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Original article (Orijinal araştırma)

Investigating insecticide resistance, *kdr* mutation, and morphology of the coastal mosquito *Aedes* (*Ochlerotatus*) *zammitii* (Theobald, 1903) (Diptera: Culicidae)

Bir kıyısal sivrisinek olan *Aedes* (*Ochlerotatus*) *zammitii* (Theobald, 1903) (Diptera: Culicidae)'nin insektisit direnci, *kdr* mutasyonu ve morfolojisinin araştırılması

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Abstract

This study aimed to assess the insecticide resistance levels and investigate the presence of the *kdr* mutation in *Aedes* (*Ochlerotatus*) *zammitii* (Theobald, 1903) (Diptera: Culicidae) mosquito populations collected from various locations within the Aegean region of Türkiye. Additionally, the study explored the morphological details of *Ae. zammitii* eggs and adults by using scanning electron microscopy (SEM). Mosquitoes were collected from their natural rocky breeding habitats from several provinces from April to October 2023 using larvae scoops at different aquatic stages. Emerged adult mosquitoes were identified using both taxonomic keys and molecular methods. The obtained images from SEM analysis revealed unique surface features that could potentially be used to identify the species. The susceptibility of adult *Ae. zammitii* to six insecticides, namely DDT (4%), fenitrothion (1%), bendiocarb (0.1%), deltamethrin (0.05%), permethrin (0.75%) and malathion (5%), was determined using the World Health Organization (WHO) susceptibility test. Results indicated that the mosquitoes exhibited varied possible resistance to the different pesticides tested. Mortality rates ranged between 72%-96%. This research confirmed the presence of *kdr* mutation associated with pyrethroid resistance in *Ae. zammitii*. The frequency of L1014F mutation ranged between 55 and 70% with the highest frequency determined in Antalya-Kaş population, followed by Karaburun and Çandarlı in İzmir. These findings significantly contribute to the understanding of insecticide resistance in *Ae. zammitii,* paving the way for developing effective mosquito control strategies in the Aegean region.

Keywords: *Aedes zammitii*, coastal mosquito, insecticide*, kdr*, SEM

Öz

Bu çalışmada, Türkiye'nin Ege Bölgesi'nin çeşitli yerlerinden toplanan *Aedes* (*Ochlerotatus*) *zammitii* (Theobald, 1903) (Diptera: Culicidae) türünde insektisit direnç seviyelerinin değerlendirilmesi ve *kdr* mutasyonunun varlığının belirlenmesi amaçlanmıştır. Ek olarak, *Ae. zammitii* türünün yumurta ve ergin morfolojileri taramalı elektron mikroskobu (SEM) kullanılarak belirlemiştir. Sivrisinekler, Nisan-Ekim 2023 tarihleri arasında doğal kayalık üreme habitatlarından larva kepçeleri kullanılarak toplanmıştır. Ergin *Ae. zammitii* örnekleri hem taksonomik anahtarlar hem de moleküler yöntemler kullanılarak tanımlanmıştır. SEM analizinden elde edilen görüntüler, türün tanımlanmasında potansiyel olarak kullanılabilecek benzersiz yüzey özelliklerini ortaya çıkarmıştır. Ergin *Ae. zammitii* örneklerinin DDT (4%), fenitrothion (1%), bendiokarb (0.1%) deltametrin (0.05%), permetrin (0.75%) ve malathion'a (5%) karşı olan duyarlılıkları Dünya Sağlık Örgütü (DSÖ)'nün duyarlılık testi ile belirlenmiştir. DSÖ duyarlılık test sonuçlarına göre ölüm oranları %72-%96 arasında değişmiş, örneklerin farklı pestisitlere karşı direnç sergilediği ortaya konmuştur. *Ae. zammitii*'de piretroid direnciyle ilişkili *kdr* mutasyonunun varlığı doğrulanmıştır. L1014F mutasyonunun sıklığı %55-70 arasında değişmekte olup, en yüksek frekans Antalya-Kaş popülasyonunda belirlenirken, bunu Karaburun ve Çandarlı takip etmiştir.

Anahtar sözcükler: *Aedes zammitii*, kıyı sivrisineği, insektisit, *kdr*, SEM

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Introduction

Mosquitoes, members of the family Culicidae, continue to pose a significant threat to global health. While certain mosquito species act as pathogen vectors of diseases like West Nile, dengue, Zika disease most of which lack effective vaccines or readily available treatments, others primarily cause annoyance and are categorized as nuisance mosquitoes. These nuisance species are capable of biting humans but lack the ability to transmit diseases (Petric et al., 2010; Heym et al., 2017; Becker et al., 2020). *Aedes* (*Ochlerotatus*) *zammitii* (Theobald, 1903) (Diptera: Culicidae) falls within the nuisance category. This species is a coastal mosquito species found in the Mediterranean region. It has a specific habitat preference, developing within the rock pool water, especially inhabiting the central and eastern regions of countries such as Italy, the Balkans, Sicily, Malta, Greece, and Türkiye (Becker et al., 2020).

Aedes zammitii is a closely related to *Aedes mariae* (Sergent & Sergent, 1903), a mosquito species that occupies the western Mediterranean coast (Mastrantonio et al., 2015; Yavasoglu et al., 2016; Robert et al., 2019). These coastal mosquitoes are morphologically similar in all stages, but *Ae. zammitti* has a more robust build and distinct coloration pattern. There is limited existing information about *Ae. zammitii's* morphology.

Aedes zammitii exhibits a highly zoo-anthropophilic blood-feeding behavior and venture beyond its typical habitats in search of blood meals, increasing the likelihood of dispersal to nearby residences. The intensity of daytime biting activity can become highly bothersome, rendering visits to these coastal areas particularly unpleasant during specific periods in late spring and summer (Mastrantonio et al., 2015; Yavasoglu et al., 2016). However, there are no reports of it transmitting diseases.

Insecticides have been a mainstay in conventional mosquito control programs, with four primary chemical classes historically employed: organochlorines (OCs), carbamates (CBs), organophosphates (OPs), and pyrethroids (PYs). The use of specific insecticides, such as malathion, deltamethrin, and permethrin, has been instrumental in managing mosquito populations. However, this approach faces a growing challenge: insecticide resistance (Liu, 2015; Naqqash et al., 2016; Touray et al., 2023). This resistance, driven by mechanisms like target site mutations (resulting in knockdown resistance) and increased insecticide metabolism, increasingly compromise the effectiveness of various insecticide classes and necessitates alternative strategies. The emergence of insecticide resistance among mosquito populations presents a significant and escalating challenge to global public health (Park et al., 2020; Clarkson et al., 2021; Yavaşoğlu et al., 2022). Pyrethroid insecticides disrupt insect nervous systems by targeting voltage-gated sodium channels (VGSCs) (Hołyńska-Iwan & Szewczyk-Golec, 2020), whereas mutations in the acetylcholinesterase-1 gene confer resistance to organophosphate and carbamate insecticides (Martinez-Torres et al., 1999; Weill et al., 2004). Mutations in the knockdown resistance (*kdr*) gene can lead to amino acid substitutions within the VGSC protein structure. These substitutions hinder pyrethroid binding, diminishing insecticide effectiveness. This necessitates increased insecticide concentrations to achieve the same lethal effect in insects (Davies et al., 2007; Bursalı, 2013; Dong et al., 2014; Uemura et al., 2024).

Mosquito control in Türkiye is a collaborative effort between the Ministry of Health and municipalities, employing both larval and adult control methods (Akiner et al., 2018; Touray et al., 2023). Monitoring insecticide resistance in various mosquito species is a global practice, and Türkiye is no exception. Extensive data exist regarding the resistance status of vector species like *Anopheles sacharovi* (Ramsdale et al., 1980; Hemingway et al., 1992; Kasap et al., 2000), *An. superpictus* (Yavaşoğlu et al., 2019), *Culex pipiens* (Akiner et al., 2009; Akıner & Ekşi, 2015; Taşkın et al., 2015; Guntay et al., 2018; Ser & Çetin, 2019), *Cx. tritaeniorhynchus* (Yavaşoğlu et al., 2022), *Ae. albopictus* (Yavaşoğlu, 2021). However, there is no study about the insecticide resistance of *Ae. zammitii* Türkiye populations. Given the widespread use of insecticides and the emergence of resistance in other mosquito species within Türkiye, investigating the insecticide resistance profile of *Ae. zammitii* populations is crucial to ensure effective mosquito control strategies. There is a critical gap in knowledge concerning this species.

This study investigated the insecticide resistance profiles of six *Ae. zammitii* populations collected from the Aegean and Mediterranean regions of Türkiye. The research evaluated the effectiveness of six insecticides commonly used in vector control programs: DDT, permethrin, fenitrothion, malathion, bendiocarb, and deltamethrin. Additionally, the study aimed to identify the presence of the *kdr* mutation, a genetic marker associated with insecticide resistance in mosquitoes. This research is expected to provide valuable insights into the current resistance status of *Ae. zammitii* populations in Türkiye. This information will be crucial for guiding the selection and implementation of effective vector control strategies, including the selection of appropriate insecticides for future control programs.

Materials and Methods

Sampling studies

Sampling was conducted across several Turkish provinces (İzmir, Antalya, Muğla, Aydın) during the summer months, from April to October 2023 (Figure 1). Samples containing different aquatic stages of *Ae. zammitii* were collected from designated locations, including rock pools, irrigation channels, and flooded farmlands using larvae scoops (Figure 2). Adult mosquitoes were sampled from barns around the coastal area using mouth aspirators. As *Ae. zammitii* exhibits exophilic behavior, they are found near their rocky breeding habitats and readily attack hosts. Larval samples were more abundant than adult samples.

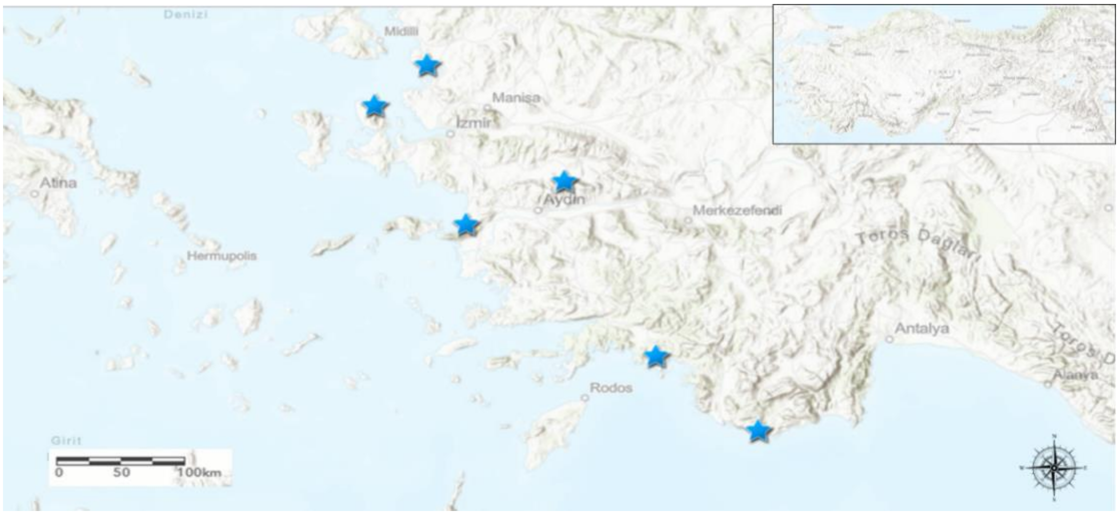

Figure 1. Sampling localities of *Ae. zammitii* populations. This map was generated with the aid of ArcGIS software (version 10.3).

The samples were placed in separate sampling tubes containing habitat water. Information about sampling time, date and coordinate information were recorded and samples were brought to the Vector Insects Research Laboratory, Aydın Adnan Menderes University, Türkiye. III.-IV. stage larvae and pupae samples collected from different localities were transferred to separate cages. Mosquitoes were reared under controlled conditions (28°C temperature, 12 h photoperiod, 60% humidity) and larval feeding with commercial fish food (Tetramin®) and allowed to develop into adults (Bursali & Simsek, 2024). Simultaneously, adult specimens obtained from animal enclosures using light traps and aspirators were maintained on a 10% sugar solution, with gravid females providing eggs for the establishment of F1 generations. Morphological identification of adult mosquitoes was performed using a stereomicroscope (Leica S8 Apo) and established taxonomic keys (Schaffner et al., 2001). F1 females, derived from both field-collected larvae and adults, were utilized in subsequent WHO insecticide susceptibility assays and molecular analyses.

Figure 2. Sampling sites showing breeding habitats of *Aedes zammitii.*

Molecular identification

Molecular methods were used to confirm morphological species identification. Four morphologically identified species from the 4 different provinces (Antalya, Muğla, Aydın,İzmir) were used (Z1-Z4). Genomic DNA was extracted from adult mosquitoes using the Invitrogen Pure Link Genomic DNA isolation kit, following the manufacturer's protocol for efficient and consistent DNA isolation. This method utilizes a multi-step process involving cell wall disruption, cellular content release, selective DNA binding, and purification. The quantity and quality of the extracted DNA will be assessed using a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop™ 2000/2000c) to ensure suitability for downstream applications. The extracted DNA was stored at -20°C. The cytochrome c oxidase subunit I (COI) genes were amplified using the primers LCO1490F (5'-GGTCAACAAATCATAAAGATATTGG-3') and HC02198R (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). The PCR mix comprised 12.5 µl PCR mix, 0.25 µl 20 µM each of primers, 1 µl template DNA and 11µl ddH20. The thermal cycling protocol comprised of an initial denaturation step at 94°C for one minute. This was followed by five cycles with denaturation at 94°C for 40 seconds, annealing at 45°C for 40 seconds, and extension at 72°C for one minute. Subsequently, there were 35 cycles with denaturation at 94°C for 40 seconds, annealing at a higher temperature of 51°C for 40 seconds, and extension maintained at 72°C for one minute. Finally, a final extension step was performed at 72°C for 5 minutes. The amplified DNA fragments were loaded onto a 1% agarose gel and visualized under UV light after electrophoresis. Visualized products were documented before purification and submission for sequencing. Sequences were edited using BioEdit software and compared to other COI sequences available in GenBank using the BLAST tool.

To infer the evolutionary relationships between the investigated organisms, a phylogenetic tree was generated using the Neighbor-Joining algorithm within the MEGA software suite. The analysis was run on 1000 replicates for inferred bootstrap consensus and the reliability of the generated tree was assessed using the bootstrap test (Tamura et al., 2007). *Culex pipiens* mtCOI sequences obtained from the GenBank database was used as an outgroup on the topology.

Insecticide susceptibility bioassays and detection of *kdr* mutation

The susceptibility or resistance levels of mosquito populations to various insecticides was evaluated using standardized protocols established by the World Health Organization (WHO, 2016). Bioassays were conducted with commercially available diagnostic susceptibility bioassay tubes. Insecticide-treated papers, containing different insecticides at specific concentrations, were obtained from a WHO collaborating center (WHOPES) located at Universiti Sains Malaysia. The selected insecticides included

DDT 1,1′-(2,2,2-Trichloroethane-1,1-diyl) bis (4-chlorobenzene) (4%), fenitrothion (1%), malathion (5%), bendiocarb (0.1%), permethrin (0.75%), and deltamethrin (0.05%). These concentrations are those commonly reported in the literature (Kushwah et al., 2015; Liu, 2015; WHO, 2016). This study was done under insectarium conditions. Each test tube included 25 unfed, 3-5 days old, F1 generation *Ae. zammitii* females and each treatment had three replicates. These adult mosquitoes were exposed to insecticide-treated papers for a defined period (1 hour for most insecticides, 4 hours for DDT) following the WHO guidelines (WHO, 2016). Subsequently, they were transferred to holding tubes and provided with a 10% sugar solution for sustenance over a 24-hour period. A control group received identical treatment but with papers impregnated only with the carrier solvent used for the insecticides, as recommended by the World Health Organization (WHO, 2016). Mortality was assessed after 24 h incubation. Populations were considered as ‘susceptible’ if the mortality rates were ≥ 98%; ‘possible resistant’ if mortality rates between 90-97%; ‘resistant’ when the mortality rates were ≤ 90% (WHO, 2016).

Total DNA was isolated from female mosquitoes belonging to each population. Three to five-day-old, unfed females were selected to investigate *kdr* mutations. The DNA extraction protocol in the Invitrogen Pure Link genomic DNA isolation kit was employed to isolate DNA from a total of ten adult females per population. The eluted DNA was subsequently amplified to detect the presence of *kdr* mutations. For the detection of *kdr* mutation in the vgsc gene, the allele-specific primers primers (C1: 5′-CCT GCC ACG GTG GAA CTT-3′/C2: 5′-GGA CAA AAG CAA GGC TAA GAA-3′) used by Liu et al., (2013) were applied in this study to assay the polymorphisms from electrophoresis of the amplicons.

The PCR protocol involved denaturation at 94°C for 5 minutes, followed by 35 cycles with each cycle consisting of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 40 seconds. A final extension step was performed at 72°C for 5 minutes to ensure complete amplification. The resulting DNA fragments were then visualized using gel electrophoresis on 1.5% agarose gels.

Scanning electron microscope (SEM) analysis

Morphological configurations of mosquito eggs and 1-day-old male body parts were determined using Scanning electron microscopy (SEM). Mosquitoes were dissected under stereoscope in 1 µl phosphate-buffered saline (PBS), pH 7.2. The antennae, the mouthparts, and the other body parts of the male were preserved in 70% ethanol. Then an ultrasonic cleaning machine was used to clean the samples twice for 10 min. Samples were serially dehydrated for 10 min in 70%, 80%, 85%, 90%, and 95% ethanol gradients, immersed in 100% ethanol for 30 min twice, and then treated with pure tert-butanol for 30 min. The samples were quickly dried and were fixed to aluminum stubs using double-sided carbon tape (Shi et al., 2021).

Samples were gold-coated in a layer of approximately 100 A° (8-10 nanometer), using a fine gold coating apparatus, with the method of magnetron sputter, ion sputtering device (Spi Supplies, SPI-MODULE Sputter Coater), and examinations of mosquito parts were carried out by a FEI-Quanta 250FEG source Scanning electron microscope (SEM) connected to an EDXS system at an acceleration voltage of 30 kV. The samples were viewed and photographed directly from the SEM video monitor. Eggs were left in-situ throughout this process.

Statistical analysis

Differences in the mosquito mortality rates after exposure to the different treatments (i.e. insecticides and control) were determined using two-way analysis of variance (ANOVA) and Tukey's HSD post-hoc test in SPSS version 23.0. Genotype frequencies, the allele frequency, and genetic conformity to Hardy-Weinberg Equilibrium (HWE) was assessed within each population using exact probability tests implemented in POPGENE software (Yeh, 1999). Statistical significance was $p < 0.05$.

Results and Discussion

The Aegean and Mediterranean regions of Türkiye offer highly suitable climatic conditions for mosquito populations. This, coupled with factors like intensive agricultural practices, tourism, and industrial activity, contributes to the proliferation of mosquito species and the potential spread of mosquito-borne diseases. In this context, understanding the levels and underlying mechanisms of insecticide resistance within these mosquito populations becomes crucial. By investigating resistance patterns, this research aims to identify the most effective insecticides for mosquito control programs in Türkiye. This knowledge is essential for guiding vector control strategies and mitigating the risk of disease transmission. This study determined the susceptibility of *Ae. zammitii,* a nuisance mosquito species found in the Aegean region of Türkiye, to various insecticide classes and identified the presence of a *kdr* mutation, which is associated with pyrethroid resistance in other mosquito species.

Aedes zammitii mosquitoes were collected from coastal breeding sites and subsequently identified using both taxonomic keys and molecular methods. Sequence analysis of the cytochrome c oxidase subunit I (COI) gene revealed high nucleotide homology (>96%) with related species sequences deposited in GenBank, as determined by BLASTn searches. The resulting phylogenetic tree (Figure 3) generated using the neighbor-joining method positioned *Ae. zammitti* within the mariae group alongside *Ae. mariae* and *Ae. phoeniciae*. This finding aligns with previous research (Mastrantonio et al., 2015; Yavasoglu et al., 2016). Notably, these three species are considered sibling species, characterized by their development in rock pools located along Mediterranean coastal regions (Urbanelli et al., 2014).

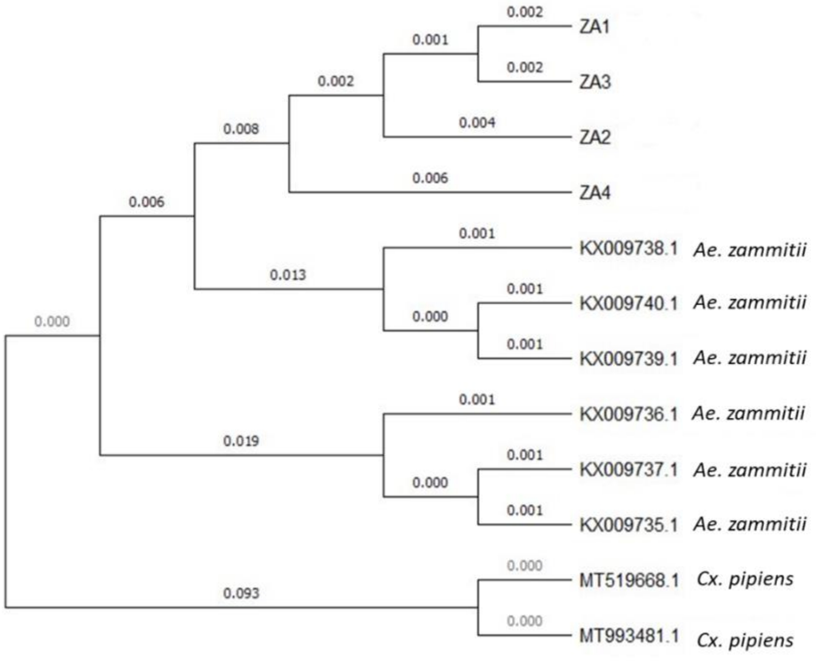

Figure 3. Phylogenetic tree created by NJ method for the COI gene region of *Aedes zammitii* (Z1-Antalya-Z2-Mugla, Z3-Aydin, Z4-İzmir).

Chemical insecticides remain the mainstay of mosquito control programs globally (WHO, 2018; Kumar et al., 2022; Duval et al., 2023). This reliance began with the discovery of DDT during World War II (Vezehegno, 2008). However, widespread use led to the development of resistance in mosquitoes and other insects, coupled with significant environmental and ecological damage (Coetzee vd., 1999). As a result, DDT has been banned in many countries. Consequently, newer chemical insecticides were developed and integrated into mosquito control strategies. Pyrethroid insecticides, for instance, are utilized in both ultra-low-volume aerial sprays for adult mosquitoes and indoor residual spraying (IRS) programs (Duval et al., 2023). Additionally, biological control agents like *Bacillus thuringiensis israelensis*, and insect growth regulators (IGRs) are widely employed for effective larval control (Özbilgin et al., 2011; WHO, 2018). During the winter, targeted treatment of potential hibernation sites is conducted. Additionally, homeowners are encouraged to adopt indoor residual spraying or insecticide-impregnated nets (Guz et al., 2020; Touray et al., 2023). The financial burden, significant risks posed to both human health and ecosystems, and limited long-term efficacy of chemical insecticides, coupled with the widespread emergence of insecticide resistance among mosquito populations, underscore the need for alternative approaches (Liu, 2015; Pimentel et al., 1992).

WHO susceptibility bioassay results indicated that the mosquitoes exhibited varied possible resistance to the different pesticides tested. The different insecticides have varying degrees of effectiveness against mosquito. Mortality rates ranged between 72%-94. All populations were resistant to DDT even though it was banned in the 1980s (Akiner et al., 2009) and had the least effects against the mosquitoes. DDT resistance has been documented in various medically important mosquito species in Türkiye, including *An. sacharovi, An. maculipennis, An. superpictus* and *Cx. pipiens* (Akıner et al., 2013; Taskin et al., 2016; Yavaşoğlu et al., 2019). This study represents the first report of DDT resistance in *Ae. zammitii* populations from Türkiye. Following the widespread withdraw of DDT in the 1970s due to resistance concerns, mosquito control programs transitioned to carbamate (CB) and organophosphate (OP) insecticides, such as malathion, fenitrothion, bendiocarb, and propoxur (Ramsdale, 1980). The extensive use of malathion in agricultural pest control creates a strong selection pressure for resistance in mosquito populations inhabiting these areas (Kasap et al., 2000). Notably, *Cx. pipiens* populations in neighboring countries like Iran, Russia, and Greece have also shown high levels of resistance to various insecticides (Rahimi et al. 2020; Vereecken et al., 2022; ECDC, 2023).

Permethrin appears to be the most effective insecticide, achieving remarkably high mosquito mortality (over 92%) across all six testing sites. Fenitrothion, deltamethrin, and bendiocarb also displayed strong efficacy, eliminating 79% to 92% of mosquito adults collected from the various locations. Malathion and DDT were the least effective insecticide overall, with mosquito mortality rates ranging from 70% to 79% across the different sites. The two-way ANOVA analysis revealed that there were clear differences between the insecticide treatments and the control group ($F(6, 84) = 1573$; $p<0.0001$); between localities from which the populations were collected ($F(5, 84) = 103.5$; $p<0.0001$) and in the interaction between the treatments and localities ($F(30, 84) = 3.711$; $p<0.0001$).There were no statistical differences in the effects of the insecticides on mosquito mortality (Figure 4). Our bioassay results revealed resistance to all tested OPs (malathion and fenitrothion) and CBs (bendiocarb) across all *Ae. zammitii* populations. This constitutes the first record of OP and CB resistance in *Ae. zammitii* populations from Türkiye. The observed resistance likely stems from the intensive use of these insecticides, particularly malathion, in agricultural pest management programs (Kasap et al., 2000) (Table 1).

This research is significant because it contributes to the understanding of insecticide resistance in *Ae. zammitii,* which is crucial for developing effective mosquito control strategies in the region. Previous reports indicate resistance in other Turkish mosquito species, including *Anopheles superpictus* (Grassi, 1899) (Diptera: Culicidae), and *Anopheles sacharovi* (Favre, 1903) (Diptera: Culicidae) (Yavaşoglu et al., 2019), *Anopheles maculipennis* (Meigen, 1818) (Bursalı & Şimşek, 2016), *Culex pipiens* (L., 1758) (Diptera: Culicidae) (Akiner & Ekşi, 2015), *Culex tritaeniorhynchus* (Giles, 1901) (Diptera: Culicidae) (Yavaşoğlu et al., 2022), *Aedes albopictus* (Grassi, 1899) (Diptera: Culicidae (Yavaşoğlu, 2021), *Aedes caspius* (Pallas, 1771) (Diptera: Culicidae) (Yavaşoglu et al., 2024) in Türkiye. These studies revealed widespread resistance to various insecticides and elevated enzyme activity, suggesting multiple resistance mechanisms. We however note that our bioassays lacked a susceptible *Ae. zammittii* population for comparison. Because of its habitat preferences, we could not rear this species in the laboratory.

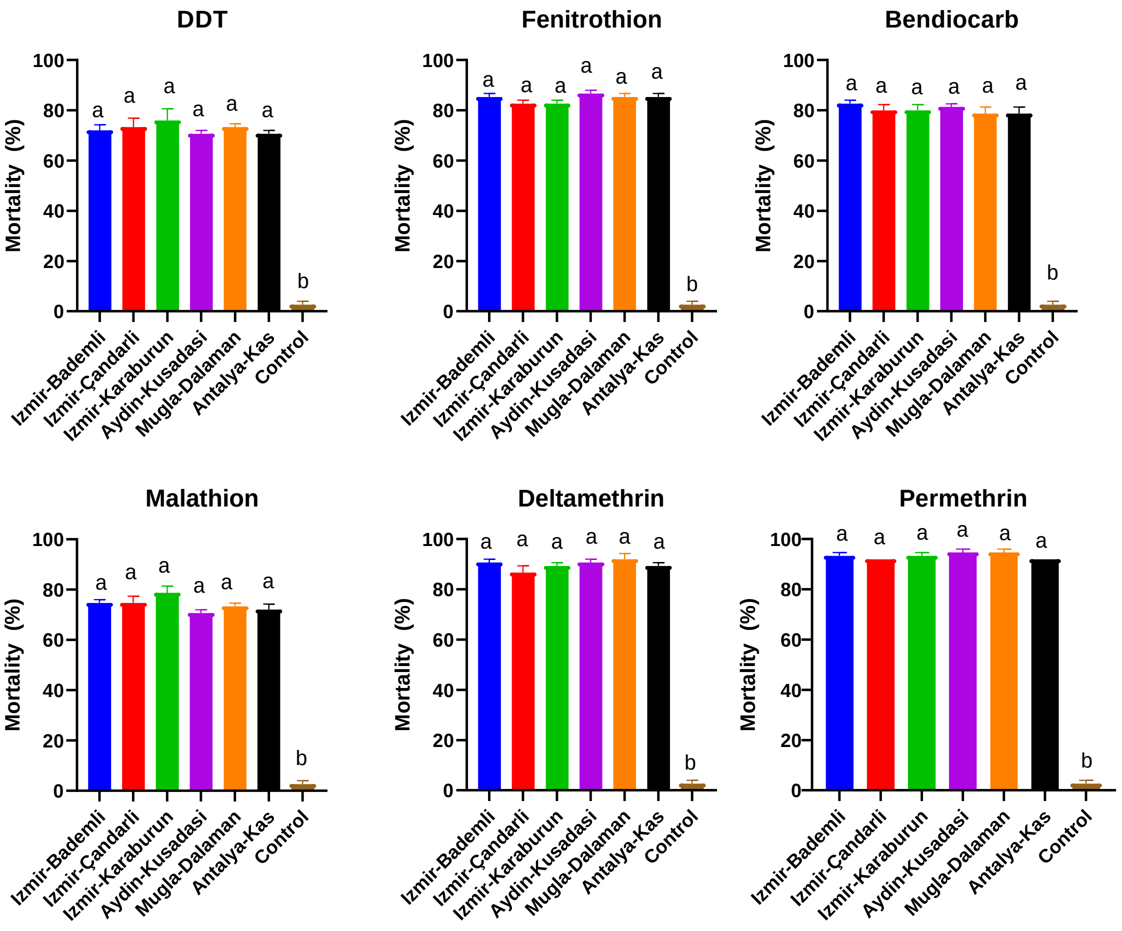

Figure 4. Insecticide resistance levels of *Aedes zammitii* collected from different localities in the Aegean and Mediterranean region of Türkiye. According to WHO susceptibility bioassay results, mortality rates ≥ 98% indicate susceptible; '90%-97% = possible resistant; mortality rates ≤ 90%resistant. The bars represent the mortality rates after exposure to treatments and error bars indicate standard deviation.

Table 1. Mortality rates (% ± standard deviation) and resistance levels of *Aedes zammitii* collected from different localities in the Aegean and Mediterranean regions of Türkiye. Mortality rates were ≥ 98% were considered as susceptible (S); '90%-97% possible resistant (PR); mortality rates ≤ 90%= resistant (R).

Localities	Resistance levels to Insecticides (% mortality)					
	DDT (4%)	Malathion(5%)	Bendiocarb (0.1%)	Fenitrothion (1%)	Deltamethrin (0.05%)	Permethrin (0.75%)
İzmir-Bademli	72±3 (R)	75±2 (R)	83±3 (R)	85±5 (R)	91±4 (PR)	93±2 (PR)
İzmir-Çandarlı	73±3 (R)	75±3 (R)	80±3 (R)	83±4 (R)	87±7 (R)	92±0 (PR)
İzmir-Karaburun	76±2 (R)	79±4 (R)	80±3 (R)	83±2 (R)	89±6 (R)	93±2 (PR)
Aydın-Kuşadası	71±2 (R)	71±3 (R)	81±3 (R)	87±3 (R)	91±2 (PR)	95±2 (PR)
Muğla-Dalaman	73±2 (R)	73±2 (R)	79±2 (R)	85±2 (R)	92±2 (PR)	95±2 (PR)
Antalya-Kaş	71±2 (R)	72±3 (R)	79±4 (R)	85±2 (R)	89±3 (R)	92±0 (PR)

The *kdr* mutation has been implicated in DDT resistance in some mosquito species worldwide (Martinez-Torres et al., 1999; Ponce et al., 2016; Saha et al., 2019; Zhou et al., 2019). However, the absence of *kdr* mutation data in our study limits our ability to determine if this mechanism contributes to DDT resistance observed in these *Ae. zammitii* populations. DNA sequence from individual mosquitoes was separated and amplified from 60 specimens of *Ae. zammitii*, using PCR. The *kdr* genotype and allele frequencies of the phenotypes, determined by the deltamethrin resistance bioassay in *Ae. zammitii,* populations of various regions, are shown in Table 2. In *Ae. zammitii*, three genotypes were identified homozygous resistance (TTT/TTT-L1014F/L1014F), heterozygous resistance (TTT/TTA-L1014F/L1014), and homozygous susceptibility (TTA/TTA-L1014/L1014). No TCA (L1014S) mutation was found *Ae. zammitii*. The frequency of L1014F mutation in *Ae. zammitii* ranged between 55 and 70% with the highest frequency determined in Antalya-Kaş population (70%), followed by Karaburun (65%) and Çandarlı (60%) in İzmir. Heterozygous genotypes were observed in all assessed populations (Table 2).

Table 2. *Kdr* genotypes and allele frequencies in *Aedes zammitii* at the study sites

Sites	Coordinates	Sample Size	*kdr* genotype			Allele frequency (%)		X^2	p
			TTT/TTT	TTT/TTA	TTA/TTA	TTT	TTA		
Antalya-Kaş	36°11'32.9"N 29°38'54.7"E	10	4	6	0	70	30	0.400	0.527
Muğla-Dalaman	36°42'20.3"N 28°43'24.0"E	10	3	5	2	55	45	1.400	0.497
Aydın-Kuşadası	37°54'20.3"N 27°16'04.8"E	10	3	5	2	55	45	1.400	0.497
İzmir-Bademli	39°02'27.5"N 26°48'45.7"E	10	4	3	3	55	45	0.200	0.905
İzmir-Çandarlı	38°56'05.1"N 26°57'02.8"E	10	5	2	3	60	40	1.400	0.497
İzmir-Karaburun	38°37'46.4"N 26°31'28.7"E	10	5	3	2	65	35	1.400	0.497

Pyrethroids are commonly used for *Aedes* control, but their widespread and sustained use has selected for resistance globally (Bursalı, 2013; Amelia-Yap et al., 2018; Melo Costa et al., 2020; Mashlawi et al., 2022; WHO, 2023). For example, *Aedes aegypti* (Linnaeus in Hasselquist, 1762) (Diptera: Culicidae), populations in Thailand displayed incipient or full resistance to various insecticides including deltamethrin and permethrin, with only a few susceptible populations found in specific areas of Songkhla and Chiang Rai provinces (Jirakanjanakit et al., 2007). These *Ae. aegypti* mosquitoes had mutations linked to pyrethroid resistance. Kushwah et al. (2015)'s study indicated resistance to DDT in both *Ae. aegypti* and *Ae. albopictus* (Grassi, 1899) (Diptera: Culicidae), with variable resistance to other insecticides. They did not detect mutations associated with pyrethroid resistance and these did not consistently correlate with phenotypic resistance. Konkon et al. (2023) investigated the susceptibility of *Ae. aegypti* and *Ae. albopictus* mosquitoes exposed to commonly used insecticides (deltamethrin, permethrin, alpha-cypermethrin, pirimiphos-methyl, and bendiocarb) in southern Benin. They observed that *Ae. albopictus* showed varying levels of resistance to alpha-cypermethrin, while *Ae. aegypti* presented widespread resistance to nearly all tested pyrethroids. Notably, resistance persisted even after pyrethroid withdrawal in specific regions Despite the cessation of their public use in 2000, resistant *Ae. aegypti* adults were detected in São Paulo during a robust monitoring a decade later (Macoris et al., 2018). Two known *kdr* mutations (Val1016Ile and Phe1534Cys) were determined with a significant decrease in the susceptible allele over time (Macoris et al., 2018). The L1014F and L1014C mutations in the *kdr* gene have been implicated in DDT and pyrethroid resistance in *Cx. pipiens* populations worldwide (Taskin et al., 2016; Fotakis et al., 2017; Tmimi et al., 2018). For instance, both mutations were identified in *Cx. pipiens* from the Aegean region of Türkiye (Taskin et al., 2016) and Greece (Fotakis et al., 2017), while *Cx. pipiens* populations in Morocco (Tmimi et al., 2018) displayed a high frequency of the L1014F allele. Variations exist in the specific *kdr* mutation responsible for resistance geographically. For example, the L1014S mutation confers resistance in *Cx. quinquefasciatus* from China (Xu et al., 2006), whereas L1014F is responsible in New Jersey (Johnson et al., 2016) and L1014C is observed in some Chinese populations (Wang et al., 2012). This study contributes significantly to understanding insecticide resistance in *Ae. zammitii*, a critical step towards effective mosquito control in the Aegean region of Türkiye.

We identified resistance or possible resistance to various insecticide classes and confirmed the presence of a *kdr* mutation associated with pyrethroid resistance. Our findings reinforce the widespread emergence of insecticide resistance among mosquito species in Türkiye, including *Ae. zammitii, Ae. caspius, Ae. albopictus,* and others. Such reports highlight the significant problem of resistance development in populations from different countries and underscore the need for diverse strategies such as utilizing standardized WHO methods to map resistance trends and identify mechanisms, investing in developing insecticides with new modes of action to counter existing resistance and exploring and implementing non-chemical control methods like source reduction, development of bio-agents, and insect growth regulators (Hancock et al., 2020; Touray et al., 2023; WHO, 2023). Bioagents, such as *Bacillus thuringiensis* and its derivatives, alongside entomopathogenic fungi, holds promise for mosquito control (Mampalil etal., 2017; Accoti et al., 2021).

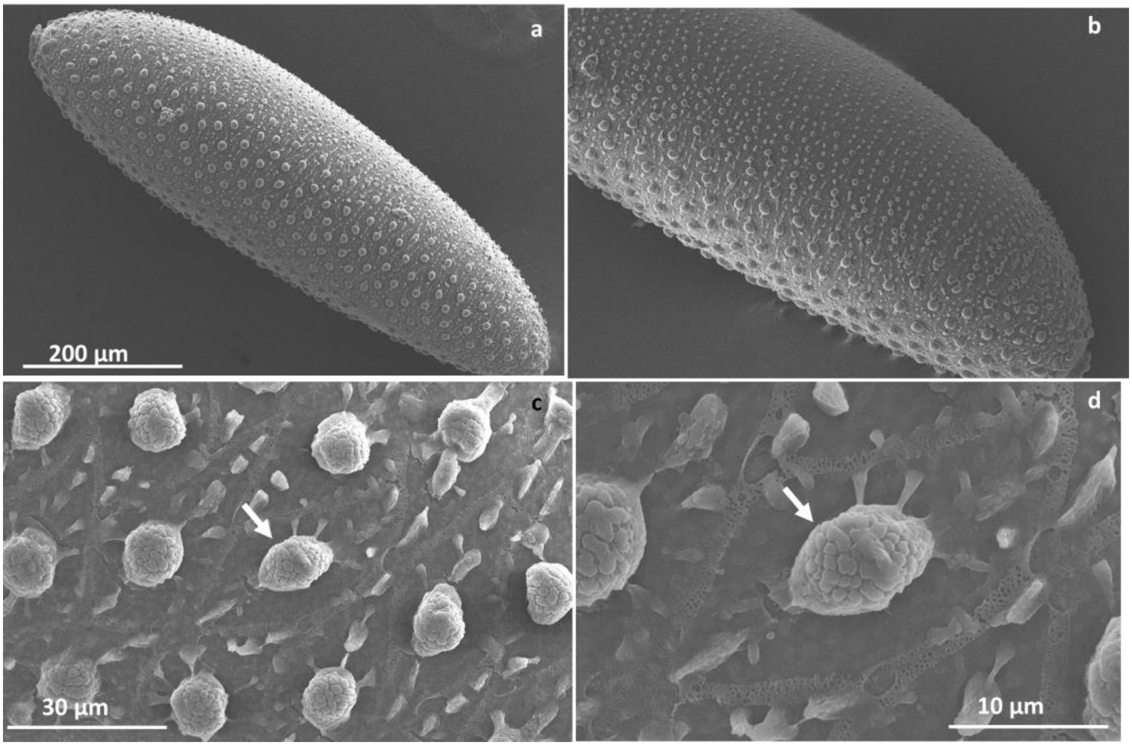

Figure 5. Scanning electron microscopy (SEM) images of *Aedes zammitii* eggs.

Identifying mosquitoes traditionally relies on microscopic analysis of morphology and molecular analysis of genes. Scanning electron microscopy (SEM) offers detailed descriptions of species such as the surface topography of adults and developmental stages (Mello et al., 2017; Kim et al., 2020). There is limited existing information about *Ae. zammitii'* morphology. While not directly connected to the genotyping work, our SEM observations can serve as valuable baseline for future research, including morphology-based identification. This study captured the SEM images of various adult *Ae. zammitii* body parts, including the head, maxillary palps, antennae, wings, scales, and abdomen. The images revealed that the adult mosquito's body is covered in numerous triangular-shaped setae and scales. These scales displayed pointed apices (emergence points) and blunt, broad distal ends. Additionally, the setae were observed to be long and backwardly bent. The abdominal scales displayed 17 longitudinal ridges interconnected by numerous small cross-ribs. The head of the male mosquito exhibited plumose antennae and long, hairy maxillary palps (Figure 6). These observations share similarities with the reported characteristics of *Ae. albopictus* and *Ae. aegypti* adults (Supriyono et al., 2023). *Ae. zammiti* eggs are characterized by their black color and cigar-shaped morphology. Females lay these eggs individually on the surface of saline water (Figure 5). The eggs measured 200 ± 11.33 µm in length and 96.23 ± 3.0 µm in width, tapering towards both ends. SEM analysis revealed a unique chorionic surface featuring an air-covering plastron network and clusters of globular tubercles of varying sizes. Notably, large oval tubercles were uniformly distributed around the eggs, while smaller, irregularly shaped tubercles filled the spaces between. These structures are believed to contribute to egg buoyancy. Previous SEM studies have explored the surface topography of numerous *Aedes* species, including *Ae. aegypti, Ae. albopictus, Ae. cinereus* (Hinton & Service, 1969; Linley, 1989a, b; Linley & Clark, 1989; Choochote et al., 2001; Alencar et al., 2003, 2008). While generally cigar/boat shaped, *Aedes* eggs exhibit variations in size, morphology, exochorionic tubercle patterns, and micropylar structures. Notably, *Ae. zammitii* eggs share some similarities with *Ae. aegypti* and *Ae. albopictus*; however, (Linley, 1989b) and (Supriyono et al., 2023) described the eggs of these latter species as having a shinier jet-black appearance and more regular, smoothly rounded large tubercles surrounded by nearly tubercle-free cell fields. In contrast, *Ae. lineatopennis* eggs, measuring 510 ± 40 µm in length and 182 ± 18.90 µm in width, possess a fragmented micropylar collar and an irregular exochorionic sculpture characterized by membrane-like walls and a mix of large and small irregular tubercles (Choochote et al., 2001) (Figure 7).

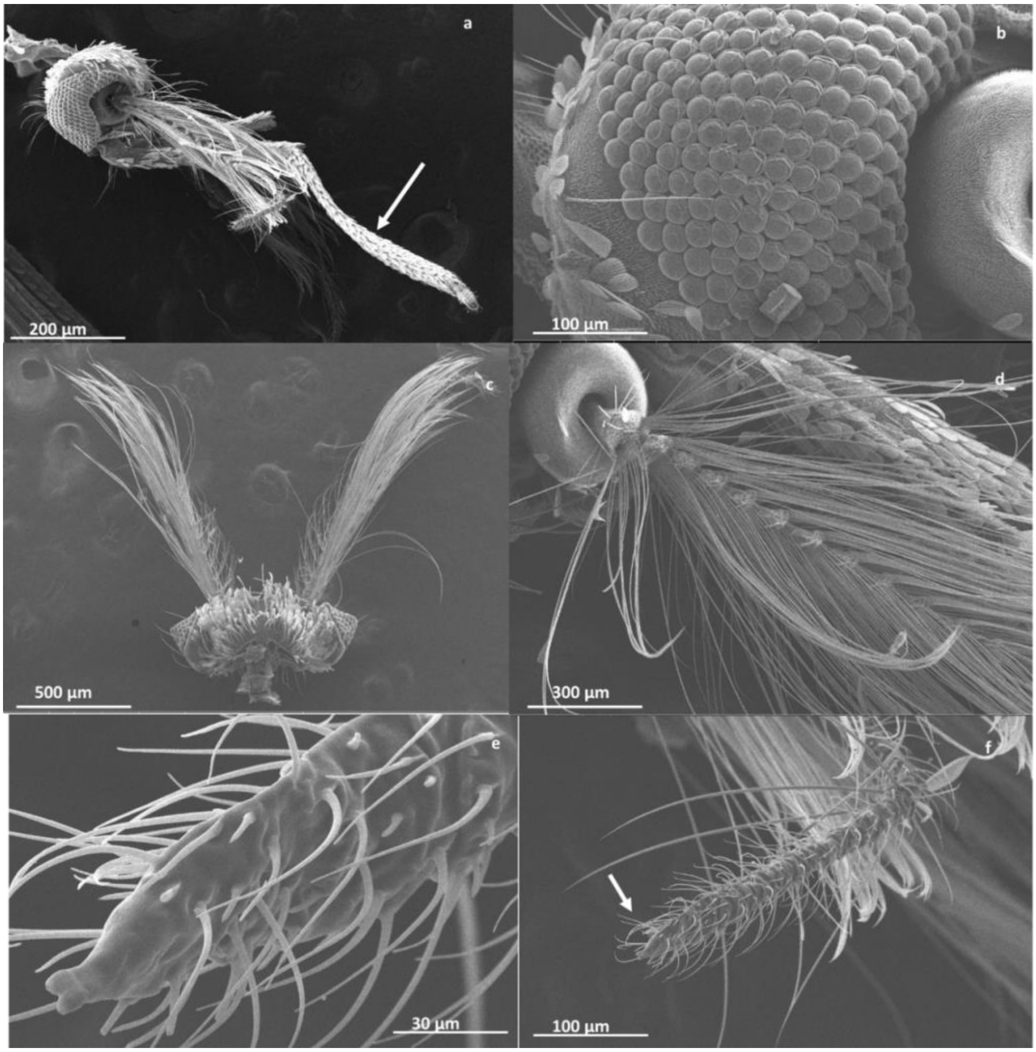

Figure 6. Scanning electron microscopy (SEM) images of scale from different head parts of the mosquito, *Aedes zammitii*. (a) Head of male showing antenna and proboscis (arrow) (b) Detail of compound eye with tiny ommatidia (c-d) Plumose antenna of male (e-f) details of the labium and labellum.

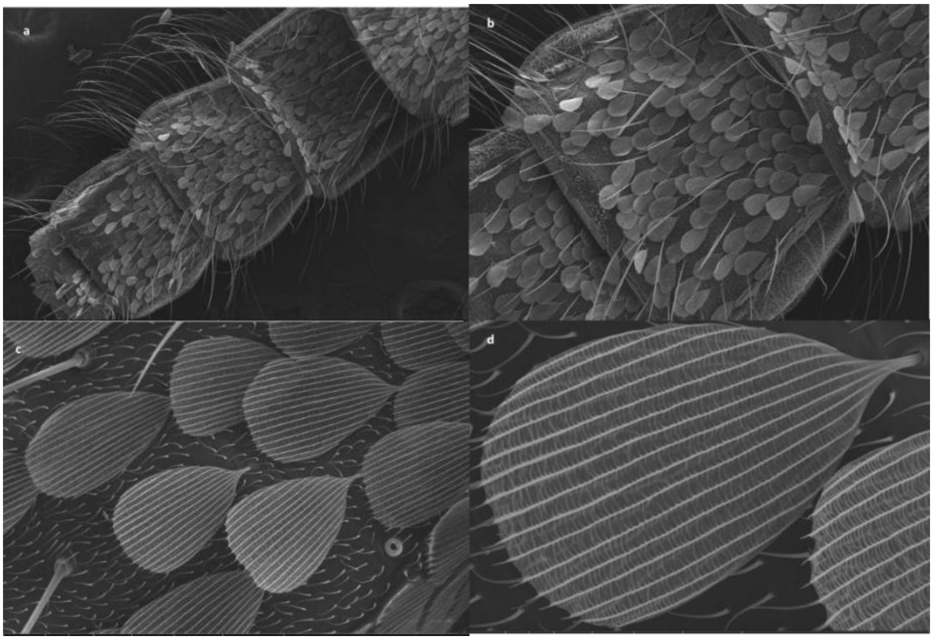

Figure 7. Scanning electron microscopy (SEM) images of scale from different abdomen parts of the mosquito, *Aedes zammitii*.

Conclusion

This study contributes to the understanding of insecticide resistance in *Ae. zammitii,* which is crucial for developing effective mosquito control strategies in the Aegean region of Türkiye. The findings highlight the need for diverse strategies to manage mosquito populations, including utilizing standardized methods to track resistance trends, developing new insecticides, and exploring non-chemical control methods. SEM analysis provided detailed descriptions of the morphology of *Ae. zammitii* eggs and adults, including their unique surface features.

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