



**T.R.
İSTANBUL UNIVERSITY
INSTITUTE OF SCIENCES**



Ph.D. THESIS

**INVESTIGATION of RESISTANCE to *BACILLUS THURINGIENSIS*
SUBSP. ISRAELENIS and *BACILLUS SPHAERICUS* USED as
LARVACIDE on MOSQUITO CONTROL in ISTANBUL**

Emine Nurhan AKIN

Department of Biotechnology

Programme of Biotechnology

ADVISOR

Assoc. Prof.Dr. Erdal POLAT

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İSTANBUL

This work is , accepted as a Ph.D. Thesis in
Department of Molecular Biology and Genetics |
Programme of Biotechnology by the following jury in 13.01.2023

Thesis Jury

[Assoc.Prof.] [Erdal POLAT] (Advisor)
İstanbul Üniversitesi-Cerrahpaşa
Faculty

[Prof. Dr.] [Mustafa SAMASTI]
İstanbul Medeniyet Üniversitesi
Faculty of Medicine

[Prof. Dr.] [İlhan ONARAN]
İstanbul Üniversitesi-Cerrahpaşa
Faculty of Medicine

[Assoc.Prof.] [Hüseyin ÇAKAN]
Çanakkale 18 Mart Üniversitesi
Fen Fakültesi

[Assoc.Prof.] [Serhat SİREKBASAN]
Çankırı Karatekin Üniversitesi,
Eldivan Sağlık Hizmetleri Meslek
Yüksekokulu

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PREFACE

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols	Explanations
%	: Percentage
Bp	: Base pair
BSA	: Bovine serum albumin
°C	: Degree Celsius
cm	: Centimeter
cm ²	: Square centimeter
CTAB	: Cetyltrimethylammonium bromide
ddH ₂ O	: Double distilled water
dNTPs	: Deoxynucleotide triphosphates
EDTA	: Ethylenediaminetetraacetic acid
gDNA	: Genomic DNA
h	: Hour Clock
H ₀	: No NULL hypothesis
H ₁	: Alternative Hypothesis
g	: Gram
g/ha	: Gram/ hectare
kDa	: Kilodalton
km ²	: Square kilometers
µg	: Microgram
µg/µl	: Microgram /microliters
µl	: Microliter
µM	: Micrometer
m ²	: Square meters
mg	: Miligram
MgCl ₂	: Magnesium chloride
mg/l	: Milligram / liter
min	: Minute
MJm ⁻²	: Millijoule per square metre
ml	: Milliliter
mm	: Millimeter

mM	: Milimolar
N	: Normality
NaCl	: Sodium chloride
ng	: Nanogram
NaOH	: Sodium hydroxide
nm	: Nanomolar
OD	: Optical density
PCR	: Polymerase chain reaction
pH	: Potential of hydrogen
Rpm	: Revolutions per minute
RT-PCR	: Reverse transcription polymerase chain reaction
Taq	: <i>Thermus aquaticus</i>
TBE	: Tris/Borate/EDTA
U/mg	: Units/milligram
UV	: Ultraviolet
V	: Volt

Abbreviations	Explanations
Ae.	: <i>Aedes</i>
AChE	: Acetylcholinesterase
An.	: <i>Anopheles</i>
ANOVA	: Analysis of variance
ANCOVA	: Analysis of covariance
B	: <i>Bacillus</i>
Bin	: Binary toxin
CDC	: Centers for Disease Control
Cry	: Crystal
Cyt	: Cytolytic
<i>C. pipiens</i>	: <i>Culex pipiens</i>
Cs	: <i>Culiseta</i>
Cpm1	: <i>C. pipiens</i> maltase 1
DNA	: Deoksiribo nukleik asit
DDT	: Dichlorodiphenyltrichloroethane
EPA	: The Environmental Protection Agency

F1	: Generation, refers to the first filial generation.
GPI	: Glycosyl-phosphatidylinositol
IGRs	: Insect growth regulators
IMM	: Istanbul Metropolitan Municipality
LC50	: Lethal concentration that kills 50 percent of the exposed larvae
LC90	: Lethal concentration that kills 90 percent of the exposed larvae
LD 50	: Lethal dosage means the compound kills 50% of the exposed larvae, expressed in mg/larva
MCs	: Methylcarbamates
Mtx	: Mosquitoicidal toxin
TE	: Transposable element
TCA	: Trichloroacetic acid
WHO	: World Health Organisation
WNV	: West Nile virus
OC	: Organochlorine
OWA	: One-Way ANOVA
Pc	: Percentage survival in the untreated control
POPs	: Organochlorine pesticides
PR	: Possibly resistant
Pt	: Percentage survival in the treated sample
PFT	: Pore-Forming Toxins
R	: Resistant
S	: Susceptible
spp	: Several species
SIT	: Sterile Insect Technique
US	: United States
USA	: United States of America.
12L:12D	: Light-dark cycle

ABSTRACT IN ENGLISH

[THESIS Ph.D.]

[INVESTIGATION of RESISTANCE to *BACILLUS THURINGIENSIS* SUBSP.
ISRAELENIS and *BACILLUS SPHAERICUS* USED as LARVACIDE on
MOSQUITO CONTROL in ISTANBUL]

[Emine Nurhan AKIN]

İstanbul University

Institute of Science

[Department of Molecular Biology and Genetics]

[ADVISOR

Assoc. Prof.Dr. Erdal POLAT]

Bacillus sphaericus and *Bacillus thuringiensis subsp. israelensis* are used as larvicides for mosquito control in Istanbul. We conducted this study from 2018 to 2020, between April and September and investigated whether the dominant *C. pipiens* species developed resistance to these bacteria in 39 districts of Istanbul. Based on the studies showing that high protease enzyme activity in mosquitoes is associated with resistance, the activity of protease enzyme in *C. pipiens* larvae was examined.

According to our empirical observations and Abbott's formula results, resistance to *Bacillus thuringiensis subsp. israelensis* type bacteria was not detected in *C. pipiens* larvae, but resistance to *Bacillus sphaericus* type bacteria was detected in 5 regions and 13 counties. The region with the highest resistance was region 1 (Bağcılar, Bahçelievler, Bakırköy, Bayrampaşa, Esenler, Fatih, Güngören, Beyoğlu, Şişli, and Zeytinburnu) with an Abbott value of 84.06%, and the region with the lowest resistance was region 6 (Adalar, Kartal, Maltepe, Pendik, Sancaktepe, Sultanbeyli, Şile, and Tuzla) with an Abbott value of 98.18%. With an Abbott value of 70.0%, the population of Esenyurt district had the highest resistance to *B. sphaericus*,

while the population of Ümraniye district had the lowest resistance with an Abbott value of 89.0%.

One-Way Analysis of Variance (OWA) for independent groups was used to detect a possible difference that may exist between regions with resistant *C. pipiens* larvae based on Abbott values tested for *B. sphaericus*, and Analysis of Covariance (ANCOVA) was applied to compare the effect value of *B. sphaericus* from the 24th and 48th hour. It was found that there was a significant difference ($p < 0,05$) in the differences between statistical analyzes. From the multiple comparison tests, Tukey HSD and Abbott 48-hour measurement, there was a significant difference between the effect values of *B. sphaericus* only in region 1 and region 6, with the Post Hoc test, and it was found that region 4 (Arnavutköy, Avcılar, Başakşehir, Sultangazi, and Küçükçekmece) had a significant difference from other regions in the 24-48 hours change.

When we look at the activity of the protease enzyme in *C. pipiens* larvae that are resistant to *B. sphaericus*; although the protease enzyme activity in *C. pipiens* larvae was higher than the control group, it was not consistent with the findings obtained by Abbott's study. In our experimental study of resistance, we have shown that there is a correlation between the development of resistance to *B. sphaericus* and protease activity, but we believe that further information can be obtained with genetic diversity and mutational analysis to explain the mechanism. As a result, according to the data we obtained in our study, it was determined that the resistance points in the regions decreased from the 1st region, the city center of Istanbul, to the rural areas.

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Keywords: [*C. pipiens*, *B. sphaericus*, *B. thuringiensis* subsp. *israelensis*, resistance.]

ABSTRACT IN TURKISH

[Ph.D. THESIS]

İSTANBUL'DA SİVRİSİNEK MÜCADELESİNDE LARVASİT OLARAK KULLANILAN BACİLLUS THURİNGİENSİS SUBSP. İSRAELENİSİS VE BACİLLUS SPHAERİCUS'A KARŞI DİRENÇ GELİŞİMİNİN ARAŞTIRILMASI

[Emine Nurhan AKIN]

İstanbul University

Institute of Sciences

[Department of Molecular Biology and Genetics]

Supervisor : [Assoc. Prof. Dr.] [Erdal POLAT]

İstanbul'da sivrisinekler mücadelesinde larvasid olarak, *Bacillus sphaericus* ile *Bacillus thuringiensis subsp. israelensis* kullanılmaktadır. 2018 ve 2020 tarihleri Nisan ve Eylül ayları arasında yaptığımız çalışmada İstanbul'un 39 ilçesinde baskın olan *C. pipiens* türünün bu bakterilere karşı direnç geliştirip, geliştirmediğinin araştırılması amaçlanmıştır. Sivrisineklerde proteaz enzim aktivitesininin yüksekliğinin direnç ile ilişkili olduğunu gösteren çalışmalardan yola çıkarak *C. pipiens* larvalarında proteaz enziminin etkinliğine bakılmıştır.

Gözlemlerimiz ve Abbott formülüne göre çıkan sonuçlarda, *C. pipiens* larvalarında *B. thuringiensis subsp. israelensis* türü bakterisine karşı direnç görülmezken *B. sphaericus* türü bakterisine karşı 5 bölgede ve 13 ilçede direnç belirlenmiştir. Direnci en fazla olan bölge 1. bölge (Bağcılar, Bahçelievler, Bakırköy, Bayrampaşa, Esenler, Fatih, Güngören, Beyoğlu, Şişli, and Zeytinburnu) olup Abbott değeri, % 84.06'dır, direncin en düşük olduğu bölge 6. bölge (Adalar, Kartal, Maltepe, Pendik, Sancaktepe, Sultanbeyli, Şile, and Tuzla) olup Abbott değeri, % 98.18'dir. Esenyurt ilçesinin Abbott değeri % 70.12 olup, *B. sphaericus*'a karşı en

fazla direncin olduğu, Ümraniye ilçesinin ise Abbott değeri % 89.90 olup direncin en düşük olduğu belirlenmiştir.

B. sphaericus'un denendiği Abbott değerlerine göre dirençli çıkan *C. pipens* larvalarının olduğu bölgeler arasındaki var olabilecek bir farkı ortaya çıkarmak için bağımsız gruplar için Tek Yönlü Varyans Analizi (OWA) ve *B. sphaericus*'un etki değerinin 24. saatten 48. saate olan değişimin kıyası için Kovaryans Analizi (ANCOVA) uygulanmış, istatistiksel analizler arasındaki farklarda anlamlı ($p < 0,05$) bir fark olduğu ortaya çıkmıştır. Çoklu karşılaştırma testlerinden Tukey HSD ile Abbott 48 saatlik ölçümde, *B. sphaericus*'un etki değerleri arasında yalnızca bölge 1 ve bölge 6 da anlamlı bir fark olduğu, Post Hoc testi ile 24-48 saat değişiminde 4. bölgenin (Arnavutköy, Avcılar, Başakşehir, Sultangazi, and Küçükçekmece) diğer bölgelerden belirgin bir farklılığa sahip olduğu belirlenmiştir.

B. sphaericus'a karşı direnç gösteren *C. pipens* larvalarında, proteaz enziminin etkinliğine baktığımızda; *C. pipiens* larvalarında proteaz enzim etkinliğinin kontrol grubuna göre yüksek olması ile beraber Abbott çalışması ile elde edilen bulgular ile uyumlu değildir. Direnç için yaptığımız deneysel çalışmada *B. sphaericus*'a karşı direnç oluşumu ile proteaz etkinliği arasında bir ilişki olduğunu göstermekle birlikte, mekanizmayı açıklamak için genetik çeşitlilik ve mutasyon analizleri ile daha fazla bilgiye ulaşabileceği kanaatindeyiz. Sonuç olarak yaptığımız çalışmada elde ettiğimiz verilere göre bölgelerdeki direnç noktalarının 1. bölge olarak İstanbul'un şehir merkezinden, kırsal kesimlerine doğru azaldığı tespit edilmiştir. |

Ocak 2023, |130| sayfa.

Keywords: |*C. pipiens*, *B. sphaericus*, *B. thuringiensis subsp. israelensis*, direnç. |

1. INTRODUCTION

Mosquitoes, vectors for many diseases, are of great importance among pests in terms of health and economy since they are almost everywhere in the world, and the disease agents they carry bring deadly results (Robinson, 2006; Reegan et al., 2015). Therefore, the mosquito population should be maintained at a level that will cause minimal human disturbance. Insecticides were used to control mosquitoes, as fast and effective results were obtained first. It is necessary to reduce the use of insecticides and increase the use of biological agents, to increase the quality of the environment, to protect human health, to protect food safety and natural flora and fauna, and to prevent the development of resistance in mosquitoes (Prabakaran & Hoti, 2008). Recently *Bacillus sphaericus* and *Bacillus thuringiensis subsp. israelensis* species from Bacillus bacteria have been preferred in biological control because they are specific to the target organism, have high larvicidal activity, and are safe for non-target organisms, mammals, and the environment (Revathi et al., 2013; Zhuang et al., 2011; Panneerselvam et al., 2013).

Bacillus bacteria, *B. sphaericus* and *B. thuringiensis subsp. israelensis*, are used in biological control against mosquitoes in Turkey, and the formation of resistance against these bacteria has not been studied before. It has been reported in the literature that resistance develops in mosquito larvae against toxins of Bacillus genus bacteria, especially *B. sphaericus* species, which are used worldwide (Darboux et al., 2007; Singkhamanan et al., 2013; Wirth et al., 2010; Su et al., 2019).

In our study, we investigated the development of resistance by dividing 39 districts of Istanbul into 6 regions, collecting the dominant species of *C. pipiens* larvae (Polat et al., 2016) from mosquito breeding areas between July and September, and testing the toxin proteins of *B. sphaericus* and *B. thuringiensis subsp. israelensis*. Comprehensive data have been collected for future resistance studies, monitoring, and comparison of resistance.

The purpose of this work was determining any resistance of *C. pipiens* larvae, which were taken from different breeding sources and different districts, against toxin proteins of *B. sphaericus* and *B. thuringiensis subsp. israelensis* and produce the districts the resistance belong to and provide important contributions to the effectiveness of the control and to review the methods applied in mosquito control.

2. GENERAL PARTS

Pests are unwanted creatures that share the same ecosystem with their bad consequences on humans, animals, agricultural products, and the goods we use. The most common pests found in urban areas are cockroaches, mosquitoes, ants, houseflies, gray silverfishes, mites, termites, spiders, and carpenter bees (İnci et al., 2014). Pests can become vectors when they transmit many pathogens of parasites, bacteria, and virus origin mechanically or biologically from vertebrate host to another (İnci et al., 2009).

Various factors around the world affect vectors and the distribution and prevalence of human pathogens; climate change, international transportation, economic exchange, and globalization, diminishing resources, expanded exchanges, and urbanization, changes in the genetics of pathogens, uncontrolled human and animal movements (İnci et al., 2009), deforestation, agricultural intensification (Hagen et al., 2012) crowded living conditions and poor sanitation (Nkya et al., 2013; Robinson, 2006).

The World Health Organization (WHO) defines the major vector-borne global diseases as malaria, dengue fever, West Nile fever, Lyme disease, chikungunya, Zika virus disease, leishmaniasis, lymphatic filariasis, yellow fever, tick-borne encephalitis and African trypanosomiasis, Chagas disease, onchocerciasis, Japanese encephalitis and schistosomiasis (Rocklöv & Dubrow, 2020).

The WHO defines many global diseases in their reports: malaria, dengue fever, West Nile fever, Lyme disease, chikungunya, Zika virus disease, leishmaniasis, lymphatic filariasis, yellow fever, tick-borne encephalitis, and African trypanosomiasis, Chagas disease, onchocerciasis, Japanese encephalitis, and schistosomiasis are defined as vector-borne global diseases (WHO, 2014).

Mosquitoes are among one the most effective invasive species and they transmit endemic or exotic diseases they are the most important arthropods that affect human health globally (Rochlin et al., 2013). One of the most important diseases transmitted by mosquitoes is malaria. According to the WHO malaria report in 2021, 247 million patients and 620,000 deaths were reported. 95% of malaria cases and 95% of deaths are from Africa, with the highest fatality rate among vector-borne diseases. The second important disease transmitted by mosquitoes is

dengue fever, and the WHO estimates the number of dengue fever cases as 5.2 million in 2019, in the same report (WHO, 2016). More than a quarter billion cases and more than half a million deaths result from vector-borne diseases each year, accounting for about 17% of all infectious diseases (WHO, 2014).

Mosquitoes have also been declared “Public Enemy Number One” as they are important public health pests in humans and other vertebrates, vectoring medically important diseases such as malaria and dengue fever (Reegan et al., 2015; Robinson, 2006).

2.1. MOSQUITOES

Mosquitoes (Culicidae) are among the insects in the order Diptera. Many mosquito species are listed as vectors for parasites, including arboviruses and Plasmodium (Vargas et al., 2014). Mosquitoes have about 3500 species (Arjunan et al., 2014), and 50–60 species among them have been found around Turkey (Şeref Gün et al., 2011). The 3 main species of mosquito that transmit diseases; Aedes, Anopheles, and Culex. According to their disease transmit; Aedes carries dengue fever, chikungunya, yellow fever, Zika, lymphatic filariasis, and Rift Valley fever, Anopheles carries Malaria and lymphatic filariasis, and Culex carries Japanese encephalitis, lymphatic filariasis, and West Nile fever (WHO, 2014).

Since *C. pipiens* mosquitoes are dominant in all districts of Istanbul, they carry a risk for West Nile virus (WNV) (Polat et al., 2016). Although WNV is seen in the Mediterranean region and Southeastern Anatolia in Turkey, its incidence is very low (Tezcan et al., 2011). WNV infection was first seen and reported as an epidemic in 2010. Kalaycıoğlu et al. according to the surveillance findings reported by the company, 47 cases were diagnosed with WNV infection in 2010 and 2011, and it was reported internationally as an epidemic (Uyar & Bakir, 2016).

Mosquitoes are holometabolous with four stages of metamorphosis; egg, larva, nymph, and adult. In the first three stages of mosquitoes; eggs, larvae, and nymphs live in water, while in the last stage; adults live in terrestrial environments. In the second stage, larvae, feed on bacteria and plankton as their food sources. Female and male mosquitoes display different behaviors when their nutritional and dispersal abilities are considered. Female and male mosquitoes consume nectar and plant extracts. Moreover, they can hydrolyze sucrose, however, only females are hematophagous. Female mosquitoes are autogenic, therefore they need blood to complete their reproductive cycle (Minard et al., 2013).

Isoe investigated the mosquito blood meal in 2009. They dried a typical 2µl mosquito blood meal to measure the ingredients. They get around ~500 µg dry material and they determined that hemoglobin (~330 µg), serum albumin (~50 µg), and immunoglobulin (~15 µg) constitute ~80% of the protein of the blood meal. They also looked at the enzymes that process the blood meal. They show that, in the midgut lumen, proteolytic enzymes are responsible for degrading the proteins from the blood meal into oligopeptides and amino acids. As soon as the mosquito is fed, these enzymes are secreted into the midgut and resulting in oligopeptides and amino acids, which are used to form other carbon-based metabolites. They also mentioned that for the maternal energy of the gonotrophic cycle, the derived nutrients from proteins play important roles. ~25% of the derived nutrients are used for egg protein and egg lipid synthesis (Isoe et al., 2009).

Lucas et al., in 2015, looked into more details on the enzymes fed into the midgut for the blood meal's digestion. They determined two main classes; endoproteases; trypsin-like and chymotrypsin-like serine proteases (SPs) represent endoproteases, and exopeptidases, that function as amino and carboxy peptidases. They also notice that blood digestion acts in a biphasic fashion by using early and late-phase digestive proteases. It turns out that the transcriptional and post-transcriptional levels are specifically regulated (Lucas et al., 2015).

Vadakkadath Meethal, in 2020, reported from various studies “Yang & Davies, 1971, Vadakkadath Meethal, 2018” that the process of the digestion of food includes a predominant enzyme in the Lepidopteran and Dipteran gut, serine proteases, that act in alkaline pH. These proteases work as important processes in many places such as in digestion, oogenesis, and metamorphosis. Targeting the major protease of the larvae's gut, that is the serine proteases, can lead to new methods for controlling the field populations (Vadakkadath Meethal, 2020).

Protease activity can be prevented with special molecules, termed protease inhibitors, that target the active or allosteric sites of proteases or their zymogens by binding to them. The action of the protease inhibitors interferes with the synthesis and secretion of midgut proteases. As a result, physiological function, growth, and development are prevented (Vadakkadath Meethal, 2018; Zhao et al., 2019). Vadakkadath Meethal, 2020, mentioned that to prevent the activity of the protease, small molecules, synthetic or naturally occurring plant protease inhibitors, or blockers of the synthesis of the protease can be used (Vadakkadath Meethal, 2020).

2.1.1. Characteristics of *C. pipiens* Larva

When we look at the adult and larval characteristics of the *C. pipiens* mosquitoes that make up the mosquito fauna of Istanbul; the *Culex sp.* larva is shown in Figure 2.1.

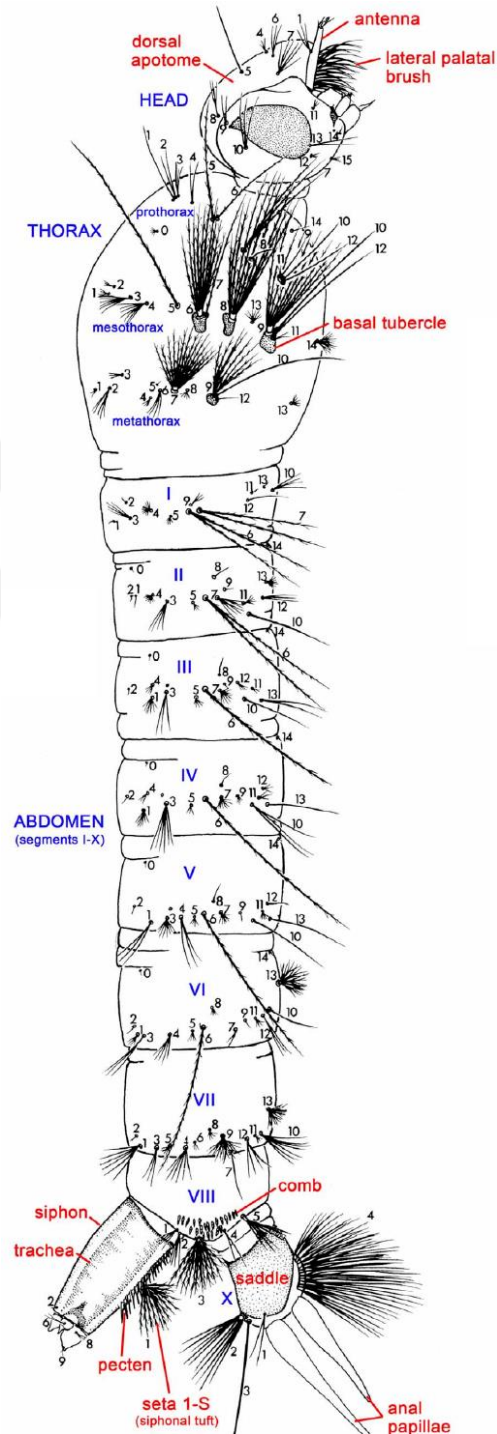


Figure 2.1: Characters of *C. pipiens* larva (Azari-Hamidian & Harbach, 2009).

The siphon is thin and tapers regularly toward the apex. The number of pecten teeth ranges from 13 to 17 and they are equally distributed up to seta 1a-S. 1a-S is usually 2-branched or single, with the size of the bottom width of the siphon or longer. Each pecten tooth is distinguished by a long pointed tip and three lateral denticles. The four far-spaced 2 pairs of branched hairs emerging from distal to the pecten in an irregular row form the siphonal tuft (1-S). It has two long anal papilla and dorsal pairs almost twice the size of the saddle, Figure 2.2, (Becker et al., 2010).

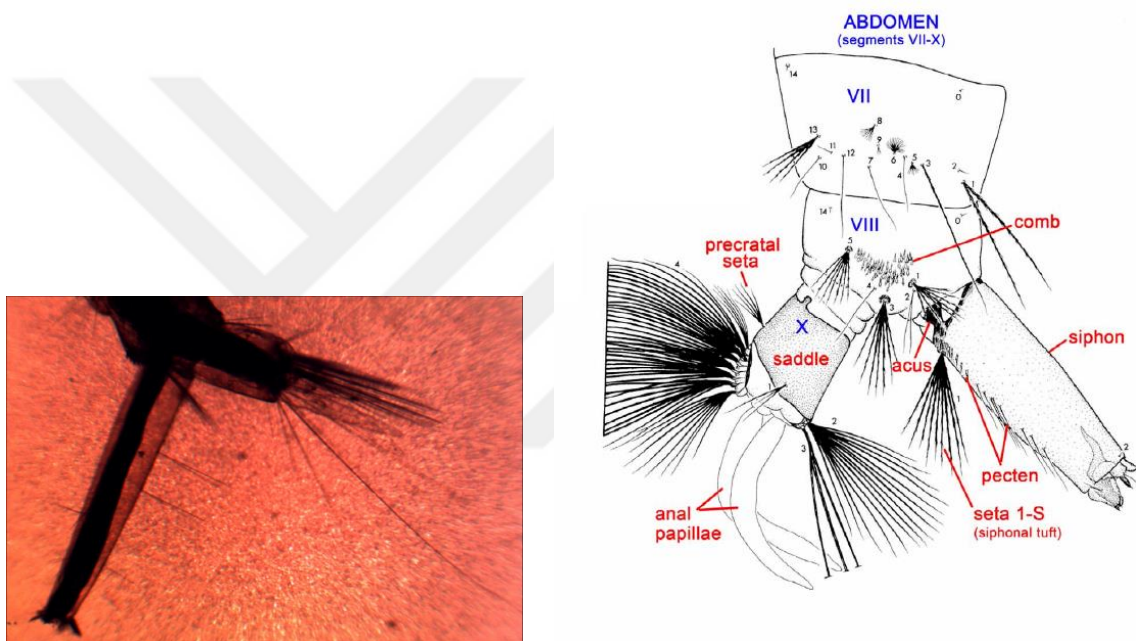


Figure 2.2: Lateral aspects of the terminal abdominal segments of a *C. pipiens* larva (Azari-Hamidian & Harbach, 2009).

2.1.2. Characteristics of *C. pipiens* Adults

Adults are 3–9 mm long with scales on their bodies and wings and have long, thin proboscis (Robinson, 2006). There are brown scales on their head. There are usually pale scales on the lateral aspect of the head and posterior margins of the eyes. They have dark antennae. A few white scales can be found in Pedicel and flagellomere I. The proboscis was brown with cream-colored scales ventrally. The female's palps were covered with brown scales. Sometimes, the middle of the apex part contains pale scales.

The mesonotum (includes the prescutum, scutum, scutellum, and postnotum) is covered with reddish-brown or yellowish-brown scales. The lateral margins of the mesonotum and the region preceding the scutellum are covered with paler scales. The scutellum has narrow, light yellow scales and dark setae. Postspiracular and prealar scales are absent, rarely a few scales.

Coxa is a small dark patch of dark scales. The femurs are yellowish at the apical border, the rest are covered with dark scales. The tibia and tarsus are dark, and the hind leg femurs are mostly covered with pale scales.

The abdominal tergum often has dark scales. Tergum II has a small base-median whitish spot. Between tergum III and tergum VII, there are narrow basal bands of pale or yellowish color that expand laterally. Sternum covered with yellowish scales, Figure 2.3

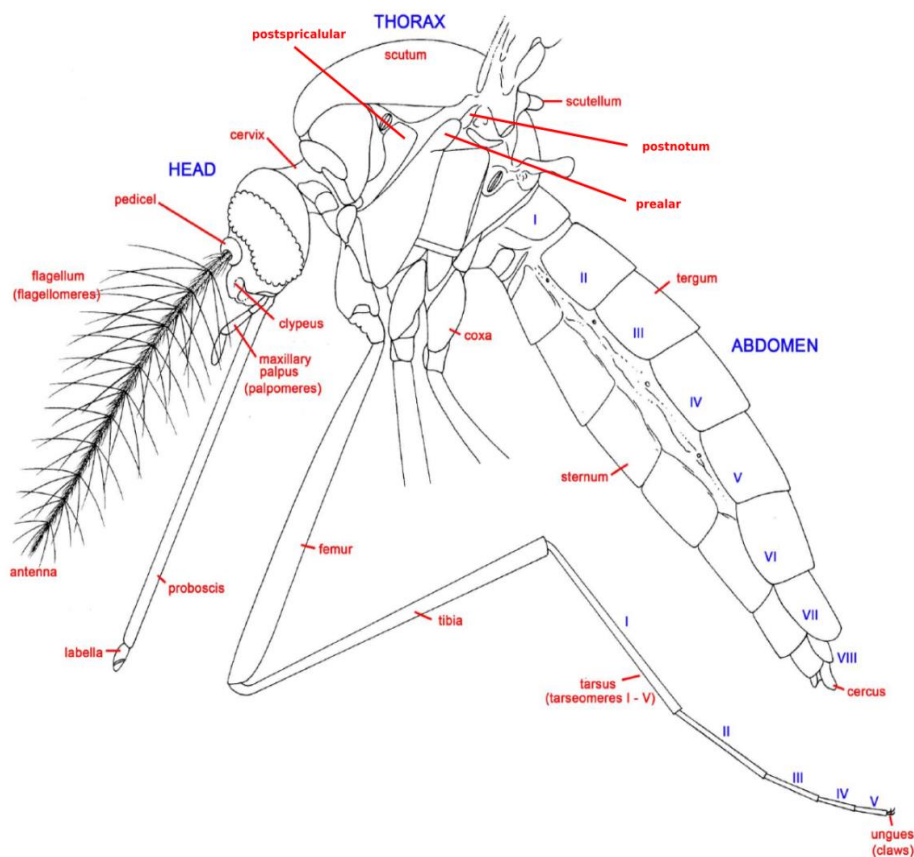


Figure 2.3: Characters of adult mosquitoes *C. pipiens* (Azari-Hamidian & Harbach, 2009).

The head part is broad, the antenna part is shorter than the head part. 1-P to 3-P prothoracic hairs are long and single. 4-P has 2 branches and are shorter than other hairs, 5-P and 6-P are

single-branched and long; 7-P 2 is branched and long. Metathoracic seta 1-T is shorter than half of the 2-T. The number of comb scales is about 40, each scale is short and even, expanding at the apex. The siphon index ranges from 4.8 to 5.0 and is thin (Becker et al., 2010).

The scales on the wings are dark in color. The subcostal vein cuts the costal vein beyond the R2+3 bifurcation line, Figure 2.4, (Öter, 2007).

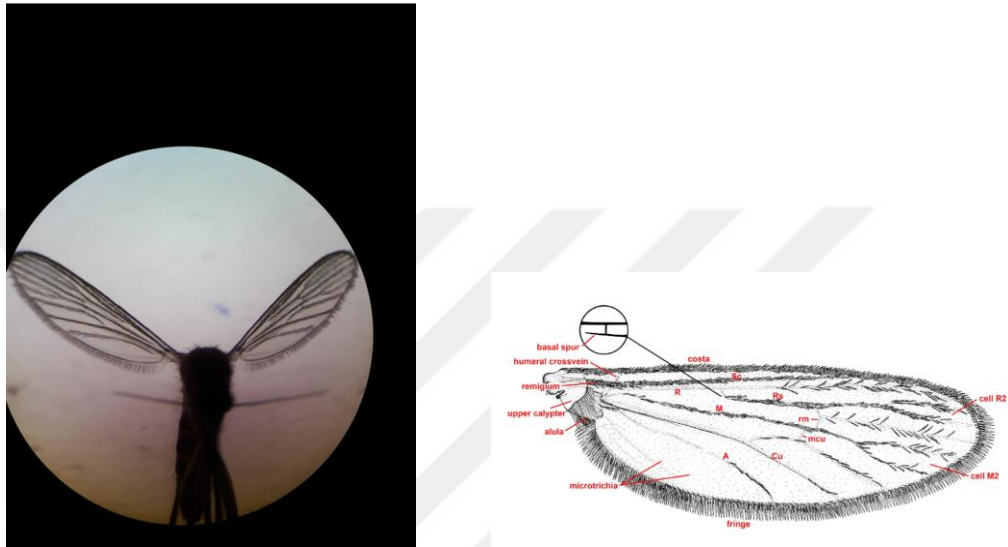


Figure 2.4: Characters of the mosquito *C. pipiens* wing (Azari-Hamidian & Harbach, 2009).

When a mosquito bites, it causes systemic and allergic reactions such as angioedema, local skin reactions, and urticaria (Revathi et al., 2013), inflammation of the mucous membrane, tissue necrosis, low blood pressure, fever, and loss of consciousness. Mosquitoes can also have significant health effects on wildlife, livestock, and pets including dogs, birds, horses, and wild cattle (Rey et al., 2012).

They can transmit a wide variety of pathogens, from viruses to parasites, to their hosts during their blood feeding (Calzolari, 2016). Arboviruses can be transmitted to a vertebrate host by the vectoring of a mosquito through two different mechanisms, mechanical or biological transmission: Mechanical transmission occurs by direct contact of the contaminated mouth parts of the arthropod vector with the vertebrate host, so it does not require the amplification of the virus in the vector. Biological contamination requires direct replication of the virus in mosquito tissue before transmission (Kenney & Brault, 2014).

Mosquitoes; live in streams, lakes, reeds, and marshes, windless and secluded places (Polat et al., 2016). Mosquitoes with their special adaptational mechanisms are capable of thriving in almost every kind of environment. It seldom to find an aquatic habitat that doesn't host mosquitoes for breeding in the World (Becker et al., 2010). Habitat types include very dirty to clean, temporary and permanent, large and small water bodies, pools, streams, ponds (less than 50 m² water area), ponds (more than 50 m² water area), water tanks, tire, and tire tracks, animal footprints, drainage, water log, gutters, open channels, household containers, and tree hollows, leaf seat buckets filled with water, flower vases, and nail marks even the smallest savings such as are potential resources (Becker et al., 2010; Bashar et al., 2016; Adeleke et al., 2008).

The metabolic processes of mosquitoes are dependent on environmental conditions such as temperature and hydrology. The mosquito density tends to increase as temperature increases. While concerns about global warming and the potential increase in mosquito-vector diseases are growing, nowhere in the world is immune to this risk and effective vaccines are not available (Arjunan et al., 2014). Tropical regions are known to be more vulnerable to the risk of mosquito-borne diseases due to climate change and intensifying globalization (Reegan et al., 2015). It has an economic impact, including the loss in commercial and labor output, especially in countries with such warm climates; (Yogananth et al., 2015) as well as disturbing pests in and around human habitats cause enormous suffering and hamper poverty reduction and restrict economic development (Matthews et al., 2011).

Each mosquito genus can find its preferred ecological preference and habitat. Mosquitoes exhibit certain rhythmic behavior patterns throughout their life cycle. For example, most *Anopheles* and *Culex* species bite at night, while some *Aedes* species bite during the day. While *Culex* and *Aedes* mosquitoes are found in water rich in organic matter, *Anopheles* mosquitoes usually live in clear water with sunlight (Minard et al., 2013).

Mosquitoes have a high reproduction rate and a relatively short life cycle, that is, the characteristics of typical r-strategists (Becker et al., 2010). Mosquito control emphasizes the need for selective and sustainable preventive measures for decreasing disease transmission. Today's vector challenge mainly includes chemical, biological, natural plant products, and environmental management (Singh & Prakash, 2012).

2.2. MOSQUITO CONTROL

2.2.1. Chemical Struggle

In the beginning, chemical-based controls were used against mosquitoes, rapid results were obtained through the harms were not known much. Later it is understood that chemical insecticides pose some dangers such as contamination of water and food resources, non-target fauna and flora poisoning, food chain condensation, and selection of resistant insect pest populations (Ramrez-Lepe & Ramrez-Suero, 2012).

2.2.1.1. *Organochlorine Pesticides*

Chemical control of mosquitoes was initially with organochlorine (OC) compounds. Organochlorine pesticides (POPs) have been used in agriculture and public health worldwide since the 1940s. They are preferred since their strong impact on pests and disease control (Wang et al., 2015). The compounds with OC are compounds that act on the central nervous system and their mechanism of action can vary according to the structure of the insecticide. Dichlorodiphenyltrichloroethane (DDT) changes the permeability of sodium and potassium channels in the nerve membrane of the insect, causing excessive nerve stimulation by providing slow closure of voltage-dependent sodium channels (Özkaya et al., 2013).

Other examples of OC insecticides are chlordane, dieldrin, lindane, mirex, hexachlorobenzene, dicofol, heptachlor, endosulfan, chlordecone, and toxaphenes (van Dyk & Pletschke, 2011). The production, the intensive agricultural and industrial use of permanent OCTs, however, have caused widespread environmental pollution. As a result of their low degradation and high organic solubility, they readily enter the food chain as contaminants. Its residues and metabolites continued to be frequently detected in freshwater soils, fish, sediments, and even the human body in recent years (Wang et al., 2015).

According to the Stockholm Convention on persistent organic pollutants (POPs), which entered into force in 2004, the contractual parties require the elimination or reduction of the release of organochlorine pesticides to the environment. Some of these pesticides, such as DDT, are still allowed in certain applications such as vector control, and can therefore continue to pollute the environment (van Dyk & Pletschke, 2011). These compounds may cause both animal

reproduction, development, and immune system, as well as carcinogenic/mutagenic potential (Wang et al., 2015).

2.2.1.2. Organophosphorus Compounds (OPs)

OPs were introduced in the 1940s and 1950s and methylcarbamates (MCs) were introduced in the 1960s to control insect pests in crops and to address other gaps in pest management. PO and MC function as acetylcholinesterase (AChE) or anticholinesterase inhibitors. These were later named nerve agents specifically for their toxic effects on the nervous system (Elsinghorst et al., 2015). AChE inhibitors include malathion, parathion, methyl parathion, tetrachlorvinphos, triazophos, phosmet, chlorpyrifos, dichlorvos, oxydemeton, azinfos methyl, and diazinon (van Dyk & Pletschke, 2011).

The presence of this enzyme in insects, fish, birds, and all mammals means that this class of pesticides is extremely toxic to undesirable targets (van Dyk & Pletschke, 2011). OPs are relatively unstable compounds that break down in the environment within weeks or months (Soloneski et al., 2015). Recent studies provide evidence that OPs exposure induces oxidative stress, a condition associated with common diseases such as cardiovascular disease and diabetes. Despite all these features, OPs and MCs are at the top of commercial insecticides (Casida & Durkin, 2013).

2.2.1.3. Pyrethroids

A class of synthetic insecticides whose chemical structure consists of naturally occurring pyrethrins found in *Chrysanthemum cineraraefolium* flowers. Gradual changes have been made to increase their insecticidal potential and reduce their sensitivity to air and light (Saillenfait et al., 2015). Pyrethroids are widely used in mosquito control programs to kill various insects and adult mosquitoes (Sarikaya & Memmi, 2013).

The primary target of pyrethroids in mammals and insects is the nervous system. Pyrethroids are generally classified into two groups of acute neurobehavioral effects in rodents, depending on the absence (Type I; permethrin, phenothrin, cismethrin, pyrethrin, tetramethrin, and bioallethrin) or the presence (Type II; deltamethrin, fenvalerate, cypermethrin) and cyano-group at the α -carbon of the alcohol moiety) are separated. Regardless of group or species, they act primarily by disrupting sodium channels in axons. In laboratory animals, Type I pyrethroids

compounds exhibit acute oral intoxication, the most common symptoms being over-arousal and whole-body tremors, while Type II pyrethroids cause choreoathetosis and salivation (Saillenfait et al., 2015; Özkaya et al., 2013).

Pyrethroids constitute major insecticides in residential, forestry, agricultural, and horticultural environments worldwide. Humans are exposed to pyrethroid insecticide by mouth, inhalation, and skin. Previous studies have revealed that metabolites of pyrethroid insecticides are detected in urine samples from unexposed general populations (Zhang et al., 2013). The US EPA (2009) classifies permethrin as "likely to be carcinogenic to humans" when taken orally (Saillenfait et al., 2015).

2.2.1.4. Insect Growth Regulators (IGRs)

Insect growth regulators (IGRs) are new insecticides that interfere with the molting and metamorphosis process. The two main target processes specific to insects are the biosynthesis of chitin in the cuticle and the activity of hormones such as juvenile hormone and insect molting hormone 20-hydroxyecdysone. In recent years, several IGRs have been developed, such as citine synthesis inhibitors (eg, flufenoxuran and diflubenzuron), juvenile hormone analogs (eg, pyriproxyfen), ecdysone agonists (eg, halofenozide), and azadirachtin-based products (eg, neem) (Sadeghi et al., 2009). Chitin synthesis inhibitors affect insects longer than hormonal IGRs. They also move faster but can affect predatory insects, arthropods, and even fish (Sarwar, 2020).

Mosquito control programs have been disrupted in recent years because mosquitoes develop resistance to synthetic chemical insecticides such as organochlorides, organophosphates, and carbamates, and insect growth regulators such as pyriproxyfen, methoprene, and diflubenzuron. Moreover, many organophosphates and organochlorides adversely affect the environment and damage biological systems (Reegan et al., 2015).

2.2.2. Environmental Management

The environmental alteration involves a physical change in potential mosquito breeding sites designed to prevent, eliminate or reduce vector habitat. Basic methods of achieving these changes include drainage, land leveling, and backfilling. Drainage operations involve creating ditches or channels to keep water moving and to remove water used as breeding grounds by

mosquitoes in a controlled manner. Remodeling projects may include straightening river banks, creating channels to increase water flow in stagnant water areas, filling small ponds or filling water collection pits, or replacing ponds to reduce mosquito populations. In some cases, marshes were drained by pumping (Mokuolu, 2016).

Physical control covers a wide range of techniques. From the use of oil in the early days of mosquito control to the use of monolayers, polystyrene beads, traps, and now lasers, the technology continues to evolve. Cemeteries are potentially important for mosquito production due to their location in urban areas and the use of many small artificial water containers. Mosquito production can be prevented by removing or following the bird drinkers (Becker et al., 2010).

When mosquitoes are active, people who are obligated to be outdoors should wear protective clothing that covers their body to protect against mosquito bites or use a variety of approved, usable, sprayable lotions, creams, and mosquito repellent products. Mosquito nets are preferred in regions where economic conditions are not sufficient because they can be obtained at a small cost compared to other protection methods (Özbel, 2017).

2.2.3. Genetic Control of Mosquitoes

Gene drivers are ideal for vector control, as the short generation times of insects allow them to propagate to fixation very quickly even if they are released from a low initial frequency. A gene driver can be designed to reduce the spread of the reproductive potential of an insect vector population. Vector-borne diseases can be eradicated if a sufficiently high proportion of the natural vector population is vaccinated or the number of insects is lowered below the threshold required to sustain disease transmission (Hammond & Galizi, 2017).

Sterile Insect Technique (SIT) is a species-specific and environmentally friendly method for controlling the insect population, radiation-mediated sterilization, based on mass rearing, and the release of large numbers of male insects. The release of insects carrying a dominant lethal gene (RIDL) offers a solution to the many drawbacks of traditional SIT while limiting its application in mosquitoes while maintaining its eco-friendly and species-specific benefit. Many decisions regarding biological security, cultural, social, and ethical aspects will need to be made at national, regional, and international levels for these vector control methods (Wilke & Marrelli, 2012).

2.2.4. Biological Control

Biopesticides, in the broadest definition, are living organisms that suppress pest populations or natural products derived from these organisms. The product considered a biopesticide can be quite diverse, such as a variety of microorganisms, including species, nematodes, plants, bacteria, viruses, and fungi (Singh & Prakash, 2012). Biological control aims to reduce the target population to an "acceptable" level and at the same time avoid negative impacts on the ecosystem (Becker et al., 2010).

More than 50 entomopathogenic bacteria, viruses, fungi, and nematodes are currently being commercially produced and increasingly used as microbial pesticides. On a global scale, microbial pesticides only make up about 1-2% of all pesticides sold; however, they have shown long-term growth over the last decade, unlike chemical pesticides whose use is constantly declining in the global market (Lacey et al., 2015).

Various microorganisms such as fungi (*Lagenidium*, *Coelomomyces*, *Entomophthora*, *Culicinomyces*, *Beauveria*, *B. bassiana*, and *Metarhizium*), viruses (*Baculoviridae* and *Reoviridae* and *Iridioviridae* and *Parvoviridae*) (Becker et al., 2010; İnci et al., 2014), (nuclear polyhidrosis (NPV), cytoplasmic polyhidrosis (CPV) and *granulosis virus* (GV) (Robinson, 2006), protozoans, (*Nosema* and *Vavraia*, *Amblyospora* and *Parathelohania* (Becker et al., 2010), Nematodes, (*Romanomermis culicivorax*) (İnci et al., 2014; Robinson, 2006), (*Steinernema spp.*, *Neosteinerinema spp.*), (*Heterorhabditis spp.*), and *X. luminescens* (Robinson, 2006) are used in mosquito control.

Other than these; in the biological control of larvae, fish (larvivorous fish *Gambusia affinis*), (Cote), insects (dragonflies, cockroaches) toxorhynchites of the genus of mosquitoes, neem oil obtained from *Azadirachta indica* tree, small crustaceans such as copepod and cyclopoid in water, some organisms such as the fern plant *Azolla*, are used directly to cover the surface of the water and prevent the development of mosquitoes (Yarsan & Çevik, 2007).

Bacillus spp., *Saccharopolyspora spinosa* (Arjunan et al., 2014), *Clostridium bifermentans* (Geetha et al., 2011), *Pseudomonas aeruginosa*, *Bacillus polymyxa*, *Bacillus subtilis* (Omoya & Akinyosoye, 2011), *Streptomyces citreofluorescens* (Singh & Prakash, 2012), *Serratia sp.*

(Patil et al., 2011), *B. alvei* and *B. brevis*, *Brevibacillus laterosporus*, *B. circulans* and *B. subtilis* (Geetha et al., 2011) bacteria are also used in mosquito control.

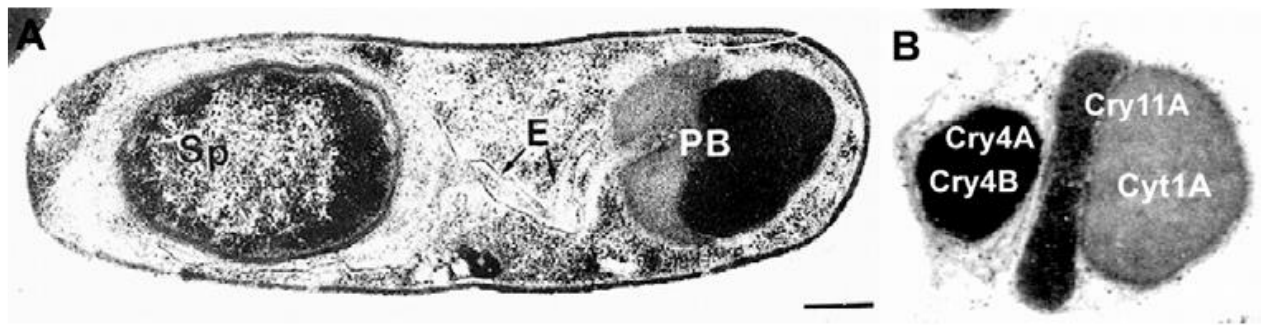
2.3. USAGE PLACES AND ADVANTAGES OF *B. THURINGIENSIS* SUBSP. ISRAELENIS AND *B. SPHAERICUS*

Mosquitoes breed in water, and it is easier to control larval-stage mosquitoes than adult mosquitoes that are widely distributed in the habitat (Becker et al., 2010). Bacterial biopesticides are considered ideal for mosquito control, that high mosquito larvicidal activity, ease of production, cost-effectiveness, safety to non-target organisms and mammals, amenability to a variety of formulations, (Prabakaran & Hoti, 2008), low environmental impact, biodegradability, (Foda et al., 2010), effective against mosquitoes at very low dosages (Revathi et al., 2013).

B. thuringiensis subsp. israelensis and *B. sphaericus* are highly effective against mosquito larvae (Patil et al., 2011). *B. sphaericus* formulations are contaminated water media, it is more effective and has a longer residual activity than *B. thuringiensis subsp. israelensis* formulations (Zhuang et al., 2011).

2.3.1. *B. Thuringiensis* Subsp. *Israelensis*

B. thuringiensis is a generally rod-shaped, Gram-positive, spore-forming aerobic bacterium. (Palma et al., 2014). *B. thuringiensis* produces an oval spore (Figure 2.5) and is easily isolated and produced on basic media such as nutrient agar (Park et al., 2010; Federici, 2013). *B. thuringiensis* has been isolated from a wide variety of environmental sources, including water, soil, grain dust, plant surfaces, dead bodies, insects, insectivorous mammals, insect excreta, trees, leaves, various conifers, and necrotic human tissues (Palma et al., 2014; Federici, 2013).



B: Spore

E: Exosporium

PB: Parasporal body

Figure 2.5: *B. thuringiensis subsp. israelensis* cell (Federici, 2013).

B. thuringiensis subsp. israelensis was first isolated from dead *C. pipiens* larvae by Goldberg and Margalit (1977). *B. thuringiensis* strains are currently classified according to flagellar (H) antigens (Elleuch et al., 2015). There are 34 recognized subspecies of *B. thuringiensis*. Some of the most commonly used include *kurstaki* (against Lepidoptera), *israelensis* (against mosquitoes and houseflies), and *tenebrionis* (against *Leptino tarsade celineata*, Colorado potato beetle) (Poopathi & Abidha, 2010).

B. thuringiensis synthesizes strains, also known as crystal (Cry) and cytolytic (Cyt) endotoxins, as parasporal crystalline inclusions at the initiation of sporulation and the stationary growth phase (Palma et al., 2014). *B. thuringiensis subsp. israelensis* crystals consist of a combination of four delta-endotoxins, Figure 2.6. (encoded by the *cry4Aa*, *cry4Ba*, *cry10Aa*, and *cry11Aa* genes) and two hemolytic factors (*cyt1Aa* and *cyt2Ba*) with synergistic interactions of six major proteins. These toxin protein genes are mainly located on the large conjugative plasmid: pBtoxis (Elleuch et al., 2015). Cry genes produce 134, 128, 78, and 72 kDa polypeptides, respectively, all of which have higher larvicidal activity than Cyt proteins (Florez et al., 2018).

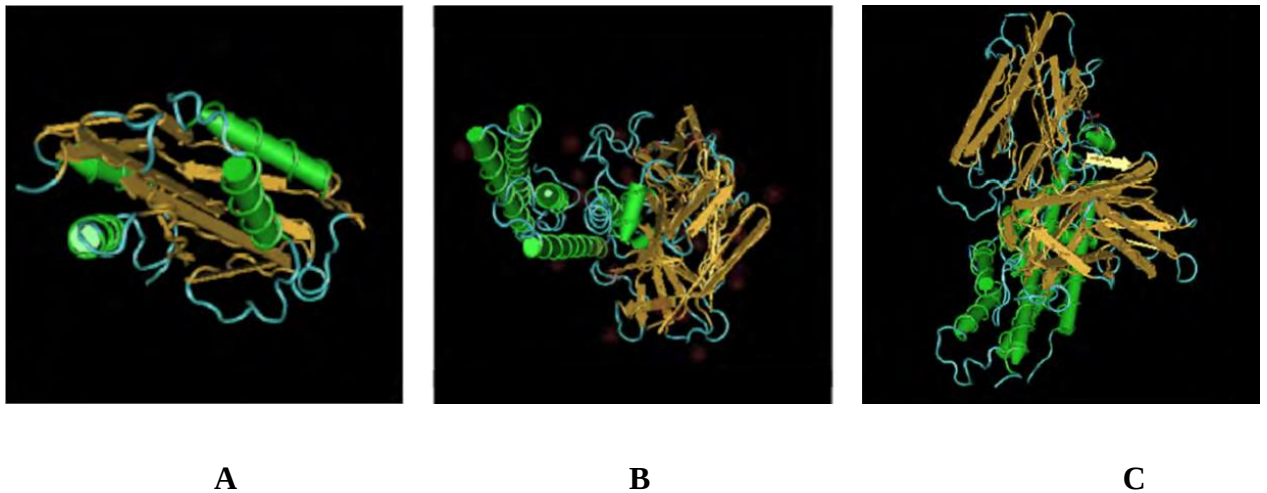


Figure 2.6: 3D Structure of Bacillus Cry proteins; A: B: Cry4Aa, C: Cry4Ba (Laurence et al., 2011).

B. thuringiensis subsp. israelensis proteins are different from each other and cause toxicity present between different mosquito breeds. For example, Cry4Aa is toxic to *Aedes* and *Culex* sp. larvae but not to *Anopheles*; Cry11Aa is highly toxic to mosquito species in *Culex*, *Aedes*, and *Anopheles* genera (Zhang et al., 2017). Cyt1Aa, on the other hand, despite its low toxicity, is highly synergistic with Cry toxins and helps overcome resistance to Cry toxins in mosquitoes (Florez et al., 2018).

Cry and Cyt toxins belong to a class of bacterial toxins that undergo conformational changes to enter the membrane of their host, known as Pore-Forming Toxins (PFT), and secreted as water-soluble proteins (Bravo et al., 2011). Cyt1Aa binds with high affinity to unsaturated phospholipids, while Cry's binds to membrane receptors (Ben-Dov, 2014; Bravo et al., 2011). Cry toxins belong to the three-domain Cry toxin family and they show clear differences in their amino acid sequences, but they are all quite similar and have a conserved three-domain structure (Palma et al., 2014).

Domain I or perforating domain consists of seven α -helix clusters located toward the N-terminal, which undergo proteolytic cleavage in all of the three-domain Cry proteins during toxin activation, and this may be responsible for toxin membrane insertion and pore formation. Domain II (central or middle domain) consists of three antiparallel β -sheets and is critical to toxin-receptor interactions. Domain III (galactose binding domain) is two antiparallel β -sheet sandwiches that are also involved in receptor binding and pore formation (Figure 2.7) (Palma et al., 2014).

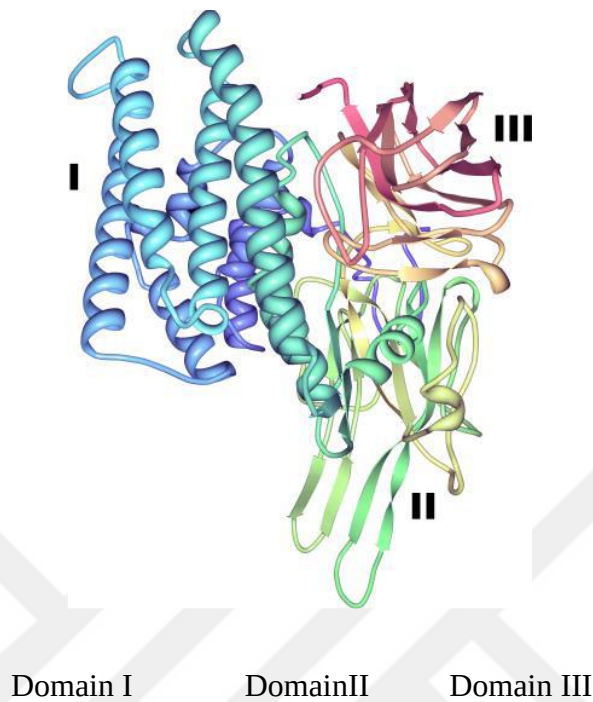


Figure 2.7: Three-dimensional structure of the Cry2Aa toxin (Palma et al., 2014).

The insecticidal process of *B. thuringiensis* delta-endotoxins begins with crystal inclusion dissolution in digestive juices under alkaline conditions in the midgut lumen. Solubilization produces active protoxin that must be activated by intestinal proteases to be harmful to insect larvae (Figure 2.8). Proteolytic cleavage occurs at both the C- and N-terminal protein end. Active Cry toxin binds to specific receptors in the larval gut, leading to membrane penetration. Later, cation-selective channels were created that caused osmotic lysis and death of larval midgut epithelial cells (Elleuch et al., 2015).

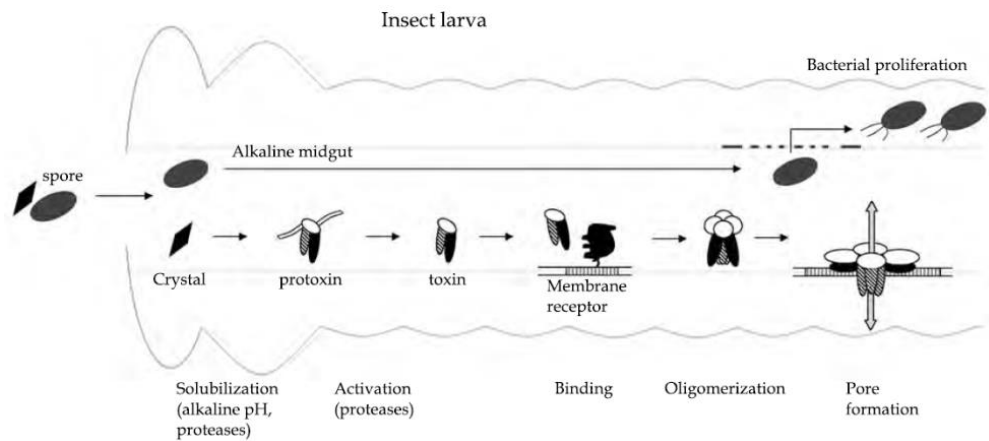
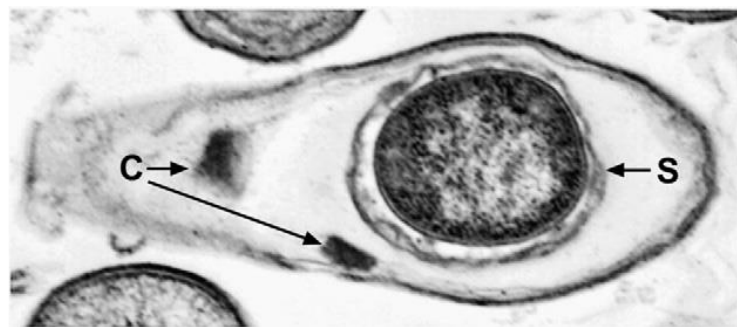


Figure 2.8: Binding of *B. thuringiensis* delta-endotoxin to a receptor protein in the midgut larva (Laurence et al., 2011).

2.3.2. *B. Sphaericus*

B. sphaericus is a rod-shaped, aerobic, endospore-forming Gram-positive bacterium (Poopathi & Abidha, 2010) that is found in various soil and aquatic habitats (Wirth et al., 2010). It produces a swollen spherical spore at one end of the sporangium, Figure 2.9. They can be easily distinguished from other *Bacillus* species (Park et al., 2010).



C: BinAB crystal

S: spore

Figure 2.9: Image of *B. sphaericus* 2362 in transmission electron micrograph (Park et al., 2010).

The *B. sphaericus* strain, which is toxic to mosquito larvae, was first described by Kellen et al. (1965) and later, more than 300 strains from worldwide have been isolated and identified (Poopathi & Abidha, 2010). Among them, strains 2362, 2297, 1597, C3-41, and IAB-59 have high larvicide activity. *Bacillus sphaericus* Neide (2362) (*Lysinibacillus sphaericus* Meyer and Neide) was found to be quite successful in combating *Culex* sp. (Su et al., 2019).

B. sphaericus strains produce two groups of proteins: binary toxin (Bin) and mosquitocidal toxin (Mtx). The Mtx protein consists of 3 proteins, 100-kDa Mtx1, 31.8-kDa Mtx2, and 35.8-kDa Mtx3 (Phannachet et al., 2010). Mtx protein contributes less to larvicidal activity while helping against the development of resistance to binary toxins (Su et al., 2019). The dual toxin is highly active against *Culex sp.*, moderately for *Anopheles sp.*, and less so or non-toxic for *Aedes sp.* (Surya et al., 2016).

The binary toxins produce parasporal inclusions during sporulation crystal binary toxins consist of 42-kDa BinA and 51-kDa BinB proteins, (Su et al., 2019) and both are required at equimolar amounts to exert maximal biological activity. After ingestion by susceptible mosquito larvae, BinA and BinB inclusions are solubilized under alkaline conditions in the larval gut. This is followed by activation by gut proteases, producing the 39 and 43-kDa active forms of BinA and BinB, respectively (Limpanawat et al., 2009). The bin operons are known to be located on chromosomal DNA (Park et al., 2010).

The active form of BinB is responsible for the host specificity, and larval toxicity occurs in synergy with the BinA protein (Phannachet et al., 2010). There is evidence to suggest that it is toxic to *Culex sp.* larvae when high doses of BinA protein alone are administered, suggesting that Bin A is a toxic subunit (Limpanawat et al., 2009).

Toxicity occurs by specific binding of the binary toxin to the apical membrane of the midgut epithelial cells of the larva, Figure 2.10. The BinB component acts as the primary binding component and directs the local binding of BinA (Limpanawat et al., 2009). The Bin toxin receptor in the midgut of the *C. pipiens* larva is a 60 kDa glucosidase, Figure 2.11, (Wirth et al., 2010) that binds to the epithelial cell membrane by a glycol anchor, and was identified as GPI (glycosyl-phosphatidylinositol)-linked α -glucosidase Cpm1 (Limpanawat et al., 2009). Because this receptor is similar to α -glucosidases and other maltase-like proteins, it was named Cpm1 for *C. pipiens* maltase 1 (Darboux et al., 2002).

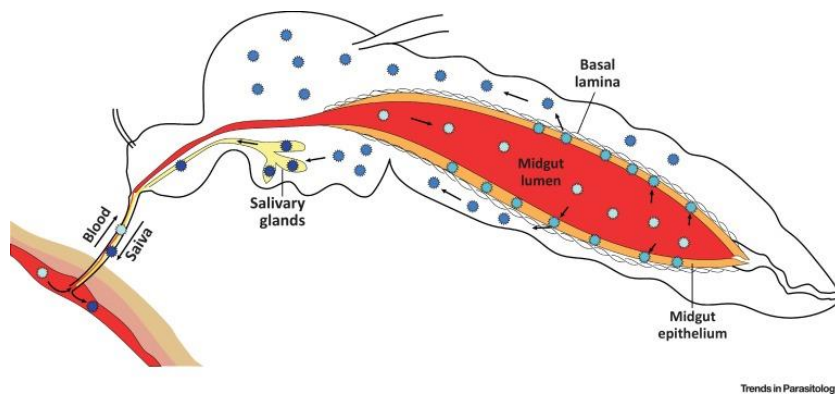


Figure 2.10: Structure of the digestive system of the larva (Rückert & Ebel, 2018).

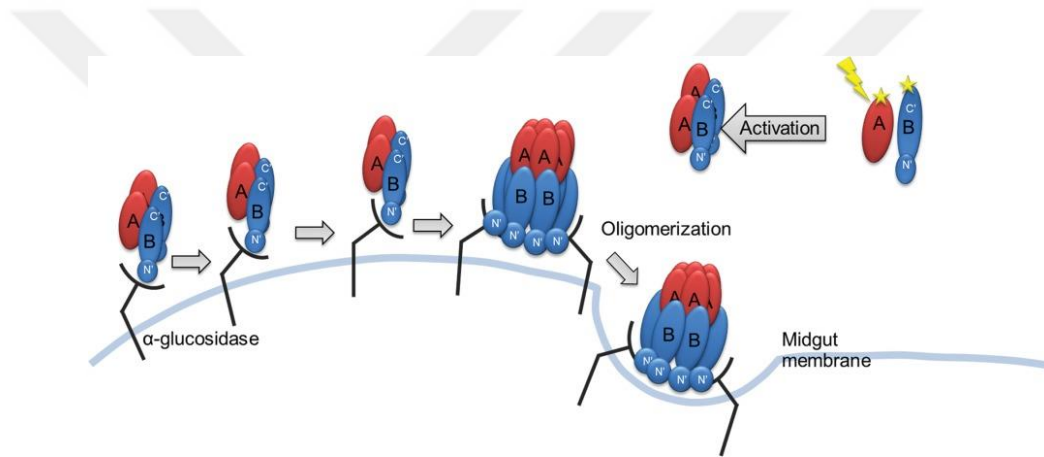


Figure 2.11: α - Glucosidase activity (Srisucharitpanit et al., 2014).

When the X-ray crystal structures (Figure 2.12) of the BinA and BinB proteins were resolved, it was observed that the amino acid sequences of BinA and BinB were highly homologous, with a similar folding structure and 25% identity and 40% identity. The N-terminal domain (trefoil domain) is highly protected against some sugar-binding proteins, while the C-terminal domain (β -sheet domain) is similar to aerolysin toxins, β -pore-forming toxins (Tharad et al., 2020).

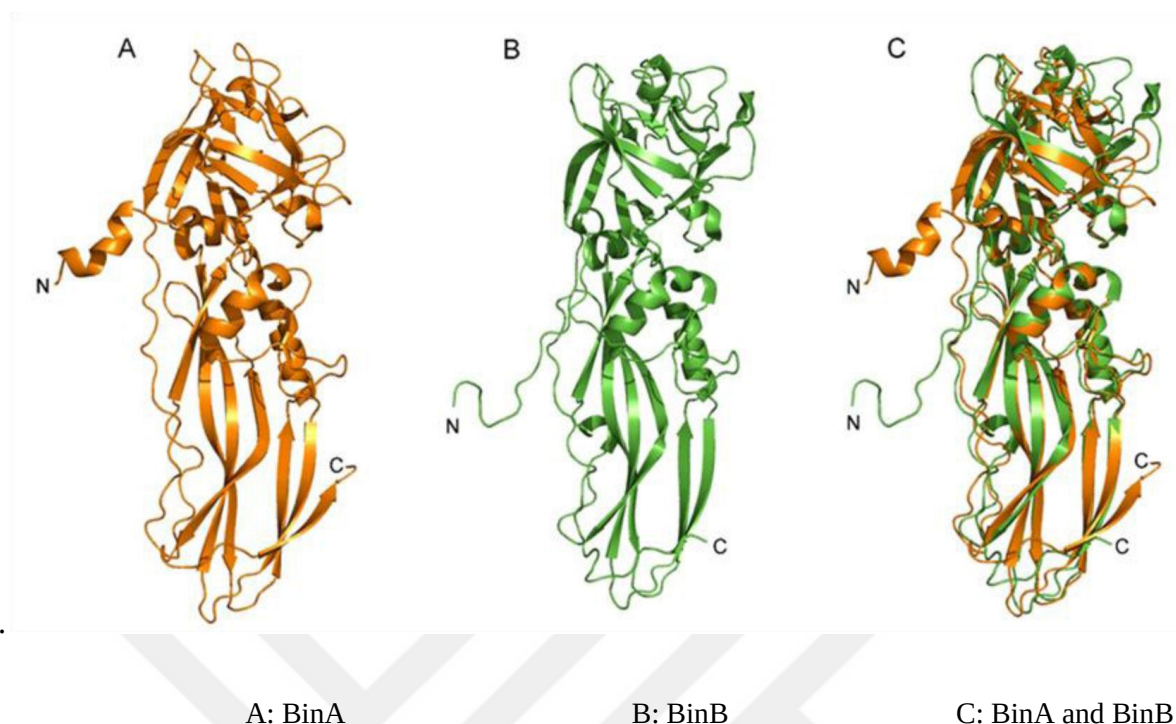


Figure 2.12: Three-dimensional crystal structure of Binary toxins (Tharad et al., 2020).

Boonyos et al. suggested that binary toxin produced by *B. sphaericus* exists as a heterotetramer consisting of 2 molecules each BinA and BinB. Studies on BinB deletion derivatives suggested that the N-terminal region is important for receptor binding, whereas the C-terminal region is responsible for the formation of the BinA-BinB complex, thus redirecting BinA to the target cells. In the same report, it was suggested that both N and C- terminal regions of BinA are important for BinA-BinB interaction (Boonyos et al., 2010).

Site-directed mutagenesis of amino acid positions 93, 99, and 104 of BinA revealed that these residues are crucial for toxicity and it was proposed that residues in this region might play a role in the formation of the BinA-BinB complex. BinA contains three conserved cysteine residues at positions 31, 47, and 195, which are required for full toxicity of the binary toxin. BinB also contains three conserved cysteine residues in the active core at positions 67, 161, and 241. Here, it was shown that C67 and C161 are crucial for the toxicity of binary toxins, possibly involved in BinA-BinB interaction (Boonyos et al., 2010).

Toxins bind to the microvilli in the midgut epithelial cells of the larvae, causing hypertrophy and lysis of the cells and ultimately the death of the larva (Federici, 2013). Mitochondrial

damage has also been noted as a prominent effect of Bin toxin, and they have recently been shown to be related to the activation of the intrinsic apoptosis pathway (Rezende et al., 2019).

Culex sp. larvae lacking or mutating the Cpm1 receptor are toxin-resistant, indicating that receptor recognition and receptor toxin binding are crucial. The specificity of the toxin for the target may also explain the differences in toxicity between different mosquito species (Tangsongcharoen et al., 2017). Because a toxin binds to a single receptor, the target larva can also cause high levels of resistance (Menezes et al., 2016; Romão et al., 2006).

2.4. RESISTANCES AND RESISTANCE TYPES OF INSECTS

The widespread use of insecticides has caused insects to adapt and become resistant to these insecticides. Because of the genetic origin of these gains, the resistance is also transferred to the next offspring. The WHO organization defines resistance as "the development of the ability of individuals in the other population of the same species to gain tolerance against a certain dose of a toxic substance found to kill most of the individuals in a normal population" (Çakır & Yamanel, 2005).

Biological, genetic, and operational factors play a role in the emergence of resistance. Biological factors include duration and number of generations, the number of offspring per generation, migration, and isolation. Genetic factors include the frequency and dominance of resistance genes, the suitability of the resistance genotype, the number of different resistance alleles, and gene interactions (Özbel, 2017). It is also important whether the genetic basis of insect resistance is dominant or recessive (Poopathi & Abidha, 2010). While there is no human effect on biological and genetic factors; operational factors such as the chemical structure and persistence of the insecticide and the method of application, time, dosage, and frequency depend on human beings (Özbel, 2017).

As the sensitivity of target organisms to a pesticide decreases, the effectiveness of that pesticide decreases. Pesticide applicators, on the other hand, generally increase the dose and or increase the frequency of application to obtain the old effectiveness. Here, in parallel with increasing doses, both the cost increases and more pesticide residues begin to accumulate in the environment. For this reason, excessive and unconscious use of pesticides causes the

development of resistance against these products in target organisms over time (Özbel, 2017; Tangsongcharoen et al., 2011).

Resistance develops faster in more persistent insecticides because their ability to stay in the environment increases the chance of toxin exposure and death to susceptible individuals and thus do not pass on their pesticide-sensitive traits to the next generation. This is more strongly selected in resistant insects because only resistant insects develop. Insect populations that migrate little to the gene pool of new, unexposed susceptible individuals also more easily develop resistance (Poopathi & Abidha, 2010).

Strong individuals, populations, and communities must genetically adapt themselves to maintain the continuity of their generations despite ever-changing conditions. For this reason, they have developed several resistance mechanisms. We can categorize resistance mechanisms into four main groups: metabolic resistance, target site resistance, reduced penetration, and behavioral resistance. Apart from these, cross-resistance and multiple resistance cases are also situations related to pests gaining resistance to pesticides (Karunaratne et al., 2018; Özbel, 2017).

2.4.1. Metabolic Resistance (Increased Detoxification)

It may occur as the detoxification of the pesticide due to the increase in the activity of one or more enzymes involved in the metabolism of a pesticide or the change in the structure of the enzyme. The main enzymes related to metabolic resistance against insecticides in insects are monooxygenases (cytochrome p450), glutathione-S-transferase (GST), and esterases (Özbel, 2017). Detoxification mechanisms are the mechanism based on the breakdown of insecticides before they reach the target area (Çakır & Yamanel, 2005).

2.4.2. Target Area

Insects acquire target region insensitivity mainly through silent point mutations within structural genes. Only a limited number of changes without disturbing the normal physiological functions of the target site can reduce insecticide susceptibility. Because the number of possible amino acid substitutions is very limited, identical mutations associated with resistance are often found in highly distant taxa. Mutations in target sites change their binding affinities to different

insecticides depending on the molecular structure of the insecticide. Therefore, altered target regions do not mediate the same level of resistance to all insecticides belonging to a particular group (Karunaratne et al., 2018).

2.4.3. Behavioral Resistance

Behavioral resistance can be defined as any behavior that an insect does to avoid an insecticide in a way that increases the chances of survival of itself or its offspring. Detecting insecticide during activities such as spawning area of the insect, stopping feeding, closing its breathing holes (stigmas), not putting it on the insecticide-applied surface, not touching or moving away from the environment, and avoiding laying eggs can reduce the exposure to insecticide (Özbel, 2017)

2.4.4. Cuticular Resistance (Reduced Penetration)

Cuticular resistance is mediated by thickening or alteration of the chemical composition of the insect cuticle to reduce the penetration of insecticides into the insect body. Less penetration can facilitate the action of metabolic enzymes by providing more time for detoxification. Cuticular resistance is thought to confer cross-resistance in many insecticides because most insecticides are lipophilic (Karunaratne et al., 2018).

2.4.5. Multiple Resistance

Multiple Resistance: It is observed when several different resistance mechanisms are present at the same time. Multiple resistances can occur when an insect population is exposed to two or more insecticides of different classes.

2.4.6. Cross Resistance

Cross Resistance: This is when an insect resistant to a particular insecticide is resistant to other insecticides of the same class or different classes of insecticides with the same mechanism of action. The best-known example of cross-resistance is that insect populations resistant to DDT are also pyrethroids-resistant (Özbel, 2017). |

3. MATERIAL and METHODS

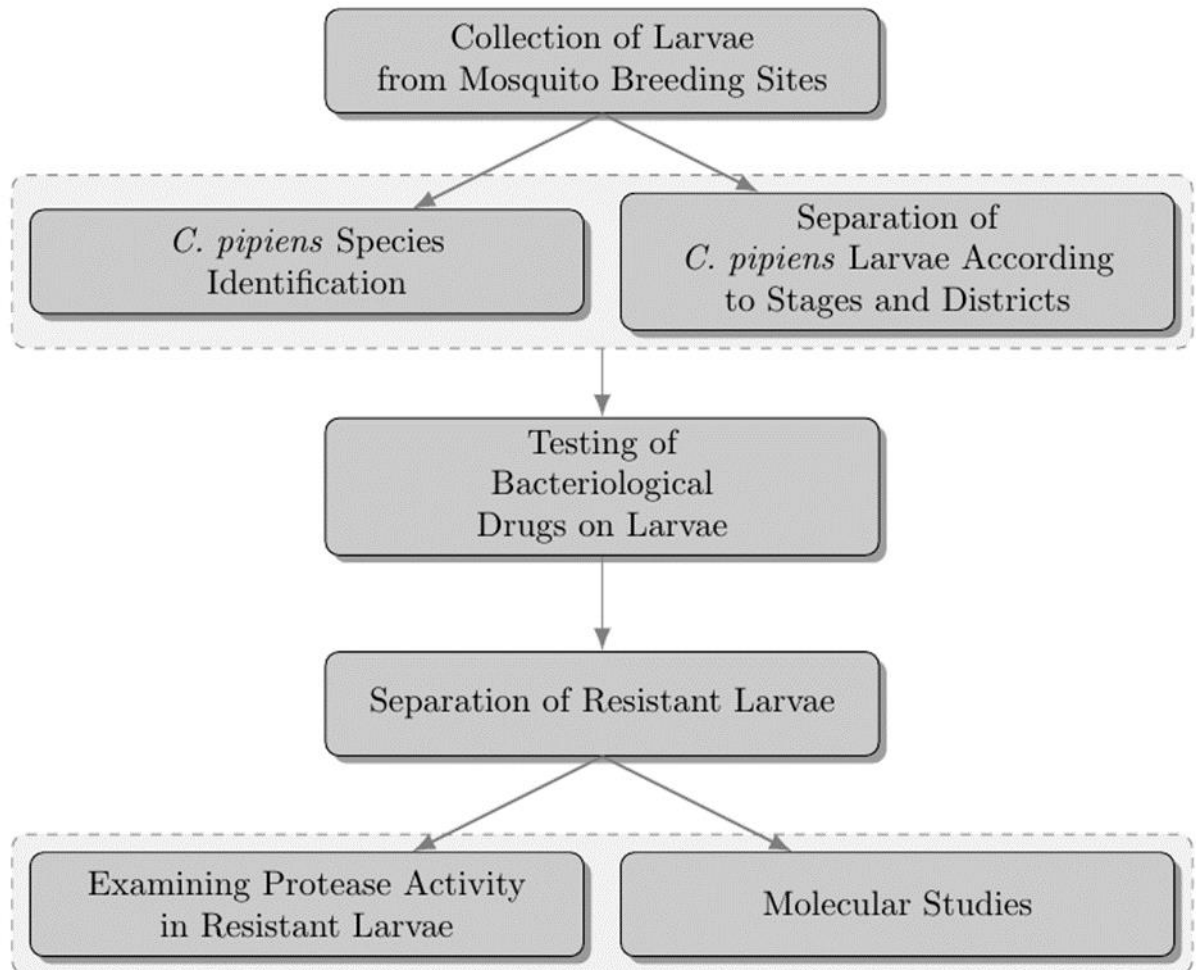


Figure 3.1: Study flowchart

3.1. GEOGRAPHICAL LOCATION and CLIMATE CHARACTERISTICS of ISTANBUL

This study was conducted within the border of Istanbul and in all its districts. Istanbul, located between 28° 01' and 29° 55' east longitudes and 41° 33' and 40° 28' north latitudes, is bordered by the high hills of the Kocaeli Mountains in the east, the water separation line of the Ergene

Basin in the west, the Black Sea in the north and the Marmara Sea in the south. It covers a total area of 5512 km².

The annual total precipitation average is 700 mm, and the monthly average temperature is 6.5 °C in winter and 21.8 °C in summer. The average solar radiation intensity in the city is 21 MJm⁻² in summer and 6 MJm⁻² in winter. Hills reach 250 meters in the city, and 2/3 of the city is covered with water basins. Istanbul is generally under the influence of the Mediterranean climate and the Black Sea climate. The winter months are mostly rainy, and the rainy period lasts from November to the end of February (Bozyazı Daylan, E. G. & İncecik S, 2002).

3.2. MATERIALS AND DEVICES USED IN THE THESIS STUDY

3.2.1. Dipper

A standard dipper with a length of two meters and a 500 ml plastic container on the end was used to collect the larvae during the field operations. The dipper has a volume of 300 ml with a top edge diameter of 13 cm, a base diameter of 6.5 cm, and a depth of 6.5 cm. The corresponding part of the interior is made with an open embouchure. This tool allows larvae and pupae collections to be performed reliably and practically. The larvae and pupae collection dipper are shown in Figure 3.4.

3.2.2. Plastic Bottles

To bring the collected larvae and pupae to the laboratory, 500 ml, 1000 ml, 1500 ml, and 5000 ml volumes of plastic pet bottles were used.

3.2.3. Larvae Feed

For feeding larvae, feeds of the type used in aquariums were given.

3.2.4. Cultivation Cages

To obtain adults from the larvae and pupae in the laboratory, 12 breeding cages with wooden slats of 30x30x30cm were built. Although there is no scientific standard regarding the size, type, and material of the cages, their durability is preferable for this study. The cages are surrounded by white tulle. The side face, which is designed to face forward, is made of a 15 cm

diameter hole and a gauze arm extending outwards through this hole to catch the mosquitoes passing from the pup to the adult period without escaping with the mouth aspirator. The breeding cage is shown in Figure 3.10.

3.2.5. Plastic Containers

When selecting the plastic container for the experiments, especially for the larvae to appear and move freely, a transparent, wide diameter and a certain height were preferred. In accordance with these specifications, transparent plastic containers with a diameter of 12 cm and a height of 4 cm were used (Figure 3.15).

3.2.6. Debouchment Aspirator

Debouchment aspirator was used to transport adult mosquitoes from the rearing cage to the Erlenmeyer flask. It is composed of three parts a glass pipe, a rubber pipe, and a glass mouthpiece. The debouchment aspirator is shown in Figure 3.11.

3.2.7. Erlenmeyer

Erlenmeyer has been used to kill adult mosquitoes. For this purpose, impregnated cotton with chloroform was placed in the mouth of the erlenmeyer.

3.2.8. Cultivation Containers

Plastic containers with 500ml volume and 26 cm x 33 cm x 6 cm in size were used to raise adults from larvae and pupae. The growing container is shown in Figure 3.16.

3.2.9. The Devices Used to Identifying Species

Stereo microscope: A Novel NSZ - 405 Binocular Zoom stereo microscope with magnification limits in the range of 14x – 60x was used for the morphological examination of adult mosquito species. Opaque white light bulb is used for illumination. The stereo microscope is shown in Figure 3.8.

3.3. COLLECTION of LARVAE

To investigate the development of resistance against biological larvicides used in the control of mosquito larvae, this study was completed in the period when mosquito activity was at its best, between April and September, between 2018 and 2020. In our study, mosquito larvae were collected from mosquito breeding source points registered to the Vector Control Unit of Istanbul Metropolitan Municipality (IMM) Health Directorate with the help of the IMM. This study includes bringing the mosquito larvae to the laboratory, (Figure 3.2) and testing the *B. thuringiensis subsp. israelensis* and *B. sphaericus* larvacides by separating the suitable ones for the study and finally investigating the resistance gene in the larvae that are considered to be resistant.

Larvae samples were collected from 39 districts in Istanbul, which are in European region Arnavutköy, Avcılar, Bahçelievler, Bağcılar, Bakırköy, Basakşehir, Bayrampaşa, Beşiktaş, Beylikdüzü, Beyoğlu, Büyükçekmece, Çatalca, Esenler, Esenyurt, Eyüp, Fatih, Gaziosmanpaşa, Güngören, Kağıthane, Küçükçekmece, Sarıyer, Sultangazi, Silivri, Şişli, Zeytinburnu, and in Anantolian region Adalar, Ataşehir, Çekmeköy, Beykoz, Kadıköy, Kartal, Maltepe, Pendik, Sancaktepe, Şile, Sultanbeyli, Tuzla, Ümraniye, and Üsküdar.



Figure 3.2: Mosquito larvae.

3.3.1. Mosquito Breeding Sites

Before collecting larvae samples, firstly, mosquito breeding sites were checked. Stagnant water with vegetation is generally known as the habitat of mosquitoes. Larvae samples were collected from different mosquito breeding habitats such as creeks, ponds, reeds, canals, treatment plants, canisters, flowerpots, wheels, open spaces, construction, septic tank, pool, elevator shaft, motor pumps, warehouses, open space grid, open space channel, tub, boat, barrel, fountain, grill, fertilizer, ornamental pond, water tank, open field pond, and closed field pond with the help of a dipper. Dipper, a standard larval scoop, is used to collect larvae and pupae found in mosquito breeding sources (Figure 3.3).





Figure 3.3: Examples of mosquito breeding sources.

3.3.2. Larval Sampling with Dipper

To take larvae samples with a dipper, different immersion and sampling methods were used since the mosquito breeding habitat's water depths varied according to vegetation and density (Figure 3.4).



Figure 3.4: Larval sampling with the dipper.

The **surface panning technique** was applied in the breeding sources where there are deep deposits such as lakes, ponds, and pools. Larvae were collected by submerging the dipper at a 45-degree angle so that the rim end of the dipper was approximately 2,5 cm below the surface of the water, and the dipper was filled as if foam balls were along the water surface.

Generally, the ‘**simple immersion**’ method has been applied in breeding sites such as depots, septic tanks, and elevator shafts. In this technique, which simply resembles getting drinking water from the creek, the dipper is completely immersed by swirling the wrist just below the water’s surface.

Where the vegetation is dense, the “**partial immersion into the water**” technique is applied. The dipper is immersed at an angle of approximately 45 degrees along shallow zones (emergent vegetation). Water is taken into the dipper quickly, however, the dipper is not moved horizontally. The dipper can be moved vertically by scraping bottom water along the edges of emergent vegetation.

In the shallow waters of the river mouths, where the length of the dipper bucket is greater than the depth of the water, the water samples were taken together with the larvae and the bottom water by pushing the dipper bucket, (Figure 3.5), (Walton, 2005).



Figure 3.5: Larval removal techniques with the dipper.

It was found that the samples coming to the laboratory frequently come from breeding sources created by human hands, such as open area deposit, can, pool, construction, closed-area deposit, open area, and wheel. When transferring the sample from the dipper into the container, sampling is carried out with a maximum of 1/3 of the sample container being empty and will provide the necessary air for the larvae to survive (Figure 3.6).



Figure 3.6: Transfer of larvae to pet bottles.

After the removal of the larvae properly, to transport the larvae to the laboratory in a healthy manner, the filled sample containers are tightly sealed. The lids of the sample cups were pierced to allow the larvae to receive oxygen. Full sample containers; to avoid overheating of the ambient water, it was placed in a cool place, and they fixed be packed vertically to prevent tipping of the sample containers in the van. Since the filled sample containers were transported within the crates, they were covered with a light-colored cover to prevent them from being damaged by direct sunlight.

Larva samples taken from various mosquito breeding habitats with dippers under suitable conditions were collected in 500 ml, 1.5, and 5 liters plastic bottles and were included in the study in Cerrahpaşa Medical Microbiology laboratories (Figure 3.7).



Figure 3.7: Arrival of larvae with pet bottles in the laboratory.

3.4. *C. pipiens* SPECIES IDENTIFICATION

To identify the larvae that were taken by using a dropper were placed on the slide to be studied under a stereo microscope and they were cut, and killed by using a scalpel between the 6th and 7th abdomen segments (Figure 3.8 and 3.9).



Figure 3.8: Looking at mosquito larvae and adults under a stereo microscope, and *C. pipiens* larvae.



Figure 3.9: The Appearance of *C. pipiens* larva in the stereo microscope.

To identify adult mosquitoes, Figure 3.10, larva and pupae samples were put in a rearing cage 30x30x30cm in size, which was made of wooden slats surrounded by white tulle.



Figure 3.10: Growing of 1st and 2nd stage *C. pipiens* larvae by giving fish food in rearing cage.

Mosquitoes were killed by placing cotton pads impregnated with chloroform in the mouth of the erlenmeyer. A mouth aspirator was used to transfer adult mosquitoes from the rearing cage to the erlenmeyer without being damaged (Figure 3.11).



Figure 3.11: Debouchment aspirator

Adult mosquitoes were attached to triangular small white cartons with nail polish in a way that their body structures would not be disturbed, and the pin dipped in the cardboard was fixed to the cork-board and separated according to their types under the stereo microscope (Figure 3.12 and 3.13).

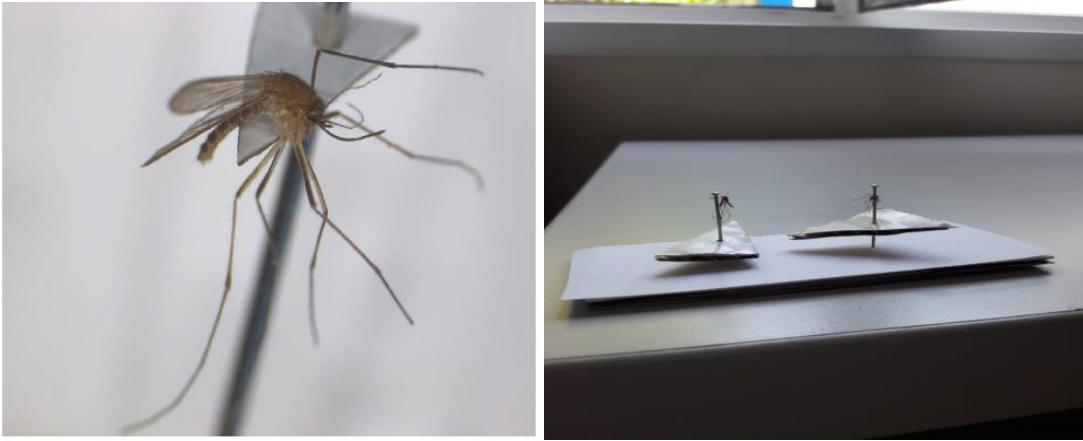


Figure 3.12: Fixation of *C. pipiens*.



C. pipiens legs

C. pipiens wings

Figure 3.13: The appearance of the *C. pipiens* under the stereo microscope.

3.5. SEPARATION of *C. pipiens* LARVAE ACCORDING to STAGES, and DISTRICTS

İMM has divided Istanbul into 6 regions according to the area of the districts, their proximity to each other, population density, and the number of pesticide applications. For the larvae collected from the regions to be in a certain order, we carried out our study according to the regional division of İMM and performed it in 6 regions.

Region 1: Bağcılar, Bahçelievler, Bakırköy, Bayrampaşa, Esenler, Fatih, Güngören, Beyoğlu, Şişli, and Zeytinburnu.

Region 2: Beşiktaş, Eyüp, Gaziosmanpaşa, Kağıthane, and Sarıyer.

Region 3: Beylikdüzü, Büyükçekmece, Çatalca, Esenyurt, and Silivri.

Region 4: Arnavutköy, Avcılar, Başakşehir, Sultangazi, and Küçükçekmece.

Region 5: Ataşehir, Beykoz, Çekmeköy, Kadıköy, Ümraniye, and Üsküdar.

Region 6: Adalar, Kartal, Maltepe, Pendik, Sancaktepe, Sultanbeyli, Şile, and Tuzla.

The larvae from the samples brought to the laboratory were divided into plastic containers according to the districts (Figure 3.14).



Figure 3.14: Separation of larvae to study in the laboratory.

The third and the fourth instar *C. pipiens* larvae were transferred to resting water with the help of a dropper (Figure 3.14).



Figure 3.15: Separation of 3rd and 4th stage *C. pipiens* larvae into plastic containers according to districts.

The first and the second stage *C. pipiens* larvae were transferred to the rearing pot, as shown in Figure 3.16. Fish food was given to them to reach the 3rd or 4th stage, and they were expected to grow at room temperature. In the meantime, if the pupae turned into adults, grown larvae were kept in cages and species were studied for adults and adult scattering around was prevented.



Figure 3.16: Larval rearing container.

Some of the larvae passed to the 3rd and 4th stages and some of them passed to the pupal stage. The larvae in the pupal stage were excluded from the experiments.

3.6. TESTING of BACTERIOLOGICAL LARVACIDE on *C. pipiens* LARVAE

Larvacide trials were carried out using commercial preparations obtained from Bayer Turkey; *B. sphaericus* [(H5a5b, Strain 2362), (VectoLex WDG, US)] and *B. thuringiensis subsp. israelensis* [(H-14, Strain AM 65-52), (VectoBac WDG, US)] powder forms have been used in the laboratory to investigate the formation of resistance (Figure 3.17).



Figure 3.17: *B. sphaericus* and *B. thuringiensis subsp. israelensis*

The larvae had been kept in water until the third stage to avoid the effect of the larval stage on the experimental results. The studied species could not reproduce under laboratory conditions therefore, bioassay could not be performed on F1 larvae, and larvae collected directly from the field were used. To limit the effects of environmental factors such as pH and pollution of the water in the areas where the larvae were taken, the larvae were first kept in fresh water for 24 hours (Tetreau et al., 2013).

Twenty-Five third or fourth-instar larvae were transferred to disposable test cups containing 100-200 ml in tap water and rested for 48 hours water with the help of a dropper. Small, unhealthy, and damaged larvae from the third stage were excluded from the experiment. The depth of water in the vessel was kept between 5 and 10 cm, as deep levels were fatal. An equal number of larvae and 1 ml of alcohol were added to the control dish. Especially in long-term experiments, when a high mortality rate was noticed in the control, the larval feed was added to the experimental dishes. For larvae, yeast extract suspended in water (1.5 mg) or ground

mouse or rabbit pellets was added at 10 mg/l to the water in the test dishes. Experiments were held at 25-28 °C for a photoperiod of 12 hours of light followed by 12 hours of darkness (12L:12D) (WHO, 2005).

When calculating the amount of use of *Bacillus thuringiensis subsp. israelensis*, and *Bacillus sphaericus*, the calculations were made according to the amounts indicated on the label on the packaging. The radius of the test vessels was 6 cm² and the surface area was 0.113 cm². The application concentration for *B. sphaericus* was 400 g/ha for 1 hectare and 200 g/ha for *B. thuringiensis subsp. israelensis* as stated in the commercial preparation of the product. The required concentration ratio for the surface area of our test vessels was 0,00026 gr for *B. thuringiensis subsp. israelensis* and 0,00045 gr for *B. sphaericus* were put into containers containing 25 larvae by using a plastic cup for each district (Figure 3.18).



Figure 3.18: Larval resistance study for *B. thuringiensis subsp. israelensis*, and *B. sphaericus*

The test results were evaluated according to the WHO guidelines;

- Bioassays should be using new solutions or suspensions.
- Dead larvae are those that cannot be induced to move when they are probed with a needle in the siphon or the cervical region.
- Moribund larvae are those incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed. (They are added to dead larvae for calculating percentage mortality).
- If more than 10% of larvae pupate, the test is invalidated because late instar larvae do not ingest 24 h before pupation, and too many larvae may have survived for a simple reason; they are too old.
- Mortality is determined at 24 h for *B. thuringiensis subsp. israelensis* and 48 h for *B. sphaericus* by counting the live larvae remaining.
- Tests with control mortality greater than 20% should be discarded. If the control mortality is between 5% and 20%, the mortalities of treated groups are calculated with Abbott's formula (WHO, 2005).

3.7. SEPARATION of RESISTANT *C. pipiens* LARVAE

After performing head and tail dissection from the larvae, which are considered resistant, from each district, the molecular method is raised to -80 to be studied.

3.8. PROTEASE MEASUREMENT

The gut of 200 3rd and 4th instar larvae of *C. pipiens* was dissected after chilling the larvae on ice. For protease assay;

- 1- 5 µl of purified protease
- 2- 10 µl of 10 mM bicarbonate
- 3- 5.2 µl of azocasein (13.3 µg/µl)
- 4- 80 µl of 5% TCA solution
- 5- 150 µl of 0.5 N NaOH

For protease assay 5 µl of the gut extract or purified protease was mixed with 10 µl of 10 mM bicarbonate buffer pH 9.0 and incubated with 5.2 µl of azocasein at 37°C for 30 min. The

reaction was stopped by adding 80 µl of 5% TCA solution. Samples were centrifuged at 9400×g for 10 min at 4°C and 50 µl of the supernatant was mixed with 150 µl of 0.5 N NaOH solution. Isolated protease values were read by spectrophotometer and the absorbance was measured at 280 nm in a microplate reader. All the assays were performed in duplicate and repeated thrice (Vadakkadath Meethal, 2020).

3.9. MOLECULAR STUDIES

3.9.1. Random Amplification of Polymorphic DNA (RAPD)

Random amplification of polymorphic DNA (RAPD) is a polymerase chain reaction (PCR) based technique for identifying genetic variation. It involves using a single arbitrary primer in a PCR reaction, resulting in the amplification of much discrete DNA. RAPD technology provides a quick and efficient screen for DNA sequence-based polymorphism at a very large number of loci. The major advantage of RAPD includes that it does not require pre-sequencing of DNA. The vast range of potential primers can be used to give the technique great diagnostic power (Kumari & Thakur, 2014).

The basic principle of the RAPD method is that a randomly selected single 9-10 bp oligonucleotide on the genomic DNA of the related species is bound randomly at low binding temperature and amplifies by PCR. The amplification product obtained in the continuation of the technique is carried out in gel electrophoresis, and the amplification products are examined by observing them as bands. The results are evaluated by the presence or absence of bands.

In molecular systematic studies, the isolation of DNA from organisms is required first. The primers used should not contain any palindromic sequences, as in other primers used in PCR, and should have a G+C ratio of 50-80%. Replication in the RAPD technique is sensitive to the length of the primer used, the GC content of the primer, and the location of a single nucleotide in the primer sequence. In this technique, the determination of the optimum conditions of MgCl₂, dNTP, and Taq DNA polymerase concentrations specific to the studied species is necessary for the reproducibility and specificity of the reaction (Öz Aydin, 2004).

3.9.2. DNA Isolation

Two methods are applied for DNA isolation; a modified version of Kidwell and Osborn's method (1992) and the CTAB method (Doyle, 1987). The larvae to be isolated from DNA were crushed and powdered with the help of liquid nitrogen, using porcelain mortar and porcelain pestle.

- 1- 500 µl of extraction buffer heated up to 65 °C for 50 mg of larvae was added to the powder, which was transferred to a 1.5 ml tube cooled in liquid nitrogen and mixed.
- 2- The tube was kept in a 60° C water bath for at least 1 hour. An equal volume (approximately 500 µl) of chloroform: isoamyl alcohol was added to the tube, which was kept on ice for 5 minutes after removal from the water bath.
- 3- The tube was inverted at least 50 times then it was centrifuged at 2000 rpm for 20 minutes at room temperature.
- 4- The supernatant (approximately 500 µl) formed after centrifugation was transferred to a new 1.5 ml tube, 0.1 volume (50 µl) of CTAB/NaCl solution was added, and the tubes were inverted several times and kept in a 60 °C water bath for 10 minutes.
- 5- The tube was removed from the water bath then it was kept on ice for 5 minutes, and an equal volume (approximately 550 µl) of chloroform: isoamyl alcohol was added to the tube.
- 6- The tubes were inverted at least 30 times then they were centrifuged at 2000 rpm for 20 minutes at room temperature.
- 7- The supernatant formed after centrifugation (approximately 550 µl) was transferred to a new 1.5 ml tube, after adding an equal volume of isopropanol (-20 °C) to the upper liquid in the tube, the tube was inverted several times and kept at -20 °C for 30 minutes. After the waiting phase, the tube was centrifuged at 13500 rpm for 10 minutes.
- 8- The upper liquid formed after centrifugation was removed with a pipette. 100 µl of 70% ethanol (-20 °C) was added to the precipitate, centrifuged at 13500 rpm for 2 minutes, and the precipitate was washed. The washing process was repeated once more.
- 9- 30 µl of sterile water was added to gDNA samples and stored at -20 °C until use.

The purity and amount of gDNA samples were determined by measuring on the NanoDrop 2000 (Thermo Scientific, N10588). The absorbances (A₂₃₀, 260, 280) of the gDNA samples were measured at 230, 260, and 280 nm wavelengths. It is known that proteins and especially

phenolic substances absorb at 230 nm wavelength. The purity of DNA samples was determined by considering the ratio of the OD260 value to both the OD280 value and the OD230 value. The fact that these ratios are lower than 1.7 indicates phenolic substance and protein contamination in the purified DNA sample. Considering that this situation may cause erroneous results in PCR studies; only gDNA samples with an OD260/OD280 value between 1.7–2.0 and an OD260/OD230 value greater than 1.7 were used. The quality of the gDNA samples was determined by agarose gel electrophoresis.

3.9.3. Agarose Gel Electrophoresis

One microliter of gDNA sample was mixed with 0.2 µl of loading buffer. The samples were run on a 1% agarose gel at a constant power of 150 V for 2 h and viewed under UV light. The gDNA samples that produced a thick, prominent, and bright band appearance of 10 Kb or more by comparison with the DNA length marker were considered unbroken and whole. The unbroken gDNA samples were used in molecular studies.

3.9.4. Real-Time PCR

In this study, RAPD primer kits (Operon Tech., Alameda, CA, USA) were used, and 10 RAPD primers, Table 3.1, were used. PCR components and PCR cycle in RAPD analysis Kılıç (Kılıç et al., 2019) were prepared according to the following protocol they used in *C. pipiens*.

Table 3.1: Primers used for the identification of *C. pipiens* in Real-Time PCR.

	Primer code	The sequence of the primer (5'- 3')	The total of bands produced	Range of bands produced (Bp)	The total of polymorphic bands produced
1	OPA-15	TTCCGAACCC	0	-	-
2	OPB-16	TTTGCCCGGA	11	500-300	9
3	OPB-19	ACCCCCGAAG	6	500-3000	5
4	OPE-17	CTACTGCCGT	8	500-3000	6
5	OPF-18	TTCCCGGGTT	0	-	-
6	OPK-15	CTCCTGCCAA	9	500-3000	4
7	OPM-12	GGGACGTTGG	12	500-3000	9
8	OPO-15	TGGCGTCCTT	10	500-3000	8
9	OPD-11	AGCGCCATTG	12	500-3000	9
10	OPL-17	AGCCTGAGCC	13	500-3000	9

RAPD Protocol: 1.5 µl 10X PCR buffer, 1.33 mM primer, 200 µM each dNTP (dATP, dGTP, dCTP, and dTTP), 2.5 mM MgCl₂, 0.2 µg/µl BSA (Bovine serum albumin), 1 unit Taq DNA polymerase enzyme, 20 ng DNA, 5.8 µl ddH₂O. After the mixture was made up to 15 µl, a drop of mineral oil was added to the PCR tubes to prevent evaporation.

In implementing the Real-Time PCR in the study, the Cepheid Smart Cycler II Real-Time PCR device and the Cepheid Smart Cycler Software V.2.0c program were used. For Real-Time PCR amplification, it was constructed from 45 cycles with the first denaturing of DNA at 94 °C for 4 min. An Eppendorf Mastercycler gradient thermal cycle was performed with 1 min denaturation at 94 °C, primary annealing at 36 °C for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 5 min.

3.9.5. Electrophoresis of PCR Products

PCR products were electrophoresed in 1 X TBE (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) on a 2% agarose gel at 110 V for 3–4 h. 100 bp DNA ladder was used as standard in all electrophoresis procedures. Then, the pictures of the gels were taken in the gel imaging unit and transferred to the computer environment.

3.10. STATISTICAL ANALYSIS

Among the *C. pipiens* larvae tested for *B. sphaericus* and *B. thuringiensis subsp. israelensis*, only those resistant to the Abbott value were evaluated statistically. One-Way ANOVA (OWA) was applied at a significance level of <0.05 to determine the resistance rates of *C. pipiens* larvae according to the Abbott value against *B. sphaericus*, the resistance rates, and the effect of the bacterial drug after 24–48 h. Tukey test was used as Post Hoc Test. Also, variance analysis was done with ANCOVA, and overall results passed to the Post Hoc test.

We used the Mann–Whitney U test to compare the protease activities of *C. pipiens* larvae treated with *B. sphaericus* and which remained alive after 48 h with those of *C. pipiens* control group larvae without *B. sphaericus* treatment. A Mann–Whitney U test was used to compare the differences between two samples when the sample distributions are not normally distributed and the sample sizes are small ($n < 30$). |

4. RESULTS

Nine hundred seventy-six bottles with larvae were brought to the laboratory for use in the experiments. The study was completed with 9345 larvae in the 3rd and 4th stages suitable for the study. The resistance-related tests were conducted for 2925 *B. sphaericus* and 1620 for *B. thuringiensis subsp. israelensis*. The Abbott values after 24 and 48 h were given in regional tables.

Walter Sidney Abbott constructed a formula (Abbott formula) in 1925 to calculate the efficiency of the insecticides by subtracting the natural deaths that occurred in the control group. The Abbott formula requires that both the treatment and the control group must have the same number of organisms at the start. If they are not of the same number, they can be simply rationed. One must, however, calculate the probability error, too. Abbott formula;

$$\text{Mortality \%} = \left(1 - \frac{P_t}{P_c}\right) * 100$$

P_c = percentage survival in the untreated control and

P_t = percentage survival in the treated sample (WHO 2005).

We can evaluate the result of this formula with these observations;

- If P_c approaches P_t then the mortality rate becomes closer to 0.
- If P_c diverges from P_t then the mortality rate becomes closer to 100.
- The Exact 100 value occurs if $P_t = 0$.

If one investigates the formula, one can see that the higher mortality rates indicate less resistance, and the smaller values indicate resistance in the group (Abbott, 1987). The resistance status of a mosquito population can be assessed by performing adult or larval bioassays according to WHO guidelines. Actual measurements can be adjusted with control measurements using Abbott's formula, only if control measurements are < 20%. Based on the survival rate, the population response is considered for a given insecticide as;

- susceptible if mortality rate >98% mortality,
- possibly resistant if the mortality rate is between 80% and 98% mortality, or
- resistant if the mortality rate is <80% mortality (WHO, 2005).

One-Way Analysis of Variance (One-Way ANOVA) and analysis of covariance (ANCOVA) at a significance level of 0.05 (p-value) were applied to independent groups to reveal a possible difference between the regions from which the *C. pipiens* larvae came, which emerged resistant according to the Abbott values in which *B. sphaericus* was tested from the data obtained in the study. One-Way Analysis of Variance the ANOVA test and ANCOVA test for differences of 24 and 48 h, Tukey's technique, one of the Post Hoc Test tests, was used to evaluate the information in the results and to determine from which groups the difference originated. The findings of the research and comments on the findings are presented in tables.

4.1. ASSAY and RESISTANCE RESEARCH with *B. THURINGIENSIS SUBSP. ISRAELENIS* and *B. SPHAERICUS* LARVACIDES on the *C. pipiens* LARVAE COLLECTED from the DISTRICTS

After dividing the districts of Istanbul into 6 regions, *C. pipiens* larvae in the 3rd and 4th stages of each district were included in the study as separate districts and tested by the label on the powder forms of *B. thuringiensis subsp. israelensis* and *B. sphaericus*. According to the Abbott values, the susceptible places and possibly resistant were found on the Istanbul map in 6 regions.

For the rest, the fallowing abbreviation and coloring is used; resistant (R) red, susceptible (S) as blue, and possibly resistant (PR) as green.

In the tables, the control status column is set to ok, if the control group achieves the WHO's guidelines < 20%, otherwise set to not ok.

4.1.1. Resistance Status of *C. pipiens* Larvae from Region 1 After 24-48 Hours Trial of *B. Sphaericus* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Bağcılar, Bahçelievler, Bakırköy, Bayrampaşa, Esenler, Fatih, Güngören, Beyoğlu, Şişli, and Zeytinburnu, which we have separated and named as 1. region, were examined for *B. sphaericus* biological larvicide and the results were evaluated at 24 and 48 h later with Abbott formula (Table 4.1).

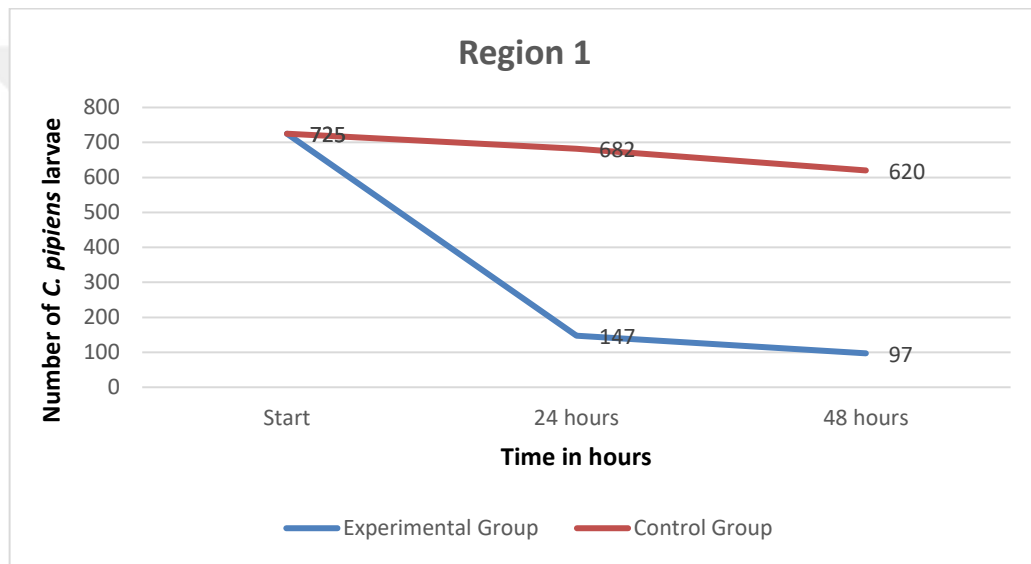
Table 4.1: Resistance status of *C. pipiens* larvae of region 1 at 24 and 48 h after *B. sphaericus* testing.

Region 1	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Bağcılar	25	6	4	25	20	76,00%	80,00%	ok	R
Bağcılar	25	4	3	23	21	82,61%	85,71%	ok	R
Bahçelievler	25	1	0	24	22	95,83%	100,00%	ok	S
Bahçelievler	25	0	0	25	21	100,00%	100,00%	ok	S
Bahçelievler	25	15	13	21	20	28,57%	35,00%	ok	R
Bahçelievler	25	9	7	25	24	64,00%	70,83%	ok	R
Bakırköy	25	3	0	23	23	86,96%	100,00%	ok	S
Bakırköy	25	2	0	20	20	90,00%	100,00%	ok	S
Bakırköy	25	3	0	24	22	87,50%	100,00%	ok	S
Bayrampaşa	25	5	2	25	24	80,00%	91,67%	ok	PR
Bayrampaşa	25	4	0	24	19	83,33%	100,00%	ok	S
Bayrampaşa	25	15	13	21	20	28,57%	35,00%	ok	R
Bayrampaşa	25	7	5	24	22	70,83%	77,27%	ok	R
Beyoğlu	25	0	0	23	21	100,00%	100,00%	ok	S
Beyoğlu	25	0	0	25	24	100,00%	100,00%	ok	S
Esenler	25	9	8	23	21	60,87%	61,90%	ok	R
Esenler	25	6	4	25	20	76,00%	80,00%	ok	R
Esenler	25	4	4	20	20	80,00%	80,00%	ok	R
Fatih	25	11	9	23	21	52,17%	57,14%	ok	R
Fatih	25	8	7	24	22	66,67%	68,18%	ok	R
Fatih	25	5	4	24	20	79,17%	80,00%	ok	R
Fatih	25	7	5	25	24	72,00%	79,17%	ok	R
Güngören	25	9	5	25	20	64,00%	75,00%	ok	R
Güngören	25	8	4	23	21	65,22%	80,95%	ok	R
Güngören	25	0	0	20	19	100,00%	100,00%	ok	S
Şişli	25	0	0	24	22	100,00%	100,00%	ok	S
Şişli	25	0	0	24	23	100,00%	100,00%	ok	S
Zeytinburnu	25	3	0	25	20	88,00%	100,00%	ok	S
Zeytinburnu	25	3	0	25	24	88,00%	100,00%	ok	S

Table 4.2: Abbott values of *C. pipiens* larvae of region 1 at 24 and 48 h after *B. sphaericus* testing.

Region	Tested with <i>B. sphaericus</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
1	Experimental Group	725	147	97
	Control Group	725	682	620
	Abbot Value	-	78.45%	84.06%

B. sphaericus was tested and 147 *C. pipiens* larvae survived after 24 h and 97 *C. pipiens* larvae survived after 48 h (Figure 4.1).

**Figure 4.1:** The graph of Abbott values of *C. pipiens* larvae of region 1 at 24 and 48 h after *B. sphaericus* testing.

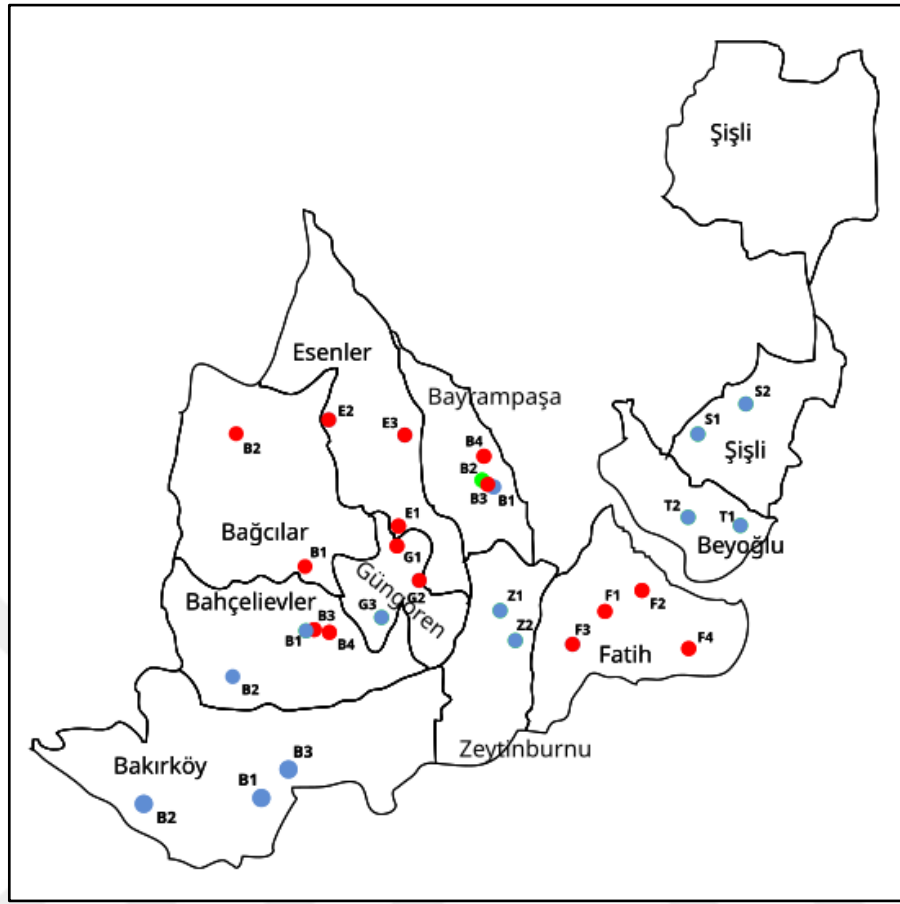


Figure 4.2: The map shows the places where the *C. pipiens* larvae of the 1st region are resistant according to the Abbott value 24 and 48 h after the *B. sphaericus* test. The color code is red for resistant, blue for susceptible, and green for possibly resistant.

According to the Abbott formula, 95 larvae, 7 from Bağcılar, 20 from Bahçelievler, 16 from Esenler, 25 from Fatih, 18 from Bayrampaşa, and 9 from Güngören, developed resistance to the toxin protein and remained alive 48 h after *B. sphaericus* was tested on *C. pipiens* larvae in the region 1. In Bakırköy, Beyoğlu, Şişli, and Zeytinburnu districts, all larvae died and no resistance was observed (Figure 4.2).

4.1.2. Resistance Status of *C. pipiens* Larvae from Region 1 After 24-48 Hours Trial of *B. Thuringiensis Subsp. Israelensis* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Bağcılar, Bahçelievler, Bakırköy, Bayrampaşa, Esenler, Fatih, Güngören, Beyoğlu, Şişli, and Zeytinburnu, which we have separated and named as region 1, were examined for *B. thuringiensis subsp. israelensis*

biological larvacide and the results were evaluated 24 and 48 h later with the Abbott formula (Table 4.4).

Table 4.3: Resistance status of *C. pipiens* larvae of region 1 at 24 and 48 h after *B. thuringiensis* subsp. *israelensis* testing.

Region 1	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Bağcılar	25	0	0	22	20	100%	100%	ok	S
Bağcılar	25	0	0	25	23	100%	100%	ok	S
Bahçelievler	25	0	0	25	23	100%	100%	ok	S
Bahçelievler	25	0	0	22	20	100%	100%	ok	S
Bakırköy	25	0	0	23	21	100%	100%	ok	S
Bayrampaşa	25	0	0	23	20	100%	100%	ok	S
Beyoğlu	25	0	0	22	21	100%	100%	ok	S
Esenler	25	0	0	24	21	100%	100%	ok	S
Esenler	25	0	0	20	20	100%	100%	ok	S
Fatih	25	0	0	24	21	100%	100%	ok	S
Fatih	25	0	0	21	20	100%	100%	ok	S
Güngören	25	0	0	20	20	100%	100%	ok	S
Güngören	25	0	0	24	21	100%	100%	ok	S
Şişli	25	0	0	23	21	100%	100%	ok	S
Şişli	25	0	0	24	24	100%	100%	ok	S
Zeytinburnu	25	0	0	23	21	100%	100%	ok	S
Zeytinburnu	25	0	0	24	21	100%	100%	ok	S

Table 4.4: Abbott values of *C. pipiens* larvae of region 1 at 24 and 48 h after *B. thuringiensis* subsp. *israelensis* testing.

Region	Tested with <i>B. thuringiensis</i> subsp. <i>israelensis</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
1	Experimental Group	425	0	0
	Control Group	425	389	358
	Abbot Value	-	100%	100%

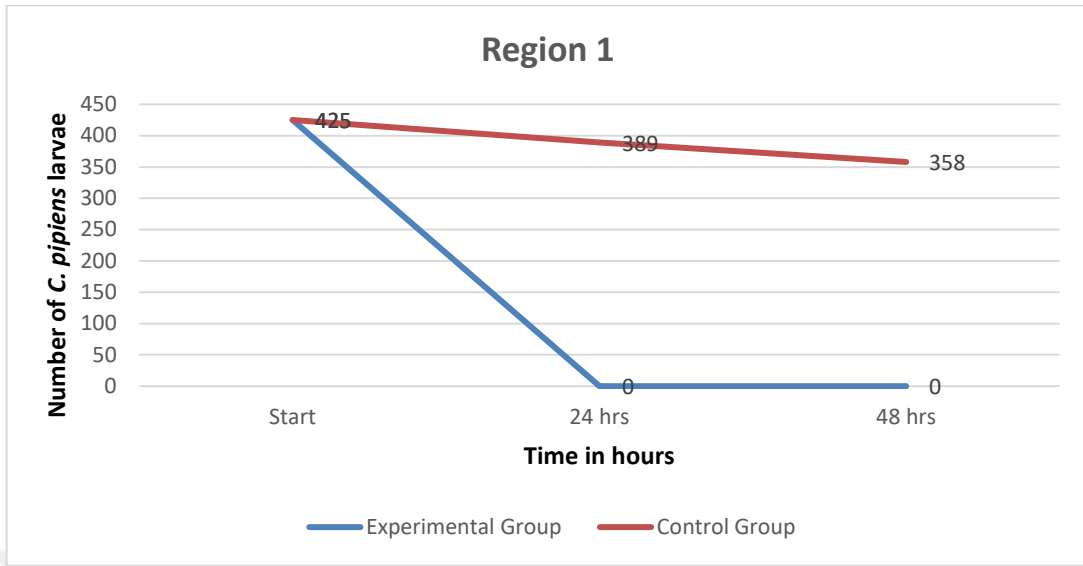


Figure 4.3: The graph of Abbott values of *C. pipiens* larvae of region 1 at 24 and 48 h after *B. thuringiensis subsp. israelensis* testing.

According to Abbott's formula, 24 h after *B. thuringiensis subsp. israelensis* was tested on *C. pipiens* larvae in the 1st region, in Bağcılar, Bahçelievler, Esenler, Fatih, Bayrampaşa Bakırköy, Beyoğlu, Şişli, Zeytinburnu, and Güngören districts, all larvae died and no resistance was observed (Figure 4.3).

4.1.3. Resistance Status of *C. pipiens* Larvae from Region 2 After 24-48 Hours Trial of *B. Sphaericus* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Beşiktaş, Eyüp, Gaziosmanpaşa, Kağıthane, and Sarıyer, which we have separated and named as region 2, were examined for *B. sphaericus* biological larvicide and the results were evaluated 24 and 48 h later with Abbott formula (Table 4.7).

Table 4.5: Resistance status of *C. pipiens* larvae of region 2 at 24 and 48 h after *B. sphaericus* testing.

Region 2	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Beşiktaş	25	7	0	23	22	69,57%	100,00%	ok	S
Beşiktaş	25	4	1	25	24	84,00%	95,83%	ok	PR
Beşiktaş	25	3	0	22	19	86,36%	100,00%	ok	S
Beşiktaş	25	0	0	21	20	100,00%	100,00%	ok	S
Eyüp	25	6	6	25	20	76,00%	70,00%	ok	R
Eyüp	25	2	1	24	22	91,67%	95,45%	ok	PR
Gaziosmanpaşa	25	6	6	24	22	75,00%	72,73%	ok	R
Gaziosmanpaşa	25	3	1	21	20	85,71%	95,00%	ok	PR
Gaziosmanpaşa	25	1	1	23	22	95,65%	95,45%	ok	PR
Gaziosmanpaşa	25	0	0	22	20	100,00%	100,00%	ok	S
Kağıthane	25	5	4	23	21	78,26%	80,95%	ok	R
Kağıthane	25	2	0	23	21	91,30%	100,00%	ok	S
Kağıthane	25	0	0	22	20	100,00%	100,00%	ok	S
Kağıthane	25	2	1	21	20	90,48%	95,00%	ok	PR
Sarıyer	25	0	0	24	22	100,00%	100,00%	ok	S
Sarıyer	25	0	0	23	22	100,00%	100,00%	ok	S
Sarıyer	25	0	0	22	18	100,00%	100,00%	not ok	S

Table 4.6: Abbott values of *C. pipiens* larvae of region 2 at 24, and 48 h after *B. sphaericus* testing.

Region	Tested with <i>B. sphaericus</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
2	Experimental Group	425	41	21
	Control Group	425	388	355
	Abbot Value	-	89.43%	94.14%

B. sphaericus was tested and 41 *C. pipiens* larvae survived after 24 h and 21 *C. pipiens* larvae survived after 48 h (Figure 4.4).

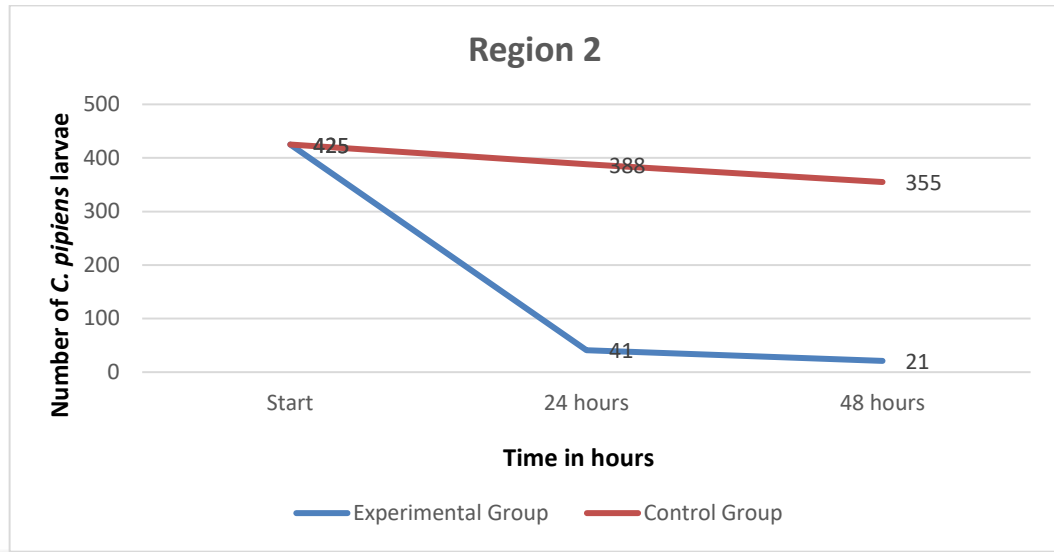


Figure 4.4: The graph of Abbott values of *C. pipiens* larvae of region 2 at 24 and 48 h after *B. sphaericus* testing.

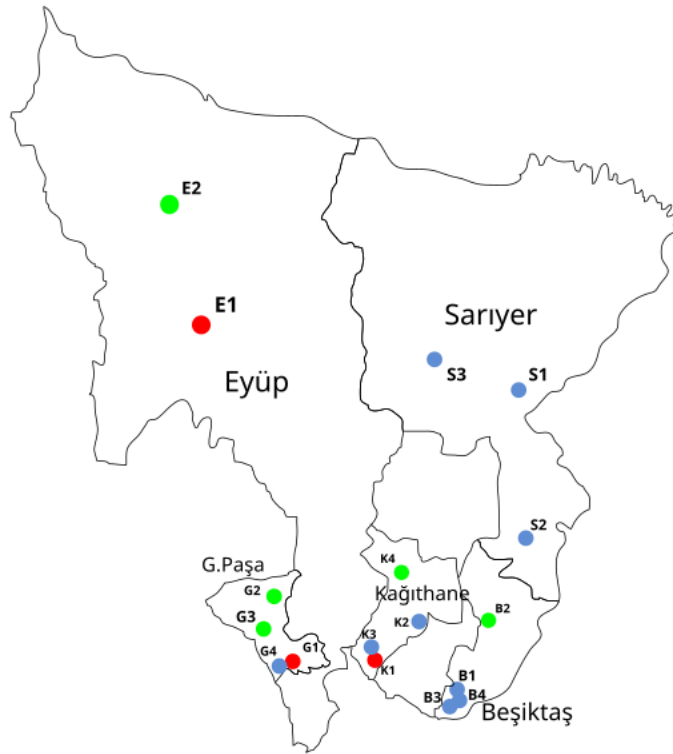


Figure 4.5: The map shows the places where the *C. pipiens* larvae of region 2 are resistant according to the Abbott value at 24 and 48 h after the *B. sphaericus* test. The color code is red for resistant, blue for susceptible, and green for possibly resistant.

According to the Abbott formula, 16 larvae, 6 from Eyüp, 4 from Kağıthane, and 6 from Gaziosmanpaşa, developed resistance to the toxin protein and remained alive 48 h after *B. sphaericus* was tested on *C. pipiens* larvae in the 1st region. In Beşiktaş, and Sarıyer districts, all larvae died and no resistance was observed (Figure 4.5).

4.1.4. Resistance Status of *C. pipiens* Larvae from Region 2 After 24-48 Hours Trial of *B. Thuringiensis Subsp. Israelensis* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Eyüp, Kağıthane, Gaziosmanpaşa, Beşiktaş, and Sarıyer, which we have separated and named as region 2, were examined for *B. thuringiensis subsp. israelensis* biological larvicide and the results were evaluated 24 and 48 h later with the Abbott formula (Table 4.10).

Table 4.7: Resistance status of *C. pipiens* larvae of region 2 at 24 and 48 h after *B. thuringiensis subsp. israelensis* testing.

Region 2	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Beşiktaş	25	0	0	25	24	100%	100%	ok	S
Beşiktaş	25	0	0	24	21	100%	100%	ok	S
Eyüp	25	0	0	24	23	100%	100%	ok	S
Gaziosmanpaşa	25	0	0	21	20	100%	100%	ok	S
Gaziosmanpaşa	25	0	0	24	34	100%	100%	ok	S
Kağıthane	25	0	0	24	21	100%	100%	ok	S
Kağıthane	25	0	0	23	22	100%	100%	ok	S
Sarıyer	25	0	0	23	21	100%	100%	ok	S

Table 4.8: Abbott values of *C. pipiens* larvae of region 2 at 24 and 48 h after *B. thuringiensis subsp. israelensis* testing.

Region	Tested with <i>B. sphericus</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
2	Experimental Group	200	0	0
	Control Group	200	188	186
	Abbot Value	-	100%	100%

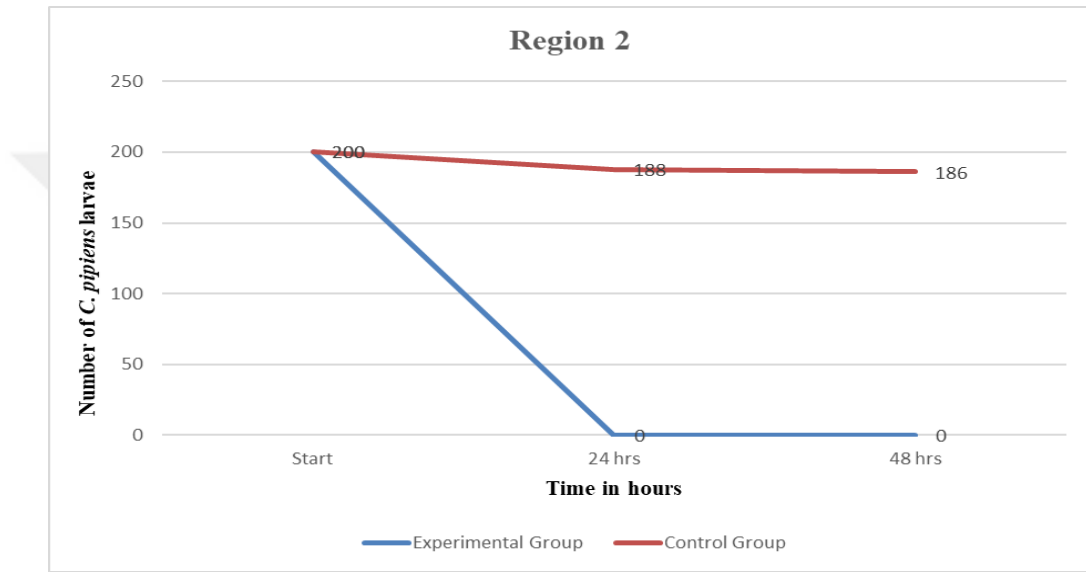


Figure 4.6: The graph of Abbott values of *C. pipiens* larvae of region 2 at 24, and 48 h *B. thuringiensis subsp. israelensis* testing.

According to Abbott's formula, 24 h after *B. thuringiensis subsp. israelensis* was tested on *C. pipiens* larvae in the 2nd region, in Beşiktaş, Eyüp, Gaziosmanpaşa, Kağıthane, and Sarıyer districts, all larvae died and no resistance was observed (Figure 4.6).

4.1.5. Resistance Status of *C. pipiens* Larvae from Region 3 After 24-48 Hours Trial of *B. Sphaericus* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Beylikdüzü, Büyükçekmece, Çatalca, Esenyurt, and Silivri, which we have separated and named as region 3, were examined for *B. sphaericus* biological larvicide. The results were evaluated 24 and 48 h later with the Abbott formula (Table 4.13).

Table 4.9: Resistance status of *C. pipiens* larvae of region 3 at 24 and 48 h after *B. sphaericus* testing.

Region 3	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Beylikdüzü	25	0	0	24	21	100,00%	100,00%	ok	S
Beylikdüzü	25	11	8	25	25	56,00%	68,00%	ok	R
Beylikdüzü	25	0	0	23	21	100,00%	100,00%	ok	S
Büyükçekmece	25	0	0	22	18	100,00%	100,00%	not ok	S
Büyükçekmece	25	2	1	24	20	91,67%	95,00%	ok	PR
Büyükçekmece	25	4	0	25	24	84,00%	100,00%	ok	S
Çatalca	25	3	1	24	21	87,50%	95,24%	ok	PR
Çatalca	25	2	0	23	21	91,30%	100,00%	ok	S
Çatalca	25	0	0	20	19	100,00%	100,00%	ok	S
Esenyurt	25	3	2	24	21	87,50%	90,48%	ok	PR
Esenyurt	25	5	5	25	20	80,00%	75,00%	ok	R
Esenyurt	25	10	6	23	20	56,52%	70,00%	ok	R
Esenyurt	25	13	11	25	20	48,00%	45,00%	ok	R
Silivri	25	4	2	23	21	82,61%	90,48%	ok	PR
Silivri	25	4	1	24	22	83,33%	95,45%	ok	PR
Silivri	25	0	0	23	19	100,00%	100,00%	ok	S

Table 4.10: Abbott values of *C. pipiens* larvae of region 3 at 24, and 48 h after *B. sphaericus* testing.

Region	Tested with <i>B. sphaericus</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
3	Experimental Group	400	61	37
	Control Group	400	377	333
	Abbot Value	-	83.41%	89.04%

B. sphaericus was tested and 61 *C. pipiens* larvae survived after 24 h and 37 *C. pipiens* larvae survived after 48 h (Figure 4.7).

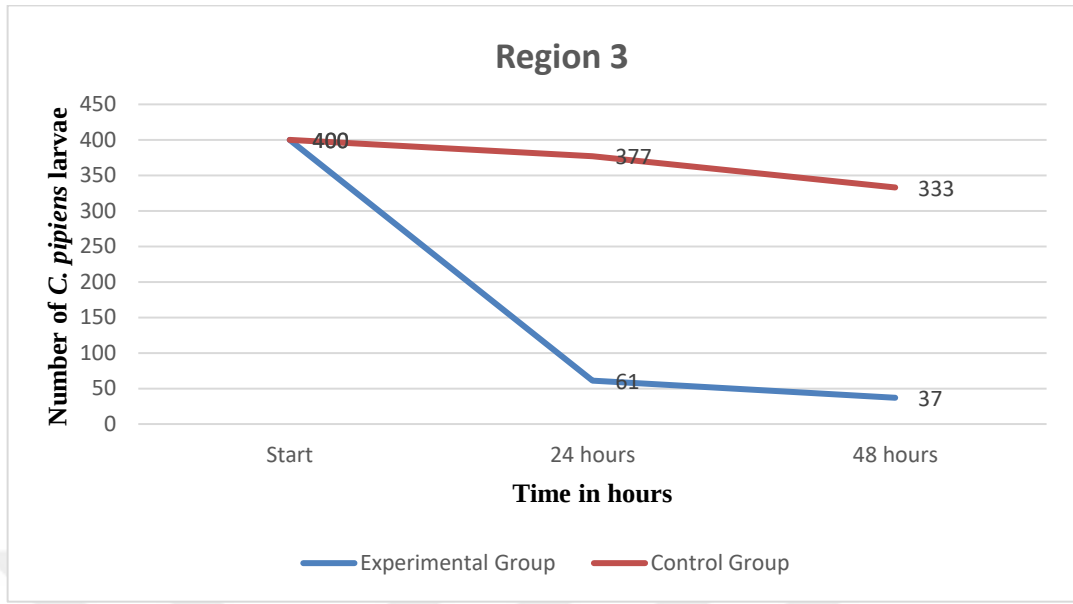


Figure 4.7: The graph of Abbott values of *C. pipiens* larvae of region 3 at 24, and 48 h after *B. sphaericus* testing.

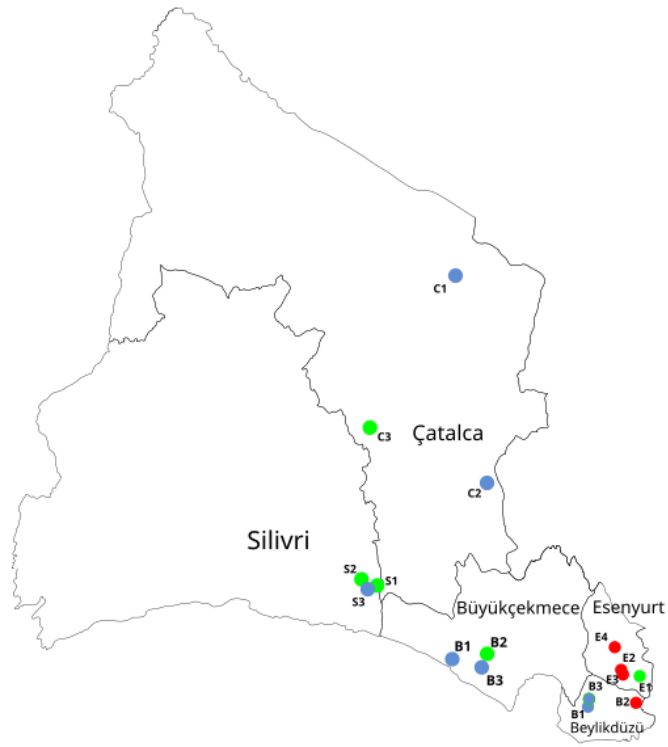


Figure 4.8: The map shows the places where the *C. pipiens* larvae of the 3rd region are resistant according to the Abbott value at 24 and 48 h after the *B. sphaericus* test. The color code is red for resistant, blue for susceptible, and green for possibly resistant.

According to the Abbott formula, 30 larvae, including 8 from Beylikdüzü, and 22 from Esenyurt, developed resistance to the toxin protein and remained alive 48 h after *B. sphaericus* was tested on *C. pipiens* larvae in region 3. In Büyükçekmece, Çatalca, and Silivri districts, all larvae died and no resistance was observed (Figure 4.8).

4.1.6. Resistance Status of *C. pipiens* Larvae from Region 3 After 24-48 Hours Trial of *B. Thuringiensis Subsp. Israelensis* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Beylikdüzü, Büyükçekmece, Çatalca, Esenyurt, and Silivri, which we have separated and named as region 3, were examined for *B. thuringiensis subsp. israelensis* biological larvicide and the results were evaluated 24 and 48 h later with the Abbott formula (Table 4.16).

Table 4.11: Resistance status of *C. pipiens* larvae of region 3 at 24 and 48 h after *B. thuringiensis subsp. israelensis* testing.

Region 3	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Beylikdüzü	25	0	0	25	23	100%	100%	ok	S
Büyükçekmece	25	0	0	23	22	100%	100%	ok	S
Büyükçekmece	25	0	0	25	23	100%	100%	ok	S
Çatalca	25	0	0	22	20	100%	100%	ok	S
Çatalca	25	0	0	24	21	100%	100%	ok	S
Esenyurt	25	0	0	25	23	100%	100%	ok	S
Silivri	25	0	0	25	23	100%	100%	ok	S
Silivri	25	0	0	23	22	100%	100%	ok	S

Table 4.12: Abbott values of *C. pipiens* larvae of region 3 at 24, and 48 h after *B. thuringiensis subsp. israelensis* testing.

Region	Tested with <i>B. thuringiensis subsp. israelensis</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
3	Experimental Group	200	0	0
	Control Group	200	192	177
	Abbot Value	-	100%	100%

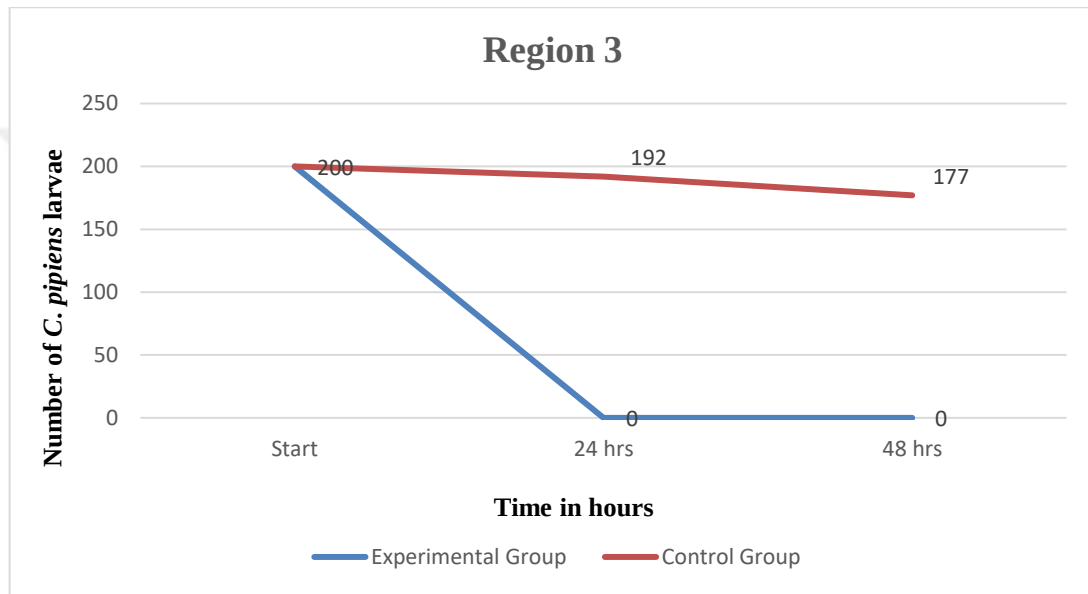


Figure 4.9: The graph of Abbott values of *C. pipiens* larvae of region 3 at 24 and 48 h after *B. thuringiensis subsp. israelensis* testing.

According to Abbott's formula, 24 h after *B. thuringiensis subsp. israelensis* was tested on *C. pipiens* larvae in region 3, in Beylikdüzü, Büyükçekmece, Çatalca, Esenyurt, and Silivri districts, all larvae died and no resistance was observed (Figure 4.9).

4.1.7. Resistance Status of *C. pipiens* Larvae from Region 4 After 24-48 Hours Trial of *B. Sphaericus* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Arnavutköy, Avcılar, Başakşehir, Sultangazi, and Küçükçekmece, which we have separated and named as region 4, were examined for *B. sphaericus* biological larvicide and the results were evaluated at 24 and 48 h later with Abbott formula (Table 4.19).

Table 4.13: Resistance status of *C. pipiens* larvae of region 4 at 24, and 48 h after *B. sphaericus* testing.

Region 4	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Arnavutköy	25	9	8	25	24	64,00%	66,67%	ok	R
Arnavutköy	25	8	7	24	21	66,67%	66,67%	ok	R
Arnavutköy	25	9	1	20	20	55,00%	95,00%	ok	PR
Arnavutköy	25	4	0	20	19	80,00%	100,00%	ok	S
Avcılar	25	6	0	25	25	76,00%	100,00%	ok	S
Avcılar	25	2	0	23	21	91,30%	100,00%	ok	S
Avcılar	25	4	2	25	24	84,00%	91,67%	ok	PR
Avcılar	25	0	0	24	22	100,00%	100,00%	ok	S
Başakşehir	25	4	2	20	19	80,00%	89,47%	ok	R
Başakşehir	25	4	0	22	20	81,82%	100,00%	ok	S
Başakşehir	25	9	1	23	23	60,87%	95,65%	ok	PR
Başakşehir	25	5	1	25	23	80,00%	95,65%	ok	PR
Küçükçekmece	25	0	0	25	21	100,00%	100,00%	ok	S
Küçükçekmece	25	4	2	20	20	80,00%	90,00%	ok	PR
Küçükçekmece	25	3	1	25	25	88,00%	96,00%	ok	PR
Küçükçekmece	25	2	0	24	22	91,67%	100,00%	ok	S
Sultangazi	25	7	0	20	18	65,00%	100,00%	not ok	S
Sultangazi	25	12	9	23	21	47,83%	57,14%	ok	R
Sultangazi	25	11	3	22	22	50,00%	86,36%	ok	R
Sultangazi	25	15	10	25	24	40,00%	58,33%	ok	R

Table 4.14: Abbott values of *C. pipiens* larvae of region 4 at 24, and 48 h after *B. sphaericus* testing.

Region	Tested with <i>B. sphaericus</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
4	Experimental Group	500	118	47
	Control Group	500	460	434
	Abbot Value	-	74.55%	89.43%

B. sphaericus was tested and 118 *C. pipiens* larvae survived after 24 h and 47 *C. pipiens* larvae survived after 48 h (Figure 4.10).

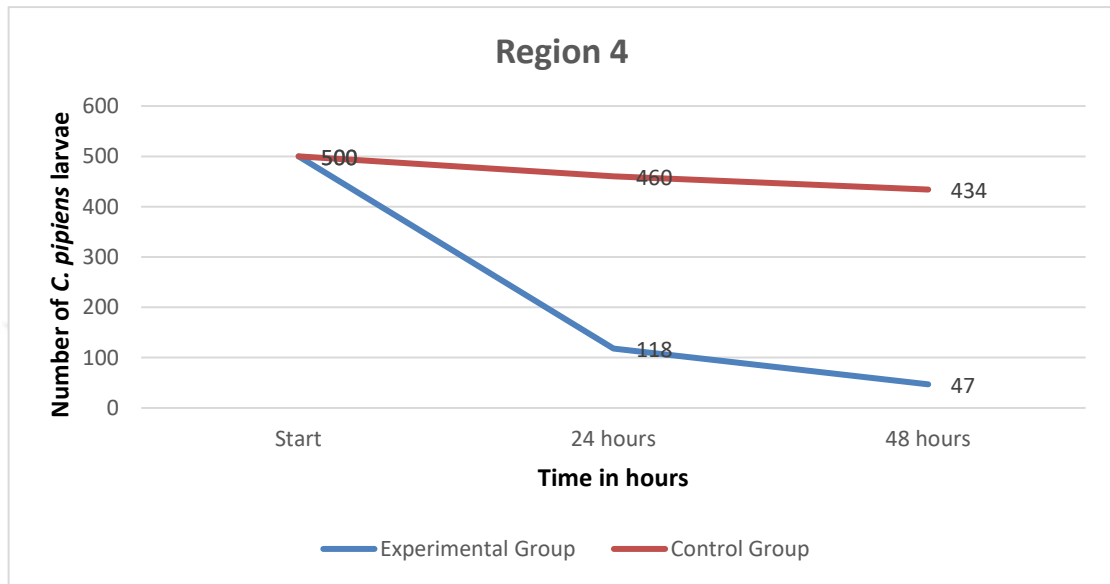


Figure 4.10: The graph of Abbott values of *C. pipiens* larvae of region 4 at 24 and 48 h after *B. sphaericus* testing.

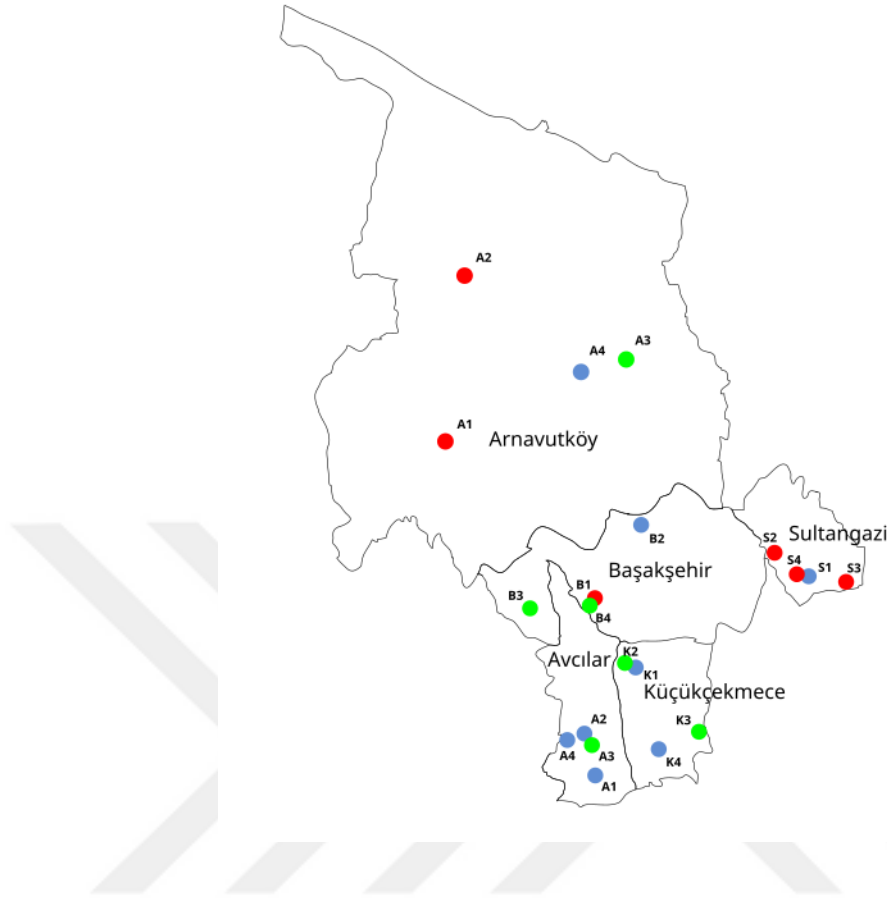


Figure 4.11: The map shows the places where the *C. pipiens* larvae of the 4th region are resistant according to the Abbott value at 24 and 48 h after the *B. sphaericus* test. The color code is red for resistant, blue for susceptible, and green for possibly resistant.

According to the Abbott formula, 59 larvae, including 17 from Arnavutköy, 4 from Başakşehir, and 38 from Sultangazi, developed resistance to the toxin protein and remained alive 48 h after *B. sphaericus* was tested on *C. pipiens* larvae in the region 1. In Avcılar, and Küçükçekmece districts, all larvae died and no resistance was observed (Figure 4.11).

4.1.8. Resistance Status of *C. pipiens* Larvae from Region 4 After 24-48 Hours Trial of *B. Thuringiensis Subsp. Israelensis* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Arnavutköy, Avcılar, Başakşehir, Sultangazi, and Küçükçekmece which we have separated and named as region 4, were examined for *B. thuringiensis subsp. israelensis* biological larvicide and the results were evaluated 24 and 48 h later with the Abbott formula (Table 4.22).

Table 4.15: Resistance status of *C. pipiens* larvae of region 4 at 24, and 48 h after *B. thuringiensis* subsp. *israelensis* testing.

Region 4	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Arnavutköy	25	0	0	24	21	100%	100%	ok	S
Arnavutköy	25	0	0	22	18	100%	100%	not ok	S
Avcılar	25	0	0	23	20	100%	100%	ok	S
Başakşehir	25	0	0	22	21	100%	100%	ok	S
Başakşehir	25	0	0	21	21	100%	100%	ok	S
Küçükçekmece	25	0	0	23	21	100%	100%	ok	S
Küçükçekmece	25	0	0	21	20	100%	100%	ok	S
Sultangazi	25	0	0	23	19	100%	100%	ok	S
Sultangazi	25	0	0	20	20	100%	100%	ok	S

Table 4.16: Abbott values of *C. pipiens* larvae of region 4 at 24, and 48 h after *B. thuringiensis* subsp. *israelensis* testing.

Region	Tested with <i>B. thuringiensis</i> subsp. <i>israelensis</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
4	Experimental Group	225	0	0
	Control Group	225	199	181
	Abbot Value	-	100%	100%

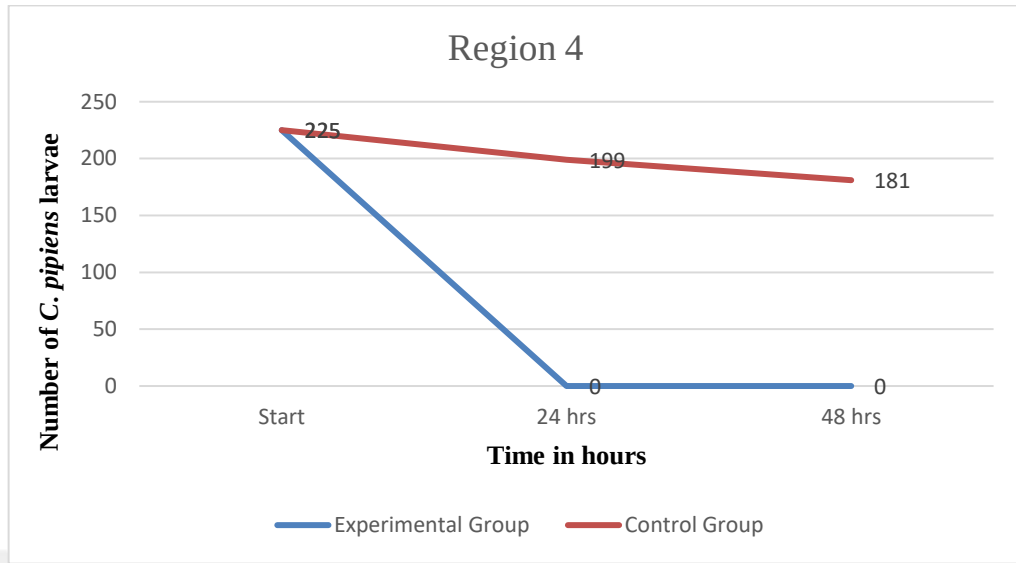


Figure 4.12: The graph of Abbott values of *C. pipiens* larvae of region 4 at 24, and 48 h after *B. thuringiensis subsp. israelensis* testing.

According to Abbott's formula, 24 h after *B. thuringiensis subsp. israelensis* was tested on *C. pipiens* larvae in the 1st region, in Arnavutköy, Avcılar, Başakşehir, Sultangazi, and Küçükçekmece districts, all larvae died and no resistance was observed (Figure 4.12).

4.1.9. Resistance Status of *C. pipiens* Larvae from Region 5 After 24-48 Hours Trial of *B. Sphaericus* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Ataşehir, Beykoz, Çekmeköy, Kadıköy, Ümraniye, and Üsküdar, which we have separated and named as region 5, were examined for *B. sphaericus* biological larvicide and the results were evaluated at 24 and 48 h later with the Abbott formula (Table 4.25).

Table 4.17: Resistance status of *C. pipiens* larvae of region 5 at 24 and 48 h after *B. sphaericus* testing.

Region 5	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Ataşehir	25	0	0	25	21	100,00%	100,00%	ok	S
Ataşehir	25	0	0	25	25	100,00%	100,00%	ok	S
Beykoz	25	5	0	23	20	78,26%	100,00%	ok	S
Beykoz	25	6	2	23	21	73,91%	90,48%	ok	PR
Beykoz	25	7	2	25	24	72,00%	91,67%	ok	PR
Çekmeköy	25	4	4	22	20	81,82%	80,00%	ok	R
Çekmeköy	25	11	0	24	22	54,17%	100,00%	ok	S
Çekmeköy	25	0	0	20	19	100,00%	100,00%	ok	S
Kadıköy	25	2	2	24	21	91,67%	90,48%	ok	PR
Kadıköy	25	3	2	21	20	85,71%	90,00%	ok	PR
Kadıköy	25	2	1	25	20	92,00%	95,00%	ok	PR
Ümraniye	25	9	4	25	24	64,00%	83,33%	ok	R
Ümraniye	25	3	0	24	22	87,50%	100,00%	ok	S
Ümraniye	25	3	3	24	22	87,50%	86,36%	ok	R
Üsküdar	25	4	3	23	21	82,61%	85,71%	ok	R
Üsküdar	25	5	4	23	23	78,26%	82,61%	ok	R
Üsküdar	25	1	0	24	19	95,83%	100,00%	ok	S

Table 4.18: Abbott values of *C. pipiens* larvae of region 5 at 24 and 48 h after *B. sphaericus* testing.

Region	Tested with <i>B. sphaericus</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
5	Experimental Group	425	65	27
	Control Group	425	400	364
	Abbot Value	-	83.81%	92.68%

B. sphaericus was tested and 65 *C. pipiens* larvae survived after 24 h and 27 *C. pipiens* larvae survived after 48 h (Figure 4.13).

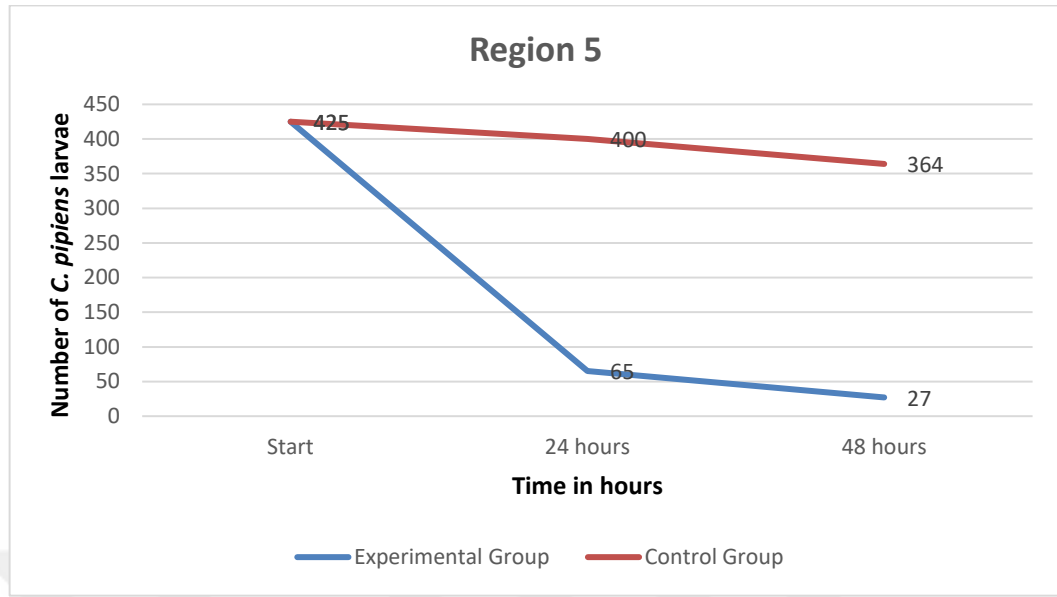


Figure 4.13: The graph of Abbott values of *C. pipiens* larvae of region 5 at 24, and 48 h after *B. sphaericus* testing.

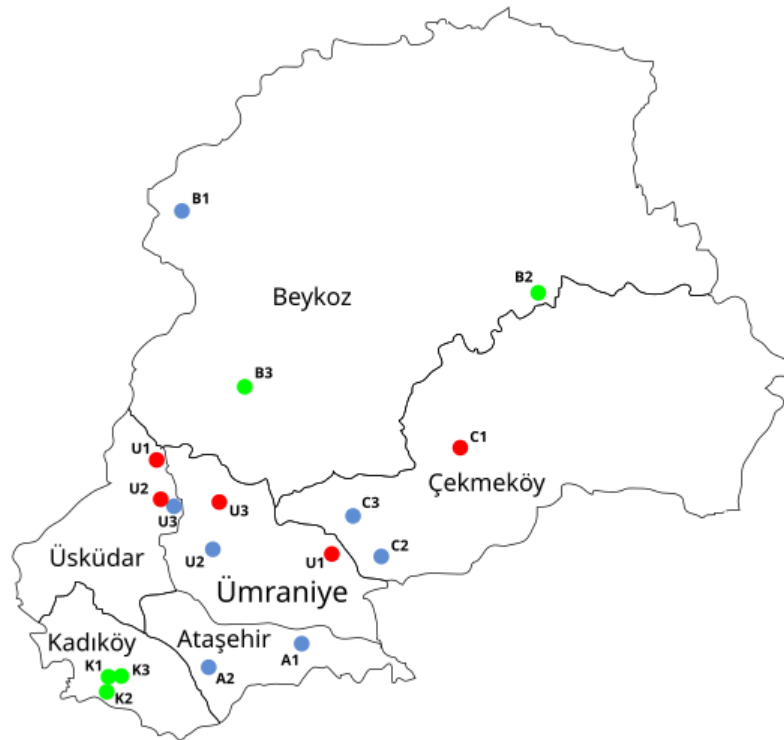


Figure 4.14: The map shows the places where the *C. pipiens* larvae of region 5 are resistant according to the Abbott value 24 and 48 h after the *B. sphaericus* test. The color code is red for resistant, blue for susceptible, and green for possibly resistant.

According to the Abbott formula, 18 larvae, 4 from Çekmeköy, 7 from Ümraniye, and 7 from Üsküdar, developed resistance to the toxin protein and remained alive 48 h after *B. sphaericus* was tested on *C. pipiens* larvae in the 5th region. In Ataşehir, and Beykoz, districts, all larvae died and no resistance was observed (Figure 4.14).

4.1.10. Resistance Status of *C. pipiens* Larvae from Region 5 After 24-48 Hours Trial of *B. Thuringiensis Subsp. Israelensis* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Arnavutköy, Avcılar, Başakşehir, Sultangazi, and Küçükçekmece, which we have separated and named as region 5, were examined for *B. thuringiensis subsp. israelensis* biological larvicide and the results were evaluated 24 and 48 h later with the Abbott formula (Table 4.28).

Table 4.19: Resistance status of *C. pipiens* larvae of region 5 at 24 and 48 h after *B. thuringiensis subsp. israelensis* testing.

Region 5	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Ataşehir	25	0	0	22	21	100%	100%	ok	S
Ataşehir	25	0	0	23	20	100%	100%	ok	S
Beykoz	25	0	0	21	20	100%	100%	ok	S
Beykoz	25	0	0	20	20	100%	100%	ok	S
Çekmeköy	25	0	0	21	20	100%	100%	ok	S
Kadıköy	25	0	0	24	24	100%	100%	ok	S
Ümraniye	20	0	0	20	20	100%	100%	ok	S
Üsküdar	25	0	0	24	21	100%	100%	ok	S
Üsküdar	25	0	0	25	21	100%	100%	ok	S

Table 4.20: Abbott values of *C. pipiens* larvae of region 5 at 24 and 48 h after *B. thuringiensis subsp. israelensis* testing.

Region	Tested with <i>B. thuringiensis subsp. israelensis</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
1	Experimental Group	220	0	0
	Control Group	220	200	187
	Abbot Value	-	100%	100%

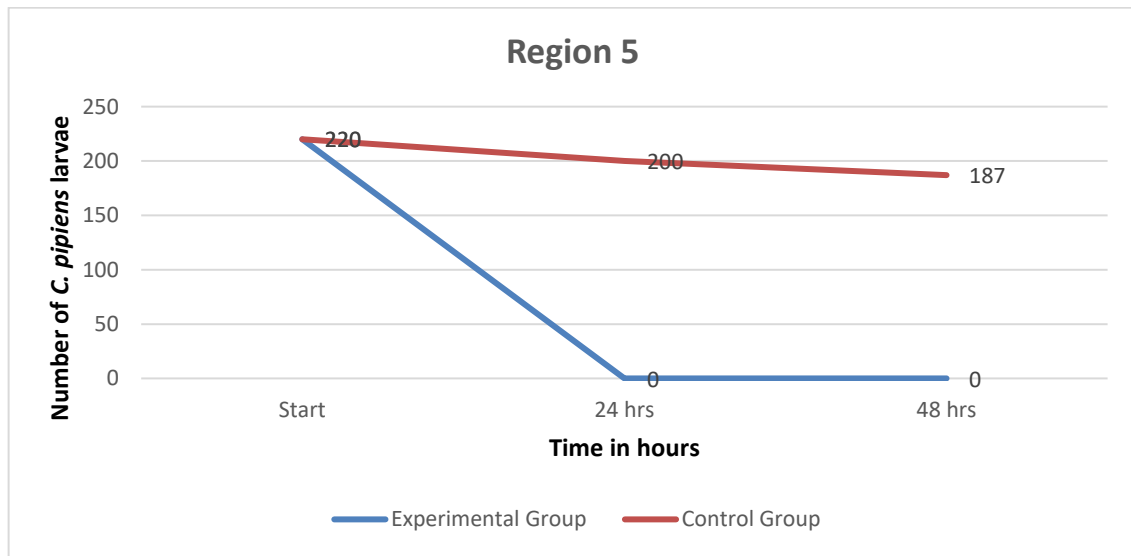


Figure 4.15: The graph of Abbott values of *C. pipiens* larvae of region 5 at 24, and 48 h after *B. thuringiensis subsp. israelensis* testing.

According to Abbott's formula, 24 h after *B. thuringiensis subsp. israelensis* was tested on *C. pipiens* larvae in region 5, in Ataşehir, Beykoz, Çekmeköy, Kadıköy, Ümraniye, and Üsküdar districts, all larvae died and no resistance was observed (Figure 4.15).

4.1.11. Resistance Status of *C. pipiens* Larvae from Region 6 After 24-48 Hours Trial of *B. Sphaericus* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Adalar, Kartal, Maltepe, Pendik, Sancaktepe, Sultanbeyli, Şile, and Tuzla, which we have separated and named as region 6, were examined for *B. sphaericus* biological larvicide and the results were evaluated at 24 and 48 h later with Abbott formula (Table 4.31).

Table 4.21: Resistance status of *C. pipiens* larvae of region 6 at 24, and 48 h after *B. sphaericus* testing.

Region 6	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Adalar	25	0	0	23	19	100,00%	100,00%	ok	S
Kartal	25	2	2	23	21	91,30%	90,48%	ok	PR
Kartal	25	2	1	20	20	90,00%	95,00%	ok	PR
Kartal	25	0	0	25	24	100,00%	100,00%	ok	S
Maltepe	25	0	0	21	20	100,00%	100,00%	ok	S
Pendik	25	1	1	25	25	96,00%	96,00%	ok	PR
Pendik	25	0	0	24	22	100,00%	100,00%	ok	S
Pendik	25	0	0	23	21	100,00%	100,00%	ok	S
Sancaktepe	25	2	1	25	24	92,00%	95,83%	ok	PR
Sancaktepe	25	1	0	24	22	95,83%	100,00%	ok	S
Sancaktepe	25	0	0	24	18	100,00%	100,00%	not ok	S
Sultanbeyli	25	0	0	22	20	100,00%	100,00%	ok	S
Sultanbeyli	25	0	0	20	19	100,00%	100,00%	ok	S
Şile	25	0	0	24	22	100,00%	100,00%	ok	S
Şile	25	0	0	25	25	100,00%	100,00%	ok	S
Tuzla	25	4	2	22	20	81,82%	90,00%	ok	PR
Tuzla	25	3	0	24	21	87,50%	100,00%	ok	S
Tuzla	25	0	0	25	24	100,00%	100,00%	ok	S

Table 4.22: Abbott values of *C. pipiens* larvae of region 6 at 24, and 48 h after *B. sphaericus* testing.

Region	Tested with <i>B. sphaericus</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
6	Experimental Group	450	15	7
	Control Group	450	419	387
	Abbot Value	-	96.4%	98.18%

B. sphaericus was tested and 15 *C. pipiens* larvae survived after 24 h and 7 *C. pipiens* larvae survived after 48 h (Figure 4.16).

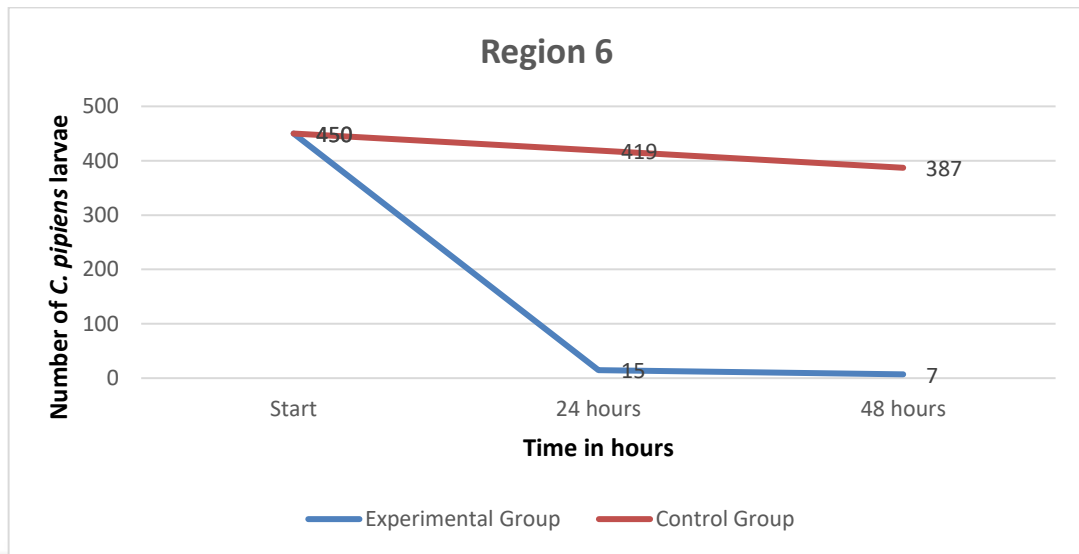


Figure 4.16: The graph of Abbott values of *C. pipiens* larvae of region 6 at 24, and 48 h after *B. sphaericus* testing.

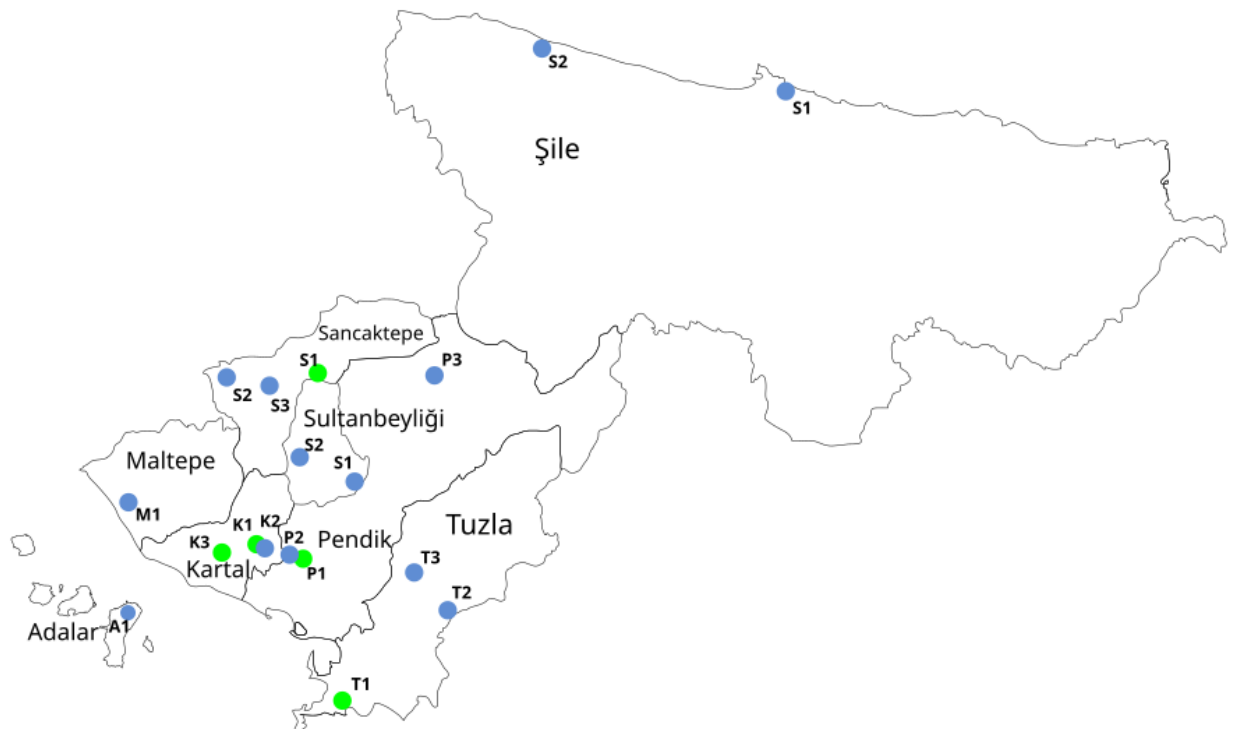


Figure 4.17: The map shows the places where the *C. pipiens* larvae of region 6 are resistant according to the Abbott value at 24, and 48 h after the *B. sphaericus* test. The color code is red for resistant, blue for susceptible, and green for possibly resistant.

According to the Abbott formula, all larvae died in Adalar, Kartal, Maltepe, Pendik, Sancaktepe, Sultanbeyli, Şile, and Tuzla, 48 h after *B. sphaericus* was tested on *C. pipiens* larvae in the 6th region, and no resistance was observed (Figure 4.17).

4.1.12. Resistance Status of *C. pipiens* Larvae from Region 6 After 24-48 Hours Trial of *B. Thuringiensis Subsp. Israelensis* Larvicide

C. pipiens 3rd and 4th instar larvae from the districts of Adalar, Kartal, Maltepe, Pendik, Sancaktepe, Sultanbeyli, Şile, and Tuzla, which we have separated and named as region 6, were examined for *B. thuringiensis subsp. israelensis* biological larvicide and the results were evaluated 24 and 48 h later with the Abbott formula (Table 4.34).

Table 4.23: Resistance status of *C. pipiens* larvae of region 6 at 24, and 48 h after *B. thuringiensis subsp. israelensis* testing.

Region 6	Total larvae (n)	Alive larvae on the test group at 24h (n)	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on 24h	Abbott value on 48h	Control status	Status
Adalar	25	0	0	23	20	100%	100%	ok	S
Adalar	25	0	0	22	21	100%	100%	ok	S
Kartal	25	0	0	20	19	100%	100%	ok	S
Maltepe	25	0	0	24	21	100%	100%	ok	S
Pendik	25	0	0	25	25	100%	100%	ok	S
Pendik	25	0	0	23	22	100%	100%	ok	S
Sancaktepe	25	0	0	20	20	100%	100%	ok	S
Sancaktepe	25	0	0	21	20	100%	100%	ok	S
Sultanbeyli	25	0	0	23	21	100%	100%	ok	S
Sultanbeyli	25	0	0	24	21	100%	100%	ok	S
Şile	25	0	0	24	20	100%	100%	ok	S
Şile	25	0	0	21	20	100%	100%	ok	S
Tuzla	25	0	0	24	21	100%	100%	ok	S
Tuzla	25	0	0	23	23	100%	100%	ok	S

Table 4.24: Abbott values of *C. pipiens* larvae of region 6 at 24, and 48 h after *B. thuringiensis subsp. israelensis* testing.

Region	Tested with <i>B. thuringiensis subsp. israelensis</i>	The number of live larvae (n)		
		Total Larvae (n)	at 24 hours	at 48 hours
6	Experimental Group	350	0	0
	Control Group	350	317	294
	Abbot Value	-	100%	100%

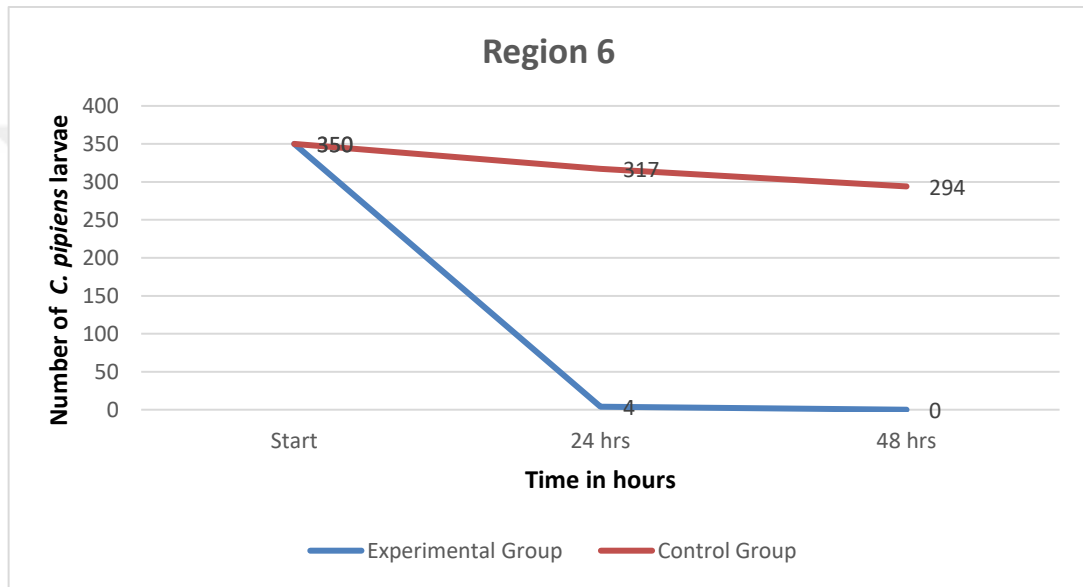


Figure 4.18: The graph of Abbott values of *C. pipiens* larvae of region 6 at 24, and 48 h after *B. thuringiensis subsp. israelensis* testing.

According to Abbott's formula, 24 h after *B. thuringiensis subsp. israelensis* was tested on *C. pipiens* larvae in the 6th region, in Adalar, Kartal, Maltepe, Pendik, Sancaktepe, Sultanbeyli, Şile, and Tuzla districts, all larvae died and no resistance was observed (Figure 4.18).

As a result of the observations of the experiments for *B. thuringiensis subsp. israelensis*, it was observed that 99.75 % of the larvae of *C. pipiens* species died on average in the first 24 h and 100% within 48 h.

As a result of the observations of the experiments in which *B. sphaericus* was tested, it was observed that 84.72% of the larvae of *C. pipiens* species died in the first 24 h and 91.93% in the 48 h.

4.2. COMPARISON of ABBOTT VALUES of 6 REGIONS

There were 198 *C. pipiens* larvae that resistance was expected according to the Abbott formula where the larvae tested with *B. sphaericus* was tested. The values are taken after 48 h and the plot is drawn according to the 6 regions and ordered with Abbot values (Figure 4.19).

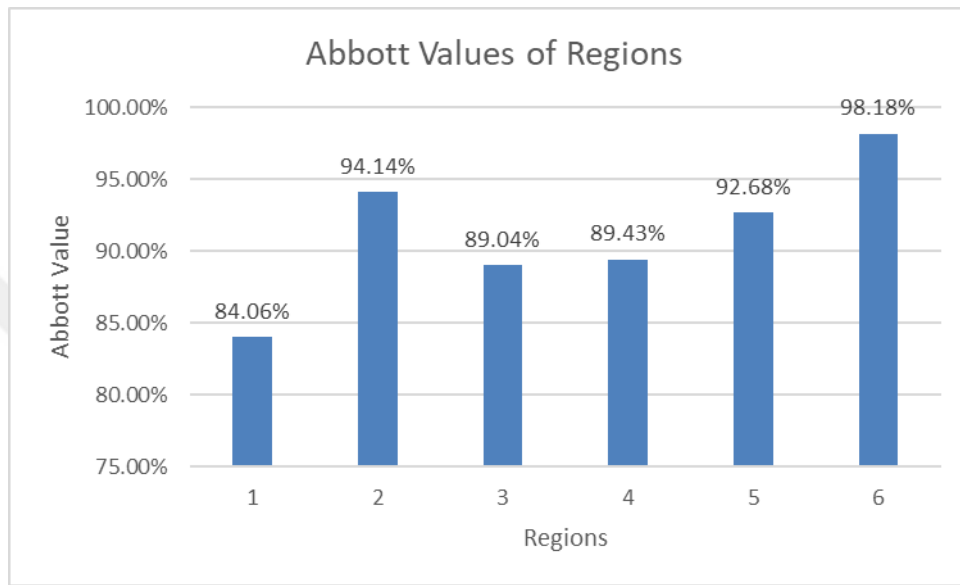


Figure 4.19: Distribution of average Abbott values of regions.

Abbott values of *C. pipiens* larvae tested for *B. sphaericus* after 48 h, region 1, 84.06 %, 2nd region 94.14%, 3rd region 89.04%, 4th region 89.43%, 5th region 92.68%, and 6th region 98.18%.

The region 1 was the most resistant region with the lowest 84.06%, Abbott value. The region 6 was the lowest resistance region with the highest Abbott value 98.18%.

The resistance rates of the districts with resistant *C. pipiens* larvae according to the Abbott values in which *B. sphaericus* was tested are shown in Table 4.25 and the graph is shown in Figure 4.20.

Table 4.25: Abbott values of the larvae that we detected resistant to *B. sphaericus* in the districts of Istanbul.

District	Number of Experiments	Total larvae (n)	Alive larvae on the test group	Alive larvae on the test group at 48h (n)	Alive larvae control on 24h (n)	Alive larvae control on 48h (n)	Abbott value on24h	Abbott value on48h	Control status
Arnavutköy	100	30	16	89	84	0,66%	82.08%	ok	Resistant
Bağcılar	50	10	7	48	41	0,79%	82.86%	ok	Resistant
Bahçelievler	100	25	20	95	87	0,73%	77.46%	ok	Resistant
Beylikdüzü	75	11	8	72	67	0,84%	89.33%	ok	Resistant
Bayrampaşa	100	31	20	94	85	0,67%	75.98%	ok	Resistant
Esenler	75	19	16	68	61	0,72%	73.97%	ok	Resistant
Esenyurt	100	31	24	97	81	0,68%	70.12%	ok	Resistant
Eyüp	50	8	7	49	42	0,83%	82.73%	ok	Resistant
Fatih	100	31	25	96	87	0,67%	71.12%	ok	Resistant
Güngören	75	17	9	68	60	0,75%	85.32%	ok	Resistant
Sultangazi	100	45	22	90	85	0,5%	75.46%	ok	Resistant
Ümraniye	75	15	7	73	68	0,79%	89.90%	ok	Resistant
Üsküdar	75	10	7	70	63	0,85%	89.44%	ok	Resistant

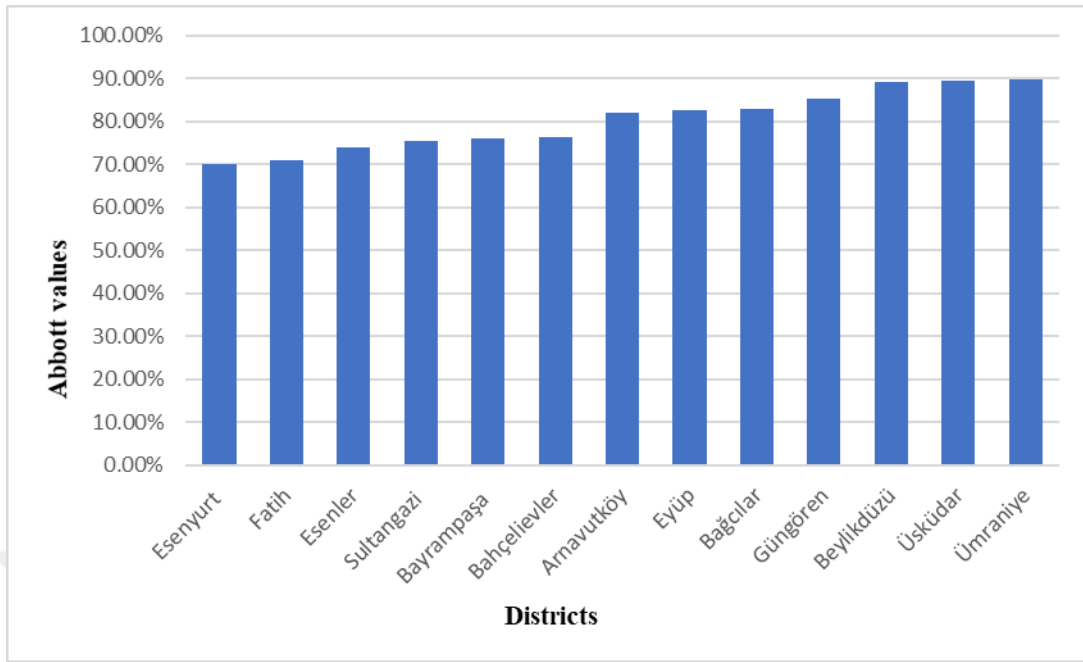


Figure 4.20: Distribution of resistance rates in districts according to Abbott values.

C. pipiens larvae, which we detected resistant to *B. sphaericus*, are seen in 13 districts of Istanbul according to Abbott values. Esenyurt's Abbott value is 70.12% and is seen as the most resistant district where *B. sphaericus* has been tested. In Ümraniye, the Abbott value is 89.90%, and the resistance rate where *B. sphaericus* was tried and is seen as the least district. Below shows the distribution of resistance in *C. pipiens* larvae against *B. sphaericus* in all districts of Istanbul according to Abbott results after 48 h, Figure 4.21.

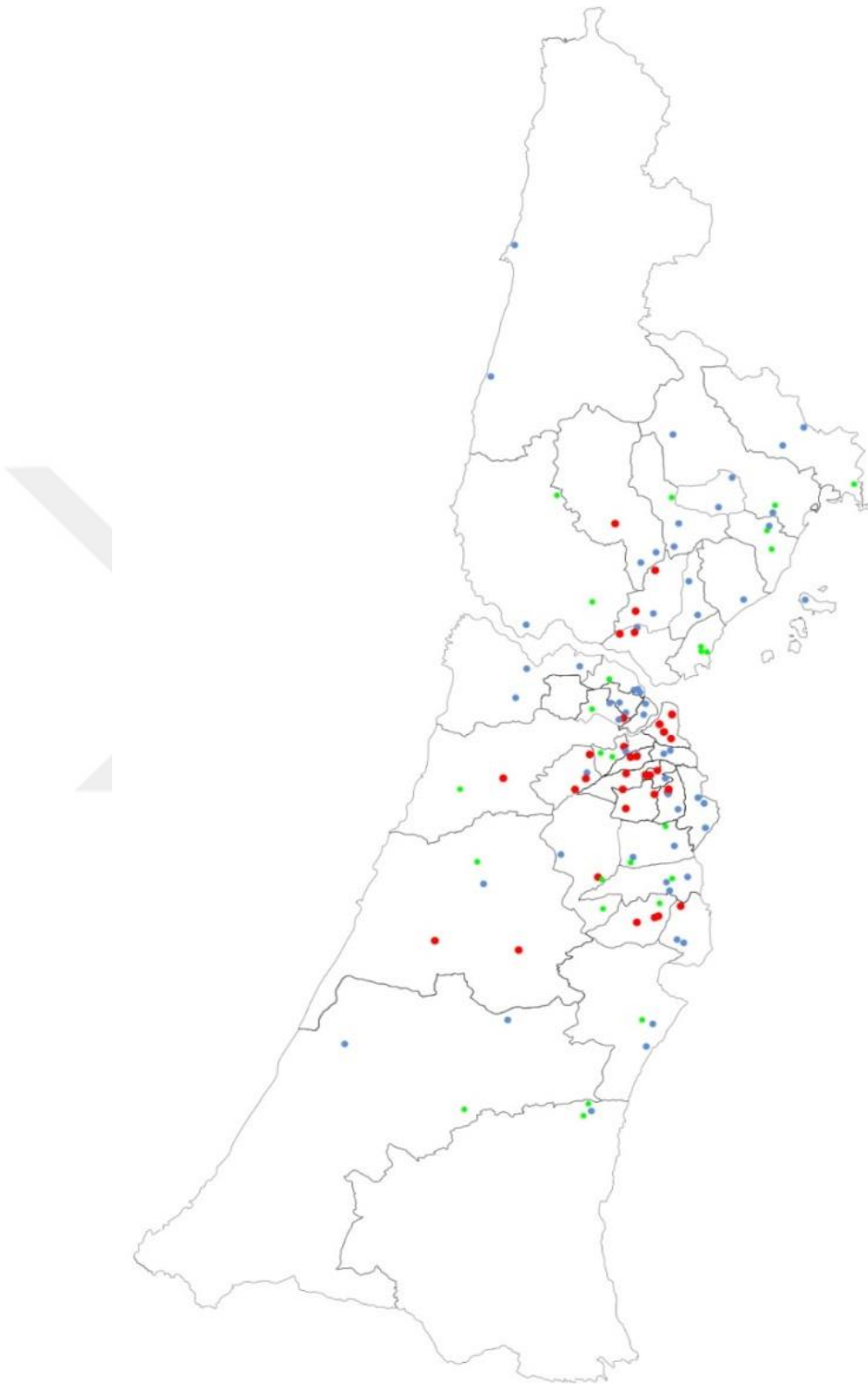


Figure 4.21: Distribution of *C. pipiens* larvae resistant to *B. sphaericus* according to Abbott results. The color code is red for resistant, blue for susceptible, and green for possibly resistant.

The distribution of the population density of 2020 by Istanbul districts is as in Figure 4.22 Istanbul neighborhoods population density map. According to the Abbott value of *C. pipiens* larvae, which we detected resistance to *B. sphaericus*, the regions with high resistance (especially region 1) and the places with population density are similar.



Figure 4.22: Istanbul district's population density map¹.

The 6th district, which includes the Adalar, Kartal, Maltepe, Pendik, Sancaktepe, Sultanbeyli, Şile, and Tuzla districts, is 1398.54 km, consists of 193 neighborhoods and has 3,072,629 population. The 1st region, which includes Bağcılar, Bahçelievler, Bakırköy, Bayrampaşa, Esenler, Fatih, Güngören, Beyoğlu, Şişli, and Zeytinburnu districts, is 154.21 km, consists of 226 neighborhoods and has 3,725,771 people^{2,3}. The districts in the 6th region are the places where there is more agriculture and animal husbandry compared to the districts in the 1st region, and the manufacturing, tourism, industry, and trade are in the districts in the 1st region. Region 1 has a lower Abbott value than region 6, so its resistance is higher (Figure 4.23).

¹ <https://www.atlasbig.com/tr/istanbul-mahalleleri-nufus-yogunlugu>

² <https://istanbultarihi.ist/85-istanbul-ilceleri>

³ https://tr.wikipedia.org/wiki/%C4%B0stanbul'un_il%C3%A7eleri

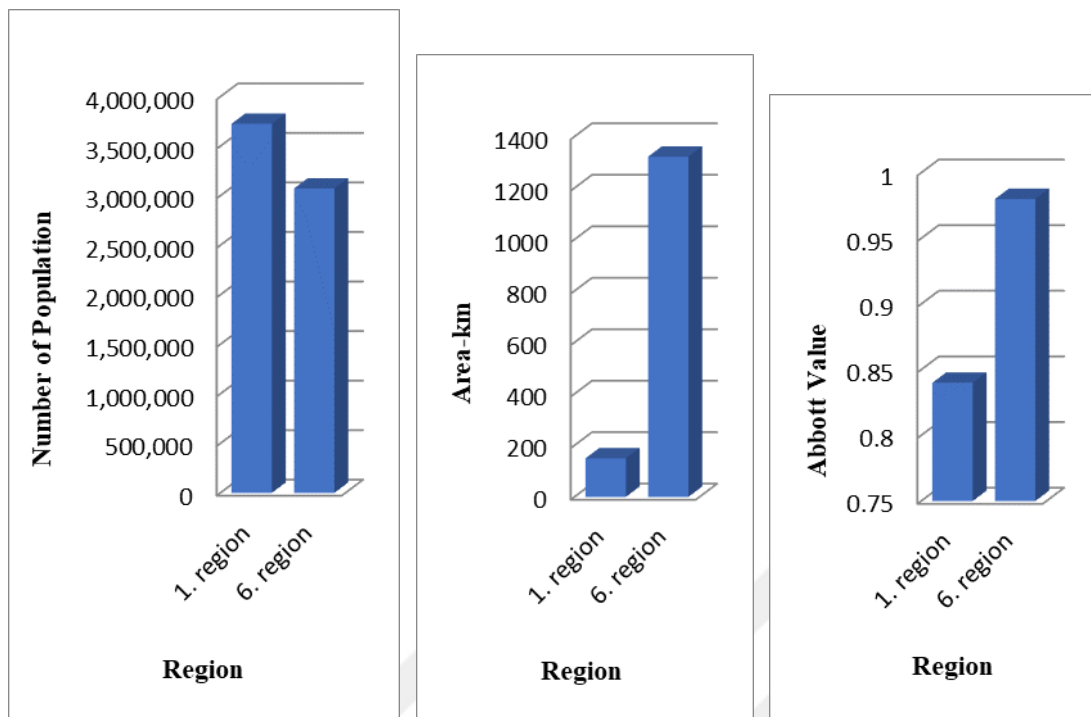


Figure 4.23: Comparison of the population numbers, the areas, and the Abbott values of the 1st and 6th regions.

4.3. ANOVA and ANCOVA ANALYSIS for the ABBOTT VALUES

Tukey Post Hoc Test was applied to evaluate the results of *C. pipiens* larvae, which detected resistance to *B. sphaericus* according to Abbott values, by applying a One-Way Analysis of Variance (One-Way ANOVA) and analysis of covariance (ANCOVA).

4.3.1. Cross Regional Analysis of the Effect of *B. sphaericus* on *C. pipiens* Larvae with One-Way ANOVA

The effects of *B. sphaericus* on *C. pipiens* were analyzed with One-Way ANOVA. The analysis includes the 24 and 48-hour effect of *B. sphaericus* on *C. pipiens* larvae (Table 4.26).

Table 4.26: Inter-regional comparison of 24 and 48 hours independently of each other.

						95% Confidence Interval for Mean			
Region	n	Mean	Std. Deviation	Std. Error		Lower Bound	Upper Bound	Min	Max
Abbott 24h	1	29	78,15%	19,29%	3,58%	70,81%	85,49%	28,57%	100,00%
	2	17	89,65%	10,24%	2,48%	84,38%	94,91%	69,57%	100,00%
	3	16	84,28%	16,87%	4,22%	75,29%	93,27%	48,00%	100,00%
	4	20	74,11%	17,25%	3,86%	66,03%	82,18%	40,00%	100,00%
	5	17	83,84%	12,91%	3,13%	77,20%	90,47%	54,17%	100,00%
	6	18	96,36%	5,54%	1,31%	93,61%	99,11%	81,82%	100,00%
Abbott 48h	1	29	84,06%	18,94%	3,52%	76,86%	91,27%	35,00%	100,00%
	2	17	94,14%	9,79%	2,37%	89,11%	99,17%	70,00%	100,00%
	3	16	89,04%	16,13%	4,03%	80,45%	97,63%	45,00%	100,00%
	4	20	89,43%	14,70%	3,29%	82,55%	96,31%	57,14%	100,00%
	5	17	92,68%	7,23%	1,75%	88,97%	96,40%	80,00%	100,00%
	6	18	98,18%	3,34%	0,79%	96,52%	99,85%	90,00%	100,00%

Table 4.27: Result of ANOVA for the Abbott values to compare 24 and 48 hours.

	F	p
Abbott 24h Between Groups	5,4811	0,0002
Abbott 48h Between Groups	2,8298	0,0192

Table 4.27, contains the calculation result of the OWA on the Abbott values of our groups for the two different measurements; counting the living larvae after 24 hours (24h) and after 48 hours (48h). The p values of both measurements are less than 0.05 with Abbott-24h having 0,0002 p-values and Abbott-48h having 0,0192 p-values.

The lower p-value indicates no NULL hypothesis in this experiment; i.e., at least one group with a mean value diverges from the other groups' means. ANOVA doesn't specify which population means are different. To classify the groups, a post-processing like Tukey HDS was needed.

4.3.2. Classification of the Result of Cross Regional ANOVA with to the Tukey HDS

Table 4.28: Multiple cross comparisons with Tukey HDS.

Dependent Variable	Region	Region	P	Dependent Variable	Region	Region	p
Abbott 24h	1	2	0,1336	Abbott 48h	1	2	0,1579
		3	0,7812			3	0,8488
		4	0,9400			4	0,7534
		5	0,8183			5	0,3100
		6	0,0014			6	0,0100
	2	3	0,9093		2	3	0,8904
		4	0,0268			4	0,9004
		5	0,8705			5	0,9996
		6	0,7752			6	0,9513
		4	0,3423		3	4	1,0000
	3	5	1,0000			5	0,9723
		6	0,1897			6	0,3762
		5	0,3736		4	5	0,9786
		6	0,0002			6	0,3621
	5	6	0,1463			6	0,8393

Table 4.28 includes two sets of results from the Tukey HSD where the left for Abbott-24h and the right for Abbott-48h. The Tukey HSD's p-values indicate that region 1 has a different experiment result than region 6 and similarly, region 2 differs from region 4, and region 4 differs from region 6 on the Abbott-24h measurement. Abbott shows only a significant difference between region 1 and region 6 in the 48-hour measurement. Since there was a significant difference between regions in the results of a one-way analysis of variance ($p < .05$), it was accepted as one of the reasons requiring the use of covariance in the study.

4.3.3. Determining the Source of Difference of the Abbott Value Between Regions with ANCOVA

The assumptions of homogeneity of variance and equality of regression slopes required to perform the ANCOVA test were examined and it was concluded that ANCOVA could be performed. Thus, the experimental and control groups were included in the analyses as 24- and 48-hour covariates to determine whether the differences between regions were actually due to experimental conditions

4.3.4. The Effect of Time on *C. pipiens* Larvae of *B. sphaericus* Between Regions

While comparing the change from the 24th hour to the 48th hour according to Abbott values of *C. pipiens* larvae, which were detected as resistant to *B. sphaericus*, and put the 24th hour into the model as a covariate (Table 4.29).

Table 4.29: Univariate analysis of covariance difference of 48h and 24h by region with base Abbott 24h.

Region	Univariate Analysis
1	29
2	17
3	16
4	20
5	17
6	18

Table 4.30: ANCOVA on the difference between Abbott 48 – Abbott 24 with Abbott 24 is the covariate.

	n	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
1	29	5,91%	5,32%	0,99%	3,89%	7,94%	0,00%	16,67%
2	17	4,50%	8,49%	2,06%	0,13%	8,86%	-6,00%	30,43%
3	16	4,76%	6,41%	1,60%	1,35%	8,18%	-5,00%	16,00%
4	20	15,32%	12,77%	2,86%	9,34%	21,30%	0,00%	40,00%
5	17	8,85%	12,53%	3,04%	2,40%	15,29%	-1,82%	45,83%
6	18	1,83%	3,61%	0,85%	0,03%	3,62%	-0,83%	12,50%

Table 4.31: The results of the 24-48 hour effects of *B. sphaericus* on *C. pipiens* larvae between regions according to Abbott values

Dependent Variable	Source	F	p
(Abbott 48h) - (Abbott 24h)	Regions	3,366	0,007

It is possible to see the effect of time on the experiments since there are two measurements at 24 h and 48 h. When the Univariate Analysis of Covariance with the difference of Abbott values of the two measurements were applied, the result had a 0.007 p-value (Table 4.31). This was far less than the Null-Hypothesis, so the effect of time is not equal on the regional groups.

The Post Hoc Test was also performed to determine which groups caused the differences in the resistant regions according to the Abbott values in which *B. sphaericus* was tested.

4.3.5. Post Hoc Test Analysis of the 24-48 Hour Effect of *B. sphaericus* on *C. pipiens* Larvae for Differences Between Regions

Table 4.32: Multiple comparisons of regions with the Tukey HSD as a Post Hoc test.

Dependent Variable	Region	Region	p
(Abbott 48h) - (Abbott 24h)	1	2	0,9946
		3	0,9982
		4	0,0041
		5	0,8790
		6	0,6218
	2	3	1,0000
		4	0,0035
		5	0,6910
		6	0,9438
	3	4	0,0058
		5	0,7576
		6	0,9224
	4	5	0,2207
		6	0,0001
	5	6	0,1698

Tukey HSD as a Post Hoc test again helps identify; region 4 had a distinctive difference from regions 1,2,3, and 6 (Table 4.32). The Abbott value of region 4 was the highest rate of change from 24 hours to 48 hours, and the closest groups with the highest change were regions 3, 1, and 2.

4.4. MEASURING PROTEASE ACTIVITY

Figure 4.24 shows the protease activities of *C. pipiens* larvae that were treated with *B. sphaericus* and survived 48 h.

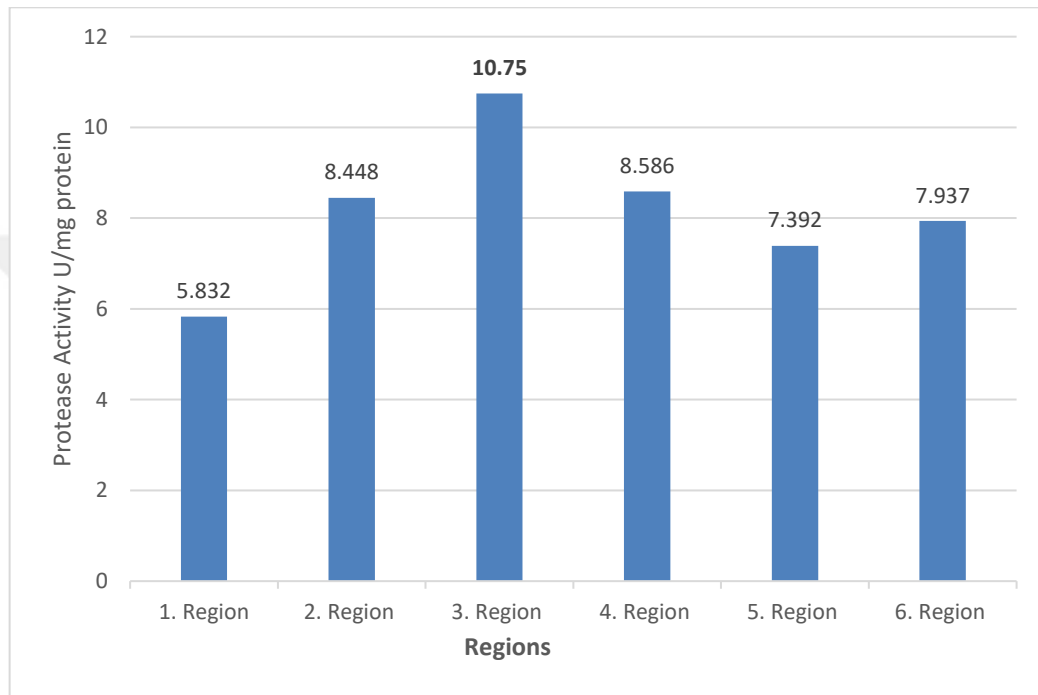


Figure 4.24: Protease activity results by region.

Region 3 with the 10.75 U/mg protein protease activity had the highest value in resistant larvae among the regions. Region 4 had 8.58 U/mg protein, region 2 had 8.44 U/mg protein, region 6 had 7.93 U/mg protein, region 5 had 7.39 U/mg protein, and region 1 had 5.83 U/mg protein protease activity.

Protease activity of the regions with the resistant *C. pipiens* larvae was compared with the control group *C. pipiens* larvae according to the Abbott values in which *B. sphaericus* were tested and the result is listed in Figure 4.25.

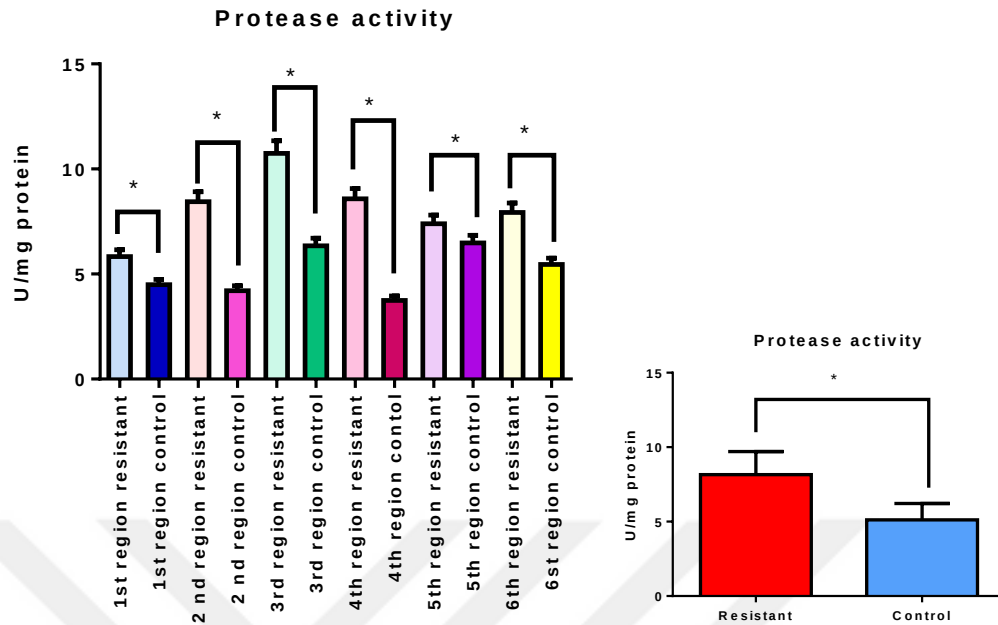


Figure 4.25: Comparison of protease activities of resistant *C. pipiens* larvae in the regions compared to control group *C. pipiens* larvae (* $p < 0.001$).

When Mann Whitney U-test results were examined in Figure 4.25, it was seen that there was a significant difference ($p < 0.001$) between the protease activities of resistant *C. pipiens* larvae and *C. pipiens* control group larvae.

Table 4.33: Difference of mean values of protease activity of *C. pipiens* larvae that appeared resistant in the regions compared to control group *C. pipiens* larvae where values were calculated by subtracting treated group value from control group value.

Region 1	Region 2	Region 3	Region 4	Region 5	Region 6
1,34	4,241	4,4	4,84	0,911	2,485

When the mean values were compared, the region 4 had the highest protease activity of the resistant *C. pipiens* larvae and the lowest was region 5, and the region with the closest value was region 3 (Table 4.33).

5. DISCUSSION and CONCLUSION

5.1. DISCUSSION

Wilke et al., in 2012 pointed out that most mosquito control measures have not been successful in fulfilling their aim, and they indicated the mosquito's large reproductive capacity and genomic flexibility as the main reasons. Additionally, they highlighted the restrictions of the Chemical control mechanism since they are potential for human toxicity, mortality in the non-target organism, environmental influences, and insecticide resistance (Wilke & Marrelli, 2012). Besides the toxic effects of many of these chemical insecticides, their long-term effects include disrupting endocrine systems in mammals, birds, and fish. The ability of insecticides to interact with estrogen receptors, thyroid receptors, androgen receptors, and other endocrine-disrupting long-term effects has been studied to a limited extent, and the total number of long-term effects are still unknown (van Dyk & Pletschke, 2011). Chemical control is increasingly restricted due to potential human toxicity, mortality in no target organisms, other environmental influences, and insecticide resistance.

Due to the known and potentially unknown side effects of synthetic chemicals, many researchers have been felt obliged to look for new methods for mosquito management that are harmless to our environment (Reegan et al., 2015). This leads to the research and development of natural larvicidal products, which mainly focus on applying larvicidal oils, botanical extracts, and microbial larvicides such as *B. sphaericus*, secondary metabolites, or costlier insect growth regulators (Patil et al., 2011).

Insecticide resistance leads to significant crop losses in agriculture. Meanwhile, they reduce the success of the fight against mosquitoes, which are carriers of important disease factors. The evolution of insecticide resistance poses an important problem for the control of pest populations (Çakır & Yamanel, 2005). The development of insecticide resistance is a complex process depending directly on genetic, physiological, behavioral, and decological factors and indirectly on the volume and frequency of applications of insecticides (Karunaratne et al., 2018).

The development of resistance can be prevented by some measures. These measures are called insecticide resistance management. While critical evolutionary factors determine the dynamics

of insecticide resistance in the field, it is important to facilitate an acceptable pest control to keep the effectiveness of resistance management central (Darboux et al., 2007; Poopathi & Abidha, 2010). Research on insecticide resistance, molecular mechanisms of resistance, and rational resistance management is of paramount importance in controlling the development and spread of resistance in vector populations (Karunaratne et al., 2018).

It has been shown that in long-term use of bioinsecticides, spores and their toxins remain in the environment for a long time and that in frequent applications, mosquitoes develop resistance, and the applications become ineffective. For this reason, excessive and illiterate use of pesticides causes resistance to these products in target organisms over time. However, selective pressure from insecticides can cause resistance in vector species (Norris & Norris, 2011).

Eritja and Chevillon, 1999 (Eritja & Chevillon, 1999), demonstrated that if the selected pesticide is removed from the field, the resistant genes can turn back to their original state very fastly. Later, in 2015, Siegwart et al. noted that the multigenic, monogenic, recessive, or dominant genetics form highly variable transmission. As a result, the fight against the resistance of the vectors is also highly variable. From the previous fact, they also confirmed that, in the future, bio-pesticide molecules will likely have various and diverse resistances, and if the biocontrol agents have more different types, there will be developed fewer resistances (Siegwart et al., 2015).

Among the biocontrol agents, *B. sphaericus* and *B. thuringiensis subsp. israelensis* have been extensively studied and deployed for mosquito control on the field. The reports before 2011 on the development of resistance to *B. sphaericus* have inspired researchers to seek alternative microbial agents or their metabolites for mosquito control (Geetha et al., 2011). Many countries continue to search and isolate new species in natural environments to reduce insect resistance to *B. sphaericus* and *B. thuringiensis subsp. israelensis* (Lee et al., 2012).

It can be expected that the diversification of molecular and biochemical insecticides may limit the emergence of resistance of the vectors. The whole complexity of the agent is retained when complete *B. thuringiensis subsp. israelensis* spores and crystals, crude neem extracts, and oregano, typically diverse virus populations, are used, and no resistance development has been observed (Siegwart et al., 2015).

The broad target spectrum of *B. thuringiensis subsp. israelensis* is due to the combination of the mosquito killer proteins Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1A produced during sporulation; the second protein synergizes the other three proteins, broadening the target spectrum and delays the development of resistance against them (Wirth et al., 2010). The selection using single *B. thuringiensis subsp. israelensis* toxins, such as Cry11Aa, Cry4Aa, and Cry4Ba, can lead to resistance (Paris et al., 2011). When resistance selection is considered, the mode of action of *B. sphaericus* displays a greater potential compared with *B. thuringiensis subsp. israelensis*. There was no reported resistance in insects against *B. thuringiensis subsp. israelensis* in 2017 (Silva-Filha, 2017).

Tetreau et al. mentioned that resistance to *B. thuringiensis subsp. israelensis* was absent in the field population of the French Rhône-Alpes region, even after only *B. thuringiensis subsp. israelensis* treatments in decades. It turns out that, even after decades of *B. thuringiensis subsp. israelensis* treatments and the mean time extensive research, there is a lack of resistance on the field populations. Although studies in natural populations reported decreased but relatively high sensitivity in some populations, however, in lab conditions, Tetreau et al. found relatively high levels of resistance to individual *B. thuringiensis subsp. israelensis* Cry toxins (Tetreau et al., 2013).

Becker et al. had observed that a remarkable property of this bacterium. Despite its extensive use in some geographical regions, such as the Rhine Valley in Germany, no resistance to *B. thuringiensis subsp. israelensis* has been reported under field conditions after many years of use (Becker et al., 2000). In this study, no resistance was found against *B. thuringiensis subsp. israelensis* in *C. pipiens* larvae.

Wirth et al. mentioned that Bin is a single-site acting insecticide that acts on a single receptor in the mosquito midgut. A single-site act is more declined to the evolution of insecticide resistance relative compared to multi-site acting insecticides (Wirth et al., 2010). The first report on the resistance to *B. sphaericus* was in 1994. It turns out that *C. pipiens* L. field populations in southern France have high resistance against *B. sphaericus* in response to repeated treatments. Su et al. listed numerous published reports on various levels of resistance developed in laboratories during research and field populations from different countries (Su et al., 2019).

During the last three decades, resistance to *B. sphaericus* has been reported on *C. pipiens*. In 1995, (Silva-Filha et al., 1995) Silva-Filha et al. found 10-fold resistance in Brazil populations. The same year, Rao et al. found 15 times more, 150-fold, resistance in the Indian populations (Rao et al., 1995). Five years later, in 2000, the fold resistance reached 10,000-fold. This was shown by Charles and Nielsen-LeRoux on the France populations (Charles and Nielsen-LeRoux, 2000). Poopathi and Abidha, in 2010, mentioned a report for 25,000-fold resistance from China and 2,000-fold from Tunisia populations. This report confirms that extensive usage of biopesticides can cause resistance to *B. sphaericus* (Poopathi & Abidha, 2010).

Just before resistance to *B. sphaericus* was reported in field studies, two different California laboratories demonstrated resistance due to repeated use, one work from Georghiou et al. with >100,000 fold (Georghiou GP, 1992) and the other one from Rodcharoen and Mulla with 37-fold (Rodcharoen J, 1994). Su et al. pointed high resistance to *B. sphaericus* in *C. pipiens* populations in Salt Lake City (USA, Utah) in 2016. They compared the resistance with a reference colony of the same species from the laboratory with the field populations. They found 20,780.0-fold at LC50 and 23,926.9-fold at LC90 resistance ratios in the field populations (Su et al., 2019).

Darboux et al. mentions two different mechanisms of resistance been observed in *Culex* populations on the Mediterranean coast of France. In 1994, Nielsen-LeRoux (Nielsen-Leroux et al., 1997) published that one of the resistance mechanisms may be related to the intracellular activity of Bin toxin after binding to the larval midgut epithelium, as the Bin protein cannot bind to the larval toxin receptor. They inserted the larva's genome and named it the SPHAE strain. In the same study, high resistance in the BP strain identified in 1997 was associated with a lack of toxin binding to the larval gut. It has been shown that the addition of a transposable element (TE) sequence to the coding region of Cpm1 on the intestinal epithelial cell membrane of the larva leads to genetic variation, changes the splicing pattern of the host mRNA, and creates a new intron. Since a nonsense mutation in the Cpm1BP allele causes the loss of the C-terminal domain of the Bin toxin protein, the receptor cannot bind properly to the cell surface and the toxic feature of *B. sphaericus* is eliminated. They showed that the cpm1BP-del allele, characterized by a TE-like sequence inserted into exon 2, produces a shortened cpm1 mRNA (Darboux et al., 2007).

Wirth et al. described mechanisms for resistance to the Bin toxin in the BP subject, known as Cpm1BP, a substitution of 5 amino acids and a nonsense mutation, and another known as Cpm1bp-del and internal deletion of 198 base pairs (Wirth et al., 2010). The alpha-glucosidase activity could not be detected in Cpm1BP and Cpm1bP-del proteins. They showed that maltase abolished its physiological function by mutations caused by the addition of a new intron from the pre-mRNA to the cpm1BP-del protein. The addition of the new intron causes a modified membrane protein expression of larvae and it does not bind with the toxin protein and thus the larva survives because the toxin protein does not act (Darboux et al., 2007).

Also in the same study, Darboux et al. observed that the resistant phenotype of *Culex* strain GEO resulted from a nonsense mutation that abolished the glycosylphosphatidylinositol (GPI) anchorage of the receptor to the larval intestinal lining, resulting in premature termination of translation (Darboux et al., 2007). They revealed that this mutation resulted from the loss of the GPI membrane anchor, which holds the -glucosidase Bin receptor to the cell membrane (Wirth et al., 2010). Wirth et al. showed that the decrease in the receptor concentration did not affect the binding to the larval gut when they looked at low-level resistant colonies from Brazil (FIELD). When they looked at the Tunisian colony (TUNIS), they did not show a loss of receptor binding in the larval gut. They also mentioned that the underlying mechanism of *B. sphaericus* resistance was unknown to date 2010. There is still no further published information than their result (Wirth et al., 2010).

Tangsongcharoen et al. investigated the deletion of some amino acids on the BinA and BinB proteins of *B. sphaericus* to investigate gene changes that may cause mosquito resistance. In their study, they showed that when the N- and C-terminal ends of toxin proteins are deleted, the toxicity disappeared. They also showed that half of the C-terminal and N-terminal ends of BinB contribute to its attachment to the larval intestinal lining (Tangsongcharoen et al., 2017). In their toxin deletion studies, they showed that the removal of 34 amino acids from the N-terminal end of BinB did not affect the binding to the larval intestine, that is, toxicity, but the deletion of more than 41 amino acids inactivated the toxin protein. They showed that the position of 30–40 amino acids of the N-terminal end of BinB plays an important role in the toxicity of the binary toxin (Singkhamanan et al., 2013).

Nielsen-Leroux reported in 2002 that the lack of Bin receptors and their inability to bind to the larval midgut was the most common resistance mechanism (Nielsen-Leroux C, 1995, Oliveira

CMF, 2004). de Melo Chalegre and others identified the inability of toxin proteins to bind to larval midgut receptors as the primary resistance mechanism. They found evidence that the *W. bancrofti* nematode vectorizes different species of *Aedes*, *Culex*, and *Mansonia*, and that this parasite is an important agent of TEs (de Melo Chalegre et al., 2015).

Melo and Wallau investigated TE elements that can cause mosquito resistance in their study. They showed that more than 40% of the mosquito genomes of *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus*, and 0.13% to 19.55% of the genomes of *Anopheles* species, are composed of TEs and that there are at least 24 super TE families. They found that mosquitoes generally have most class I TEs from TEs. They predicted that TEs have been extensively exchanged between mosquitoes by horizontal transfer over the past 30 million years. They revealed that the mosquito vector *Wuchereria bancrofti* nematode, which facilitates the spread of TEs among mosquitoes by horizontal transfer, affects the presence and genome size, and variability of the mosquito (de Melo & Wallau, 2020).

Paul et al. reported resistance levels in populations collected from Albany and Syracuse, and New York State, for mosquito control, for 4 larvicides (for methoprene, phenotrin, *B. sphaericus*, and *B. thuringiensis subsp. israelensis* and 1 registered adult killer (phenothrin)). High levels of resistance were found only to *B. thuringiensis subsp. israelensis* only at one site, Syracuse. Resistance levels to the other insecticides were less than 10-fold. Given the large difference between the *B. thuringiensis subsp. israelensis* resistance in Syracuse and Albany, it appears that these populations of *C. pipiens* do not rapidly mix, leading to the localization of resistant populations (Paul et al., 2005).

Raval-Nelson et al. decided to determine whether the catch basins are breeding sites for mosquitoes or not in Pennsylvania, where there are 70,000 catch basins, and if so they want to test the efficacy of *B. sphaericus* larvicide in these basins. They determined that catch basins were ideal places for mosquitoes, *C. pipiens*, and *B. sphaericus* was an effective larvicide. They concluded that the larvicide was effective if the calculated p-value was below 0.05. The resulting Chi-square value was 40.9412 and the p-value ≤ 0.001 . According to the research results, *C. pipiens* larvae were assumed to be killed by *B. sphaericus* application (Raval-Nelson et al., 2005).

In this work, *C. pipiens* larvae collected from the districts of Istanbul were classified as districts. It was determined that *B. thuringiensis subsp. israelensis* killed 99.75% of *C. pipiens* larvae in the first 24 h and 100% within 48 h. It was determined that *B. sphaericus* killed *C. pipiens* larvae, 84.72% in the first 24 h and 91.93% in 48 h. Real-Time PCR is performed by isolating DNA from locally separated *C. pipiens* larvae.

Kılıç et al. investigated the genetic characterization of *C. pipiens* populations, which is the dominant species. They collected 25 different sub-locations belonging to 6 provinces in the Aegean region of Turkey by using the random amplified polymorphic DNA (RAPD) method. Twenty reproducible and clear band-producing polymorphic primers were studied, and a low level of genetic variation and a high level of gene flow between populations were found as a result of the study (Kiliç et al., 2019).

In our study, the RAPD method was studied to determine whether there is a genetic differentiation between *C. pipiens* larvae that are resistant and non-resistant to *B. sphaericus* according to the regions they come from. However, in the DNA purification stage, which is the first process for the samples selected for this purpose, a sufficient amount of DNA could not be reached for the PCR method, which could not be resolved by different optimization studies and the reason could not be understood. Due to the limited duration of the project, finding an answer to the related question has been included in the project as an aim of future studies.

In the evaluation of the experiment according to the Abbott value, it was observed that *C. pipiens* larvae developed resistance to *B. sphaericus*. When the 6 regions were evaluated, where *C. pipiens* larvae develop resistance to *B. sphaericus*, the region with the lowest resistance rate was the first region with the lowest 84.06% Abbott value. According to the Abbott values, the resistance rates decrease to 89.04% in the 3rd region and 89.43% in the 4th region, 92.68% in the 5th region, 94.14% in the 2nd region, and 98.18% in the 6th region. In the 6th region, there was no resistance according to the Abbott value. When 39 districts of Istanbul were evaluated on a district basis according to the Abbott value, the larvae that we detected resistant to *B. sphaericus* were seen in only 13 districts of Istanbul. The Abbott value of the Esenyurt district was 70.12% and it was determined to be the most resistant district against *B. sphaericus*. The Abbott value of Ümraniye was 89.90% and it was determined that it was the district with the lowest resistance rate against *B. sphaericus*.

One-Way ANOVA test was applied with a 0.05 significance level (p-value) to the resistant regions according to the Abbott values of the *C. pipiens* larvae in which *B. sphaericus* was tested. In the One-Way Analysis of Variance ANOVA test, the p-value was below 0.05. In the experiment, the p-value of the living larvae after 24 h was 0.0002 and the p-value of the larvae that lived 48 h later was 0.0192. From the increase of the p-value from 0.0002 to 0.0192 in time, we may conclude that, in some regions, the *B. sphaericus* needs time to affect the larvae. According to the ANOVA test results, the Tukey HSD technique was used to determine between which groups the difference originates, and Abbott 24-hour measurement, region 1 from region 6, region 2 from region 4, and region 4 from region 6 different p values. Only region 1 and region 6 showed a significant difference in the Abbott 48-hour measurement. In 48h measurement, only region 1 and region 6 showed a difference. When we look at the Abbott results, we see that the 2nd and 5th groups were possibly resistant near the 6th region. Therefore, close to the 6th group, these groups have less resistance than the 1st, 3rd, and 4th groups.

The population density of the 1st region is slightly higher than the population density of the districts in the 6th region, therefore, the number of mosquito sources produced and the number of spraying demands will increase, as a result the larvae will be exposed to spraying more. Since the total area of the districts in the 6 regions is almost 10 times larger than the districts in the 1st region, the amount of pesticides falling on a larger area will be less and the formation of resistance will be less.

There was a significant difference ($p < 0.05$) between regions in the results of a One-Way analysis of variance, to further investigate the covariance test was performed and the Post Hoc Test was used to determine from which groups the differences in the resistant regions originated. Tukey HSD as a Post Hoc test again helps identify these; region 4 has a distinctive difference from regions 1, 2, 3, and 6. Abbott is the 4th region with the highest rate of change from 24 h to 48 h. Other close groups with the highest variation are region 3, region 1, and 2. In our evaluation according to the Abbott value, the resistance of *C. pipiens* larvae only against *B. sphaericus* is the fastest in the 4th region, followed by the 3rd region, the 1st region, and the 2nd region, respectively.

While the southern coasts of Istanbul and the vicinity of the Bosphorus stand out as the regions with the highest population density, the parts with the lowest population density are the northern coasts². The result of this work is striking that the highest resistance exists in the center of

Istanbul, the old city walls, and the resistance decreases outwards in every direction. Region 1 (Bağcılar, Bahçelievler, Bakırköy, Bayrampaşa, Esenler, Fatih, Güngören, Beyoğlu, Şişli and Zeytinburnu) is the center of manufacturing and industry, where population density and urbanization are higher than other regions. However, it is seen that the population with income and social economic level is more dominant.

Yeşiltepe neighborhood of Zeytinburnu is the most densely populated neighborhood of Istanbul with a population of over 96 thousand per square kilometer. All 3 neighborhoods with the lowest density are in Şile and have populations of only 5, and 6 people per km². The Fatih neighborhood of Çatalca is the lowest density neighborhood on the European side with a population of 7 people per km². On the European side, there is an agricultural area of 83,906 decares in the Çatalca district and 9.867 hectares in the Arnavutköy district. On the Anatolian side, there is an agricultural area of 2.932 hectares in the Şile district and 14.766 decares in the Beykoz district². The northern part of Istanbul is the place where the population density is less than in rural areas, and agriculture and animal husbandry are more prominent than other districts. It can be thought that the population density, cultural and social economic level, increases in the number of request spraying, and the mosquito larvae that are constantly exposed to the insecticide develop resistance. For this reason, the resistance rate may have been higher in region 1 compared to other regions.

Survival of a larva resistant to *B. sphaericus* has a significant impact on the emergence of resistance in the mosquito population. When the larvae are exposed to the insecticide, they do not die thanks to their resistance genes and they have an advantage over other larvae. Studies show a decrease in the frequency of resistance alleles if the insecticide is not used or its use is interrupted. In studies related to this, they showed that in Brazil, the resistant larva population became susceptible after 16 months if the insecticide was not used, whereas, in China, the resistance quickly reversed after 6 months. (Wirth et al., 2010, Silva-Filha MH, 2007, Yuan et al., 2000).

Charles and Nielsen-LeRoux et al. showed that mosquito resistance occurs more rapidly in closed breeding areas. In Recife (Brazil), they reported that they found a 10-fold resistant population when exposed to toxin protein 37 times in two years in mosquito breeding areas such as open ducts and closed cesspools. In Cochin (India) they reported that resistance emerged when they were exposed to toxin protein 35 times in various open breeding sites, again

about two years later, and 36 times in Dongguan (China) eight years later. Time, dose, and selection pressure in population genetics seem to play a role in the emergence of mosquito resistance (Charles and Nielsen-LeRoux, 2000).

Gong et al. in a deltamethrin-resistant strain of the *C. pipiens pallens* mosquito species, found that two serine proteinase genes (Trypsin and chymotrypsin) were expressed at least three times more in resistant *C. pipiens* than in susceptible *C. pipiens*. They showed in their study that *C. pipiens* survived after deltamethrin exposure, that two serine proteinase genes were higher in deltamethrin-resistant mosquitoes and were associated with insecticide resistance (Gong et al., 2005).

Mechanisms involving the overlapping functions of the maltase may be activated to compensate for the absence of Cpm1 in BP mosquitoes. This phenomenon has already been observed in herbivorous insects feeding an artificial diet with high endogenous proteinase inhibitor levels (Darboux et al., 2007). The biochemical characterization of these enzymes may provide important clues for developing new control strategies by either using peptidases as targets or interfering in the production of these enzymes (Borges-Veloso et al., 2012). The type of proteases present in the gut of mosquito larvae can be identified using such specific synthetic protease inhibitors (Vadakkadath Meethal, 2018).

As serine proteases represent the major proteases in the gut of mosquito larvae, targeting the protease isolated from the gut of the larvae of *Ae. albopictus* will be a promising strategy for the control of mosquitoes (Vadakkadath Meethal, 2020).

The 3rd region with the highest protease activity measurement values of the regions to which the *C. pipiens* larvae resistant to *B. sphaericus* belong had 10.75 U/mg protein, respectively, the 4th region; 8.58 U/mg protein, region 2; 8.44 U/mg protein, region 6; 7.93 U/mg protein, region 5; 7.39 U/mg protein; and region 1; It was measured at 5.83 U/mg protein. It was observed that *C. pipiens* larvae in region 3 protease activity at a higher rate compared to other regions, whereas *C. pipiens* larvae in region 1 protease activity at a lower rate. The mean values of the protease activity were found to be 4.84 U/mg protein at the 4th region at most in the *C. pipiens* larvae that we tested, compared with the control group. Respectively, 4.4 U/mg protein in region 3, 4.24 U/mg protein in region 2, 2.48 U/mg protein in region 6, 1.34 U/mg protein in region 1, and 0.91 U/mg protein in region 5 mg measured.

Protease inhibitors kill the mosquitos by blocking the mosquito's protease activity. In this study on protease activity, when the resistant larvae are compared with the control group, the protease enzyme activity is increased in the resistant larvae compared with the control group. The elevation of the protease enzyme activity supports the resistance results we obtained from the regions.

To find any resistance on mosquito larvae, they were tested during their most active duration starting from April to September. This was what achievable during this limited time frame with current capabilities. Considering the demographic structure and area measurement of the districts worked on, the resistance test could have been executed accordingly and had comparisons and analysis.

To study the resistance of mosquitoes to the toxin proteins of *B. thuringiensis subsp. israelensis* and *B. sphaericus* used in mosquito control in Istanbul, we could select 2 or 3 districts for resistance testing. To search for resistance genes, it would be better if we could perform genetic assays on larvae from a single district instead of combining larvae from different districts and collecting them in a pool.

This study will provide an opportunity for a comparison that will form the basis for future tracking of mosquito resistance. My next goal, with the support of the project and the team, is to select 2 or 3 counties, conduct resistance testing, track resistance, and show genetic variation in resistance not only in *Culex* larvae but also in *Aedes* species that are increasing in the mosquito population.

5.2. CONCLUSION

In the fight against a major vector, mosquitoes, landscaping, and eliminating mosquito breeding areas are very effective and do not have any adverse effects in natural environments. Physically placed window screens, mosquito nets, light traps, and fly-killing devices are important natural methods for combating mosquitoes.

In addition to these methods, biological methods have been started to be used in the fight against mosquitoes due to the resistance developed against insecticides, which are widely used in the control of mosquitoes, and due to their harmful effects on the environment. Biological methods include the youth hormones, sterilization, predatory flies, fish fed with larvae, fungi, and bacteria consumed by the larvae as food.

Many researchers have shown that *C. pipiens* larvae develop resistance to *B. sphaericus* and *B. thuringiensis subsp. israelensis*, which are used to combat mosquito larvae. With this study, it was demonstrated for the first time that *C. pipiens* larvae developed resistance against *B. sphaericus*, which is used as a larvicide in the control of mosquitoes in Turkey. No resistance was observed in *C. pipiens* larvae against *B. thuringiensis subsp. israelensis*.

When it is observed pointwise on the map, there is a similarity in the places marked as resistant according to population density. Depending on the population density, it is observed that there is resistance because the spraying services are more intense compared to the increasing spraying demand. It is striking that the resistance points spread from the center to the countryside. Different biological drugs should be used instead of *B. sphaericus*, mostly in centers, or they should be rotated, or their use should be suspended and resistance should be monitored.

This study also considered the protease activity, it was determined that the protease enzyme was increased in the resistant regions of *C. pipiens* larvae against *B. sphaericus* compared with the control group. As the protease enzyme rises in the larvae, resistance development will be observed in the larva. Biological drugs can be developed to inhibit the protease activity of the larvae related to protease inhibitors.

Insecticide applications should be made in more limited areas where vectors are concentrated, rather than in large areas. Insecticides should be used only during the breeding season of mosquitoes and during periods of overgrowth of adults, rather than all seasons. In vector

control, adult and pre-adult stages should struggle simultaneously. For different life stages of the target organism, products from different classes, such as herbal, microbial, and synthetic origin with different mechanisms of action should be used. During the struggle, products with different mechanisms of action and in suitable formulations can be used by mixing. However, while preparing the mixture, care should be taken to ensure that pesticides do not reduce the effects of each other and that they do not cause toxic effects on non-target organisms or any safety problems.

To prevent the formation of resistance, insecticides or biological agents used in mosquito control should be tested for resistance, and research institutions should develop rapid and reliable resistance testing techniques. In drug licensing, LD50 or LC50 values should be determined for pests for which drugs are licensed, and a more effective system should be established to ensure the traceability of pesticide use. Larvaside control should be done on time and adequately. |

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CURRICULUM VITAE

Personal Information	
Name Surname	Emine Nurhan Altuner
Place of Birth	Istanbul
Nationality	☐ T.C. ☐ Other:
E-Mail Address	
Web Page	

Education Information	
Undergraduate	
University	Istanbul Üniversitesi
Faculty	Cerrahpaşa Tıp Fakültesi
Department	Medical Biological Sciences
Year of Graduation	18.06.1996

Masters	
University	Istanbul Üniversitesi
Name of Institute	Cerrahpaşa Tıp Fakültesi
Department	Medical Microbiology
Program	Microbiology

Ph.D.	
University	Istanbul Üniversitesi
Name of Institute	Fen Bilimleri Enstitüsü
Department	Department of Molecular Biology and Genetics
Program	Programme of Biotechnology

Articles and Publications	
Akın, E.N., Yücel, N., Bingöl, N., 2020, İş Sağlığı ve Güvenliği kapsamında biyosidal ürün çalışanlarında meslek hastalıklarının önlenmesi, <i>Sağlık Bilimlerinde Multidisipliner Araştırmalar</i> 12, 165.	

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