not statistically significant. Interestingly, *CACTUS* mRNA levels in PAA_{0m}-ds*CACTUS* treated group were 27.4% significantly lower than in PAA_{4m}- ds*CACTUS* treated one (unpaired two-tailed t-test; $p<0.05$).

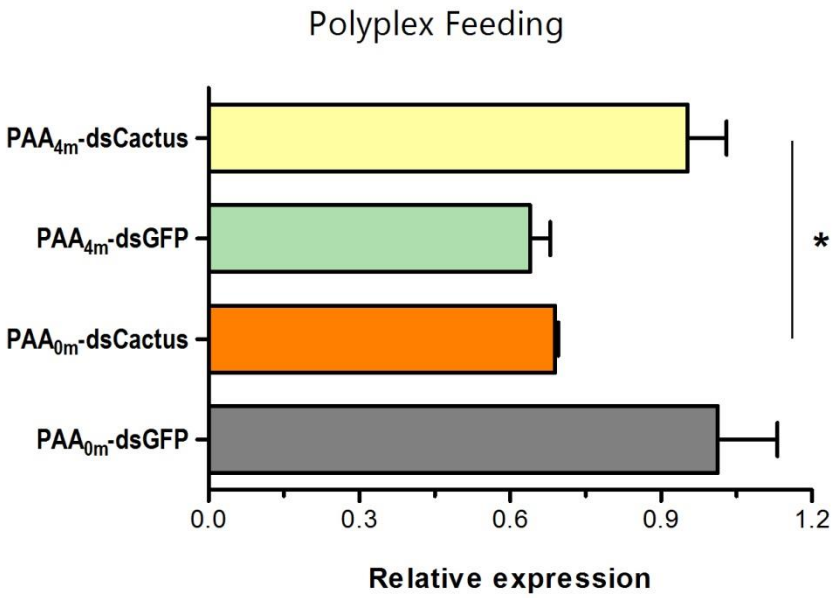


Figure B7: *CACTUS* silencing in female *An. gambiae* midguts, after 4 days of feeding on nanoparticles. The unpaired two-tailed t-test was used to compare the differences in mRNA (*; $p<0.05$). Expression analysis was determined by real-time PCR, in cDNA pools of 7-8 same-treated mosquitoes, in 3 biological replicates. Results were analyzed in GraphPad Prism v.5

DISCUSSION

Synthetic insecticides are the most powerful tool for mosquito vectors control, until now. However, the most important species transmitting pathogens (arboviruses, malaria parasite, etc) have developed alarming rates of insecticide resistance worldwide. This condition along with the limited number of available insecticide formulations constitutes a risk for human health and determines the necessity of applying new control approaches incorporating biological, environmental and/or genetic methods.

RNA interference (RNAi) is a conserved immunity mechanism of eukaryotic organisms; besides, it has been widely used as a gene silencing strategy for pest control. Here, it is proposed as a malaria control strategy blocking parasite transmission through the mosquito. This method has the advantage of being specific and mosquito-firendly, without detrimental effects on the ecosystem and unpredictable side-effects, as it targets the parasite inside the mosquito and not the mosquito itself.

However, the instability of nucleic acids, especially RNA, during and/or after the introduction poses a crucial problem [143]; a delivery method is required to ensure that dsRNA will be protected from extra- or intra-cellular degradation (for example, by nucleases) and will be efficiently released also in the tissue of interest (probably among other tissues) to inhibit specific protein production. Synthetic vectors consist a common delivery system for dsRNA [70]. They include non-toxic cationic polymers that spontaneously bind to anionic oligonucleotides via electrostatic interactions and the resulting positively charged polyplexes easily enter the cell via endocytosis (the cellular membrane is negatively charged) [70]. Polyethylenimine and chitosan are among the most widely used polymers to generate nanoparticles for dsRNA delivery [56, 65, 84].

Here, we investigated whether polyamidoamines (PAAs) could be efficient carriers for dsRNA in adult female *Anopheles gambiae* mosquitoes. Genes selected as dsRNA targets are *CACTUS* and *ESP* (*epithelial serine protease*), both involved in mosquito immunity against pathogens and playing a fundamental role in *Plasmodium* parasite invasion of the midgut [118, 122].

The first step was to ensure that ds_1*ESP* and ds_2*CACTUS*, generated for this study, efficiently knock-down the endogenous genes expression: ds_2*CACTUS* and ds_1*ESP*

reduced *CACTUS* and *ESP* mRNA levels by 62% and 74%, respectively, in mosquito midguts 3 days post-injection. Moreover, 8-10 days after a *P. berghei*-infective blood-meal, decreased number of oocysts was observed in the midguts of ds*CACTUS* - and ds*ESP*- injected female mosquitoes, compared to the control ds*GFP*- injected group. Our result is in line with the already published data of Rodrigues et al., 2012 [118] and Frolet et al., 2006 [73], regarding the role of these two genes in the parasite transition from ookinete to oocyst stage.

Nevertheless, the number of midguts examined in the fluorescent microscope was relatively small, in spite of the experiment repeats. It should be noted that, every time there was a percentage of injected mosquitoes that died a few days post-injection (~10 dead out of 25 injected mosquitoes). A possible explanation is, that injected mosquitoes gain a wound in their thorax; after the infective blood-meal, they were kept for 8-10 days at 19^{o}C (temperature suitable for parasite development), under appropriate humidity. These conditions are favorable for pathogens (e.g. fungi, bacteria) to infect them. Indeed, many times fungi had developed in the cup where injected mosquitoes were kept and this could be the reason why fewer individuals survived for up to 8-10 days (until the midgut dissection). Additionally, the fact that ds*ESP* and ds*CACTUS* both act against genes that are important for mosquito immunity could play an additional role in the imminent death post-injection (however, almost the same percentage of ds*GFP*-injected mosquitoes died every time). Another problem arisen during this experiment is the significant percentage (28%) of ds*GFP*- injected mosquitoes with no oocysts in their midguts; this could be attributed either to low mouse parasitemia levels (<10%) or to some mosquitoes not getting enough amount of blood-meal, a parameter that is out of our control. As this phenomenon is usual in this kind of complicated experiments, which implicate and synchronize the host, the vector and the parasite, many biological replicates are needed using high numbers of female mosquitoes to ensure significant results. Thus, although we should perform additional studies, the fact that we have never observed large number of oocyts in ds*ESP* or ds*CACTUS* as opposed to ds*GFP* is encouraging, favoring our hypothesis that this is mainly because of RNAi-mediating silencing.

PAAs used are non-toxic polymers, with 0 or 4 methyl groups adjacent to the disulfide bond. The disulfide linkages make the polymers very stable under physiological conditions, but rapidly degradable in the reductive cytosol environment [132]. PAA-dsRNA nanoparticles were formed by self-assembly of opposite charges (cationic polymers and anionic oligonucleotides), when mixed [70]. The first step for the evaluation of PAAs as suitable dsRNA carriers was to determine the polymer/dsRNA weight ratio that offers the maximum encapsulation of the genetic cargo, so that dsRNA is efficiently protected in the extracellular environment. It was overall observed that increased dsRNA binding occurs in increased w/w ratios for both PAA_{0m} and PAA_{4m} polymers, as previously shown in Elzes et al., 2016 [70], as

well. In detail, dsRNA molecules bind efficiently to PAA_{0m} polymers already from 6.25 or 12.5 w/w ratio and on, while to PAA_{4m} polymers at 50 w/w and 100 w/w ratios. PAA_{4m}-dsRNA complexation might be permissible in higher polymer weight, probably due to the steric hindrance.

Subsequently, successful gene delivery requires unpacking and release of the dsRNA. In the cell interior, polyplex decomplexation happens either due to polymer degradation or to polyanion exchange [108]. Here, the former was investigated exposing the polyplexes to DTT and Hydrazine and the latter using Heparin. PAA_{0m} polymers dissociated quickly in presence of low DTT concentrations, but PAA_{4m}-dsRNA polyplexes displayed high stability, having remained intact even at approximately 1 M DTT; polymer disassembling occurred only in high Hydrazine concentrations, which is a very strong reductant. This result indicates that hindering methyl groups around the disulfide bond increase the polyplex stability, in accordance to previous findings of Elzes et al., 2016 [70]. The degradation behavior of PAA_{0m}- dsRNA and PAA_{4m}- dsRNA polyplexes in presence of Heparin was almost the same.

After monitoring the PAA- dsRNA polyplexes formation and degradation behavior by agarose gel electrophoresis, the next step was to introduce them in adult female *An. gambiae* mosquitoes, both by injecting and feeding them. PAA_{0m}-ds*CACTUS* nanoparticle injections to adult mosquitoes resulted in slight decrease of the targeted gene mRNA levels in the midgut, due to the small quantity of ds*CACTUS* included in the injection (described in detail in Results- paragraph No. 5). A possible way to overcome this limitation could be to perform two back-to-back injections of 69 nl each to individual mosquitoes; however, it is doubtful whether an adult mosquito tolerate this final volume intrathoracically.

Feeding on PAA_{0m}-ds*CACTUS* polyplexes led to a 32% silencing of *CACTUS* expression in the midgut, a result of marginal significance, compared with the control group. Remarkably, PAA_{0m}-ds*CACTUS* - fed mosquito group exhibited higher knock-down effect on *CACTUS* than PAA_{4m}-ds*CACTUS*- fed group; it is high likely that the stability of PAA_{4m} polymers prevented intracellular dissociation and release of ds*CACTUS*. Furthermore, the addition of 4 methyl groups to the monomers turns the polymer very hydrophobic. Despite hydrophobicity being a factor facilitating gene delivery, it is also a known cause for cytotoxicity that could have decreased mosquito cell viability, after 4- day feeding on PAA_{4m}-dsRNA. In Elzes et al., 2016 [70], after the assessment of PAA_{0m}, PAA_{2m} and PAA_{4m} as polymeric vectors, it was concluded that PAA_{2m} moderate steric hindrance around the disulfide bonds is the most favorable for polyplex stability, efficient release of the genetic payload and low cytotoxicity.

It should also be mentioned, that some practical problems arose in the polyplex-feeding experiment. First of all, after 4 days of feeding on nanoparticles (in sucrose solution), 5-8 out of the 25 mosquitoes of each group died. Given that temperature and humidity conditions were appropriate, a possible explanation for the high mortality percentage is that mosquitoes did not eat adequately. Probably, the filter-syringe system adjusted to the cups did not let every mosquito have enough amount of food during these 4 days. Another uncontrolled parameter is how much of the nanoparticle-sucrose meal each mosquito consumed. After the midgut dissections, mosquitoes of each group were randomly divided into 3 pools of 7-8 mosquitoes for the subsequent *CACTUS* expression analysis; it is self-evident that not every individual ate the same food amount. Such inequalities among the mosquitoes of each pool give a relatively high standard deviation in *CACTUS* mRNA levels detected in the midgut and, finally, not statistically significant differences between the experimental groups, in most cases.

Another important note is that silencing efficiency depends on several factors, e.g. transcript and protein turn-over rates, dsRNA uptake efficiency by cells, etc. While setting up the experiments, it should be taken into account that dsRNA silencing begins already from day 1 post-injection and lasts for up to 6 days. Hence, the subsequent steps (e.g. midgut dissections, RNA extraction, transcript detection via real-time PCR) should be synchronized within this turnover period.

Some suggestions for optimization of the next experiments are given:

- ✓ A smaller filter could be adjusted to the syringe, providing less volume but more concentrated food solution
- ✓ Each experimental group should contain more mosquitoes, e.g. 2 cups x 30 mosquitoes each
- ✓ Labeled nanoparticles (e.g. PAA-GFP) might be used to ensure that they reach the mosquito midgut, among other tissues
- ✓ A dsRNA construction against a housekeeping mosquito gene expressed in the midgut, leading to a lethal phenotype, when silenced, could be included as a control to verify that PAAs are spatiotemporally efficient carriers of dsRNA
- ✓ Synthesize PAA_{2m} polymers and evaluate their potential for dsRNA delivery, following the same approach

In conclusion, here we reported some preliminary results on polyamidoamine-mediated dsRNA delivery in *An. gambiae* midgut, in order to prevent *Plasmodium* parasite development inside the mosquito and block transmission to humans. Our findings indicate that polyamidoamines are promising dsRNA carriers and enhance the potential of genetic methods for mosquito-vector control. The present study

could be used as groundwork, but optimization/ modification of some protocols is necessary for meeting the future goals.

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