STUDIES ON THE MECHANISM OF RESISTANCE AGAINST PYRETHROIDS IN *HELICOVERPA ARMIGERA*: MOLECULAR AND PROTEOMIC APPROACH

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STUDIES ON THE MECHANISM OF RESISTANCE AGAINST PYRETHROIDS IN *HELICOVERPA ARMIGERA*: MOLECULAR AND PROTEOMIC APPROACH

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ABSTRACT

STUDIES ON THE MECHANISM OF RESISTANCE AGAINST PYRETHROIDS IN HELICOVERPA ARMIGERA: MOLECULAR AND PROTEOMIC APPROACH

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Helicoverpa armigera is an insect, causes important economical losses in crops. To reduce this loss, chemical insecticides such as pyrethroids have been commonly used against *H. armigera* in farming areas all over the world. However, excess and continuous usages of them cause resistance development in *H. armigera*. Insects develop resistance against applied insecticides by following three main mechanisms; by reducing the amount of insecticide entering into the insect body, developing insensitivity of the insecticide effective site and increasing detoxification metabolism of insecticides such as increased metabolism of them in midgut tissue of *H. armigera*. Therefore, changes in differentially expressed midgut proteins were analysed at protein level with two-dimensional gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) together with examine biochemical activity changes of certain detoxification enzymes such as esterases (EST) and glutathione S-transferases (GST). Moreover, transcriptional level analysis of certain genes from EST and GST systems together with cytochrome P450 monooxygenases (CYP450) system were done with quantitative real-time PCR method, too.

According to the comparative proteome analysis, it was found that *H. armigera* field samples overcome pyrethroid stress mainly by increasing energy metabolism related proteins expressions such as ATP synthase, Vacuolar ATPase A and B and arginine kinase proteins. Furthermore, certain detoxification enzymes such as thioredoxin peroxidase and NADPH cytochrome P450 reductase were up-regulated in Mardin population, suggesting that they were actively participating in response to pyrethroid stress. NADPH cytochrome P450 reductase could play a role in detoxification of toxic pyrethroid metabolites such as 3-phenoxybenzaldehyde. However, while glutathione S-transferases (GSTs) were not found up-regulated in the comparative proteome analysis, biochemical assays (GST-CDNB, GST-DCNB and GST-PNBC) showed significant increases in enzyme activities in the Adana and in the Mardin field population, as compared to the susceptible strain. Furthermore, GST-DCNB and GST-PNBC activities showed significant increase in Çanakkale population. As overcoming energy crisis may lead to an increase in oxidative stress, detoxification enzymes (GSTs and thioredoxin peroxidase) might be involved in pathways for eliminating toxic reactive oxygen species such as H_2O_2. Similarly, although esterases (EST) were not found as differentially expressed, biochemical assays for ESTs showed significant increases in enzymatic activities in the Adana and the Mardin field populations. Thus, ESTs are also proposed to be involved in developing resistance as an initiator of pyrethroid metabolism in *H. armigera* from Turkey.

Quantitative real-time PCR results showed that while CYP9A14 gene expression was up-regulated in all analyzed field populations, CYP9A12 gene expression was up-regulated in both Çanakkale and Mardin populations. CYP4S1 gene expression was also up-regulated only in Mardin field population. However, while CYP6B7 gene expression together with CYP9A12 and CYP4S1 genes expressions were down-regulated in Adana population, CYP6B7 gene expression was not significantly changed in both Çanakkale and Mardin populations. In addition, GST, GSTX01 and ESTX018 gene expressions were not significantly changed in all field populations in comparison to susceptible population. Therefore, CYP9A14, CYP9A12 and CYP4S1 genes proposed to be involved in detoxification of toxic pyrethroid metabolites possibly through regulation of NADPH cytochrome P450 reductase.

In conclusion, it is suggested that one of the main mechanisms of resistance development is increased energy metabolism in the midgut tissue of *H. armigera* which may be a general prerequisite for compensating the costs of energy-consuming detoxification processes.

Key words: *Helicoverpa armigera*, Glutathione S-transferases, Esterases, Cytochrome P450 Monooxygenases, Mass Spectrophotometry, Specific Activity, Quantitative Real-Time PCR.

ÖZ

HELICOVERPA ARMIGERA'NIN PİRETROİDLERE KARŞI GELİŞTİRDİĞİ DAYANIKLILIĞIN MEKANİZMASI ÜZERİNE ÇALIŞMALAR: MOLEKÜLER VE PROTEOMİK YAKLAŞIM

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Pamukta yeşil kurt (*Helicoverpa armigera*) tarımsal ürünlerde önemli ekonomik zaralara neden olan bir böcektir. Dünyadaki tarım alanlarında bu zararı azaltmak için *H. armigera*'ya karşı piretroid gibi kimyasal böcek ilaçları kullanılmaktadır. Bununla birlikte, sürekli ve yoğun ilaç kullanımı *H. armigera*'da direnç gelişimine neden olmaktadır. Böcekler kendilerine karşı kullanılan ilaçlara karşı direnç gelişimini aşağıda belirtlien üç ana mekanizma ile sağlarlar; böcek vücuduna giren ilacın miktarını azaltarak, ilacın etkilediği bölgede ilaca karşı duyarsızlık oluşturarak yada örneğin ilacın mide dokusundaki detoksifikasyon metabolizmasının hızını artırarak gerçekleştirirler. Bu yüzden, mide dokusundaki proteinlerin ekspresyonlarındaki değişimler protein seviyesinde iki boyutlu jel elektroforezi (2D-PAGE) ve MALDI-TOF kütle spektrofotometresi (MALDI-TOF-MS) araştırılırken ayrıca estraz (EST) ve glutatyon S-transferaz (GST) gibi detoksifikasyon enzim sistemlerinin biyokimyasal aktivitelerindeki değişimlerle birlikte incelendi. Ayrıca EST ve GST sistemlerinden seçilen bazı genlerin transkripsiyon seviyesindeki değişimleri sitokrom P450 monooksijenazlar (CYP450) enzim sisteminden seçilen genlerle birlikte gerçek zamanlı polimeraz zincir reaksiyonu metodu ile analiz edildi.

Karşılaştırmalı proteom analizine gore, *H. armigera* tarla örnekleri piretroid stresinin üstesinden gelebilmek için ekspresyonunu artırdıkları başlıca proteinler ATP sentaz, Vakuolar ATPase A or B ve arjinin kinaz gibi enerji metabolizması ilgili proteinlerdir. Ayrıca, tiyoredoksin peroksidaz and NADPH sitokrom P450 redüktaz gibi bazı detoksifikasyon enzimlerinin ekspresyonu Mardin populasyonunda artması bu proteinlerin piretroid stresine karşı aktif olarak yer aldıklarını göstermektedir. NADPH sitokrom P450 redüktaz 3-fenoksibenzaldehit gibi toksik piretroid metabolitlerinin detoksifikasyonunda rol oynayabilir. Bununla birlikte, *H. armigera*'nın Adana ve Mardin populasyonlarının hassas populasyonla karşılaştırmalı proteome analizine gore GST'lerinin ekspresyonunda artış olmazken, GST (GST-CDNB, GST-DCNB ve GST-PNBC) aktivetelerinde anlamlı artışlar gösterdi. Ayrıca, *H. armigera* Çanakkale populasyonu da GST-DCNB and GST-PNBC aktivitelerinde anlamlı artışlar gösterdi. Enerji krizinin üstesinden gelmeye çalışılırken oksidatif stresi artmaktadır, detoksifikasyon enzimleri (GST ve tiyoredoksin peroksidaz) oksidatif stres sırasında oluşan H_2O_2 gibi toksik reaktif oksijen türlerinin eleminasyon yollarında yer almaktadır. Benzer şekilde, esterazlar ekspresyonu artan EST proteini belirlenmediği halde, biyokimysal EST aktiviteleri Adana ve Mardin populasyonlarında anlamlı artışlar gösterdi. Bu nedenle, EST'lerin Türkiyedeki *H. armigera*'daki dayanıklılık oluşumunda piretroid metabolizmasının başlatıcısı olarak rol oynadığı ileri sürüldü.

Gerçek zamanlı polimeraz zincir reaksiyonu sonuçları CYP9A14 geninin ekspresiyonun analiz edilen tarla populasyonlarının tümünde arttığını gösterirken, CYP9A12 geninin Çanakkale ve Mardin populasyonlarında arttığını gösterdi. CYP4S1 geninin ekspresyonuda sadece Mardin populasyonunda arttı. Bununla birlikte, CYP6B7 geninin ekspresyonu CYP9A12 ve CYP4S1 genlerinin ekspresyonları ile birlikte Adana populasyonunda azalırken, CYP6B7 geninin ekspresyonu Çanakkale ve Mardin populasyonlarında değişiklik göstermedi. Ayrıca, tarla populasyonlarının tamamında GST, GSTX01 ve ESTX018 genlerinin ekspresyonlarında hassas populasyona göre anlamlı bir değişim göstermedi. Bu nedenle, CYP9A14, CYP9A12 ve CYP4S1 genlerinin toksik piretroid

metabolitlerinin detoksifikasyonunda NADPH cytochrome P450 redüktazın regülasyonuyla yer almış olabilecekleri öne sürülmektedir.

Sonuç olarak, dayanıklılık oluşumunda ana mekanizmalardan birinin *H.* armigera'nın mide dokusundaki enerji metabolizmasındaki artış olduğu öne sürülmektedirki bu artış enerji gerektiren detoksifikasyon işlemleri için gerekli enerjinin sağlanmasında genel bir ön koşul olabilmektedir.

Anahtar kelimeler: Helicoverpa armigera, Glutatyon S-transferazlar, Esterazlar, Sitokrom P450 Monooksisjenazlar, Sentetik piretroid'ler, Spesifik aktivite, Kütle Spektrofotometrisi, Gerçek Zamanlı Polimeraz Zincir Reaksiyonu.

To my lovely family…

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ABBREVIATIONS

DDT – Dithiothreitol

IAA – Iodoacetamide

CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

PMSF – Phenylmethylsulfonyl fluoride

NL – Non-linear

MALDI – Matrix-assisted laser desorption/ionization

ToF – Time of flight

IPG – Immobiline pH gradient

TFA – Trifluoroacetic acid

CHCA – α-cyano-4-hydroxy-cinnamic acid

KCl – Potassium chloride

EDTA – Ethylenediaminetetraacetic acid

BSA **–** Bovine serum albumin

CDNB – 1-chloro-2,4-dinitrobenzene

DCNB – 3,4-dichloronitrobenzene

PNBC – p-nitrobenzylchloride

EPNP – 1,2-epoxy-3-(p-Nitrophenoxy)propane

GSH – Reduced glutathione

α-NA **–** α-naphthyl acetate

PNPA **–** p-nitrophenylacetate

GST**–** Glutathione S-transferases

EST– Esterases

CYP450 – Cytochrome P450 monooxygenases

DEPC – Diethyl Pyrocarbonate

q-RT-PCR – quantitative-Real time - polymerase chain reaction

CHAPTER I

INTRODUCTION

Helicoverpa armigera (Hübner), cotton bollworm, is a polyphagous insect. As polyphagous insects are feeding wide range of plant under different plant families (Bernays and Chapman, 1994), it is a major pest for crops such as cotton, maize, sorghum, pigeon pea, chickpea, soybean, groundnut, sunflower, and a range of vegetables. Therefore, it causes very serious losses in these crops all over the world (Sequeira and Playford, (2001); Sekulic *et al.,* (2004); Horvath *et al.,* (2004)). In addition, *H. armigera* moth has ability to fly long distances; it can cover large geographical areas (Fitt, 1989). Consequently, it occurs throughout much of Africa, Asia, India, Indonesia, Australia, Turkey and recently some Europe countries such as France and Spain.

One of the most common problems during production of the economical important crops such as groundnut, tomatoes and cotton is destruction of these crops by insect invasion. In order to control its proliferation in cultures of agricultural crops, there are four main different types of insecticides; organochlorine, carbamate, organophosphate and pyrethroid (synthetic analogue of pyrethrins), used in different parts of the world. Since organochlorine, carbamate and organophosphate type insecticides are highly toxic for mammals including man, pyrethroids were introduced into the market around the 1980s. As pyrethroids are so effective at low doses without toxicity for mammals, they have been favoured by farmers and used extensively in agricultural areas. However, common usage of pyrethroids has caused development of resistance, as it was reported from different countries Kranthi *et al.,* (2001); Martin *et al.,* (2002); Y. Yang *et al.,* (2004); Grubor *et al.,* (2007); Djihinto *et al.,* (2009); Achaleke *et al.,* (2009)], including Turkey (McCaffery, (1998); Ugurlu *et al.,* (2007).

1.1. Taxonomy of *Helicoverpa armigera*

Family: Noctuidae

Subfamily: Heliothinae

Species: *Helicoverpa armigera*

The moth currently called *H. armigera* has most commonly been referred to in the past as: *Bombyx obsoleta F.*, *H. obsoleta F.* and *Noctua barbara*. It has also been reported as *Heliothis armigera* and *Heliothis armiger* (Zalucki *et al.*, 1986).

1.1.1. Life Cycle of *Helicoverpa armigera*

H. armigera usually completes 6-7 generations per year under appropriate temperature conditions (**Figure 1.1**). During its lifetime, a single female may oviposit from 500 to 3,000 eggs, averaging close to 1,000. The eggs hatch in 3 to 7 days in warm weather, and larvae are mature after 2 to 3 weeks. Fully developed larvae move to the soil where they form an earthen cell 2-10 cm below the surface. When the chamber is dug, an exit tunnel is also dug, to ensure the emerging adults can escape **(Figure 1.2)**. The pupal period generally lasts from 8-21 days depending on temperature. Diapausing pupae can over-winter in the soil in more temperate areas for long periods (> 175 days). In tropical areas, diapauses can be induced by drought. Adults, those are mainly nocturnal, active in night while sleeping during the day, in their mating and egg laying activities period, can live as long as 2-3 weeks (Jallow and Zalucki, 1998).

H. armigera is highly migratory, and can fly long distances (Fitt, 1989). It tends to over-winter as pupae in the soil of local cropping area. As moth emerged from these over-wintering pupae, it may take several generations to build to high numbers of pest. Therefore, crop damage by *H. armigera* is commonly happened during the later parts of summer season (Deuter *et al.*, 2000). *H. armigera* has been preferentially feeding on buds, flowers and fruits. However, larvae may not always tend to feed on the plant structure on which they hatched, or be able to continue feeding once they have started. The preference for fruiting structures and the tendency to move from one fruit to another, often without consuming each fruit completely, is the main reason why extensive damage often results to crops even when the number of large larvae is relatively low (Zalucki *et al*., 1986).

In Turkey, *H. armigera* usually found at the Black Sea, Marmara, Aegean, Mediterranean, Central Anatolia and South-eastern Anatolia region. In late summer, their larvae mature in 10-15 days at Çukurova. Fully developed larvae move to the soil where they form an earthen cell 1-6 cm below the surface. The pupal period generally lasts from 9-12 days in summer. It completes 5 generations per year, third of which are on cotton. The plants, first generation of *H. armigera* develop on and last generation of it fed on, are important host plants for *H. armigera*. Host plants for the first generation are cotton, chickpea, corn, okra, potato, tomato, eggplant, and bean. The last generation hosts on cauliflower, bean, pepper, eggplant, spinach, okra, cabbage, maize (Zirai Mücadele Teknik Talimatı, 1995).

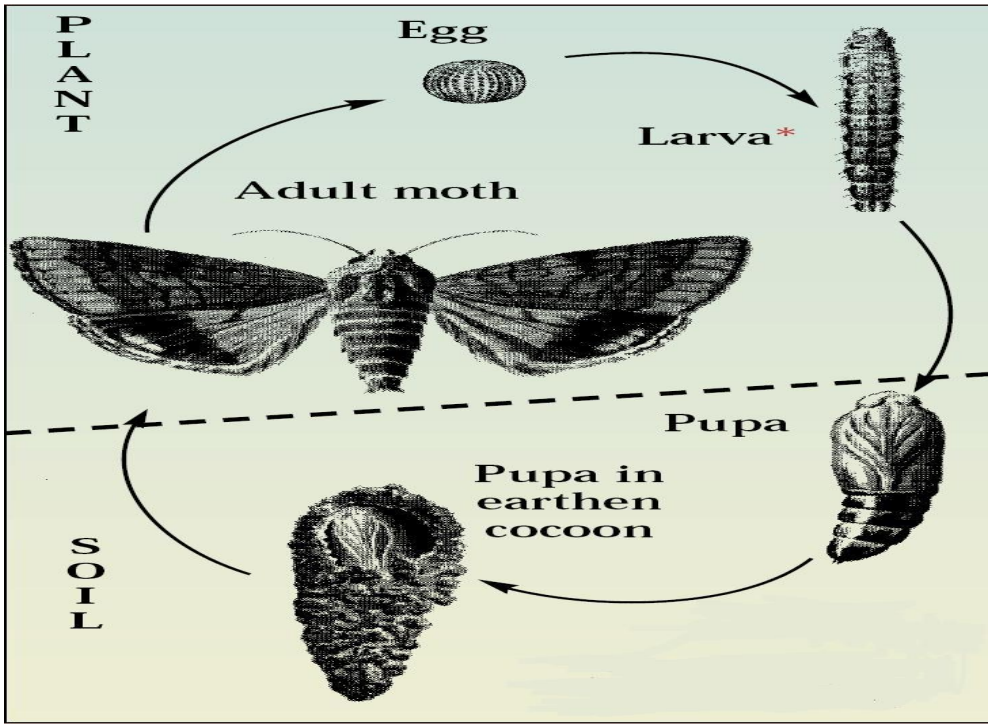


Figure 1.1. Life Cycle of *Helicoverpa armigera* (I.M.P.G., 2000)

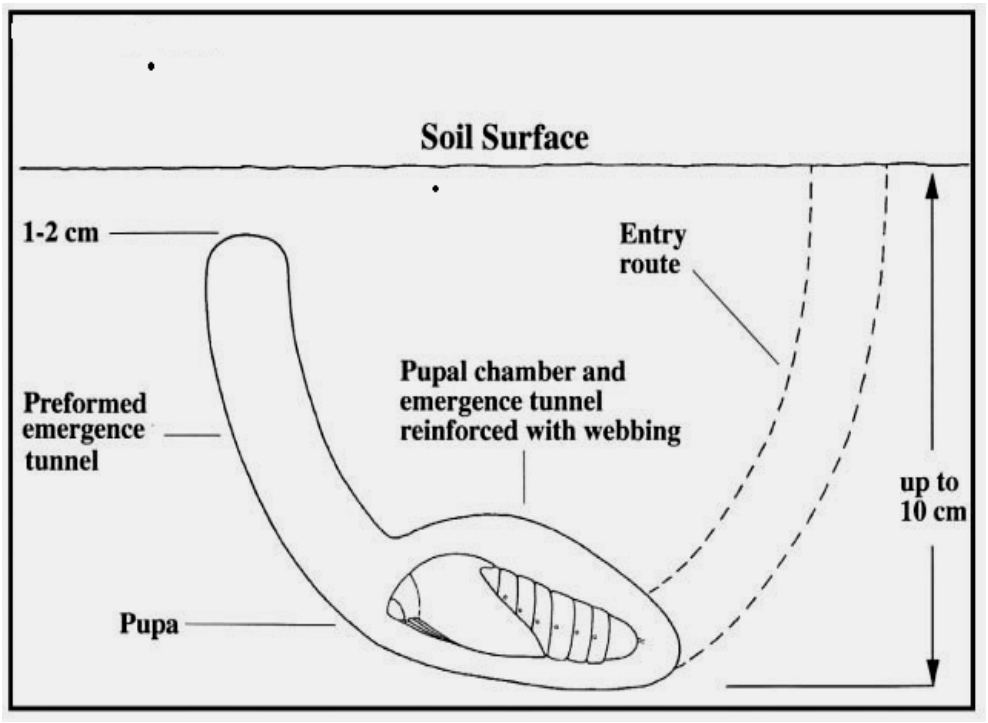


Figure 1.2. *Helicoverpa armigera* Pupa in Soil (Cotton CRC information and identification guide)

1.1.2. Morphology of *Helicoverpa armigera*

1.1.2.1. Morphology of *Helicoverpa armigera* Egg

Eggs are dome-like with a ribbed surface. The eggs are small (approximately 0.5 mm in diameter; about half the size of a pinhead) and sub-spherical (dome shaped with a slightly flattened bottom) in shape. Eggs are usually laid singly, making detection difficult near buds, flowers, fruits, or on leafy plant parts. They are initially pale green, sometimes with black dots, and they later change to cream and then brown (CPC, 2002; Deuter *et al*., 2000; DEFRA, 2001; Zalucki *et al*., 1986).

1.1.2.2. Morphology of *Helicoverpa armigera* Larva

Early instars are predominantly green and appear spotted because of dark spiracles and tubercle bases. Larvae pass through four, five, or sometimes even six instars, and ultimately reach 30 to 40 mm in length, and they usually display striped patterns and may vary in colour from light green to brown to black and have distinct hairs when held up to the light **(Figure 1.3)**. There is a good deal of colour variation in the larvae. For example, larvae may have white, instead of black spots. Superimposed on the dorsal bands are numerous lighter longitudinal lines, which are wrinkled or wavy. There are often dark, raised spots on the back, at the base of fine hairs. In *H. armigera,* there is a dark triangular area on the back of the first abdominal segment of the third, fourth and fifth instars of the larvae. Larvae have a posture when disturbed characteristic of a number of species in this family: it lifts its head and curls it under the front of the body. If even more disturbed, it lets go and drops, rolling into a spiral (CPC, 2002; Deuter *et al.*, 2000; DEFRA, 2001; Zalucki *et al*., 1986).

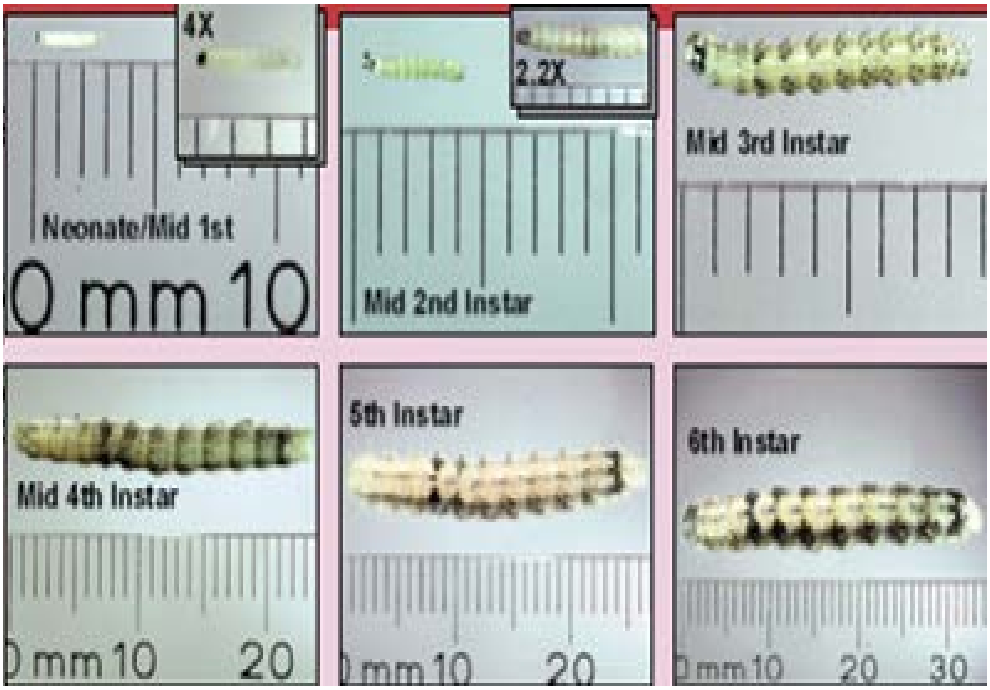


Figure 1.3. *Helicoverpa armigera* Larval Stages (I.M.P.G., 2000)

1.1.2.3. Morphology of *Helicoverpa armigera* Pupa

Pupae are 14-20 mm long, pale brown in colour with a tinge of green, turning darker brown as the adult develops within. Pupae can be separated reliably by measuring the distance between the outer edges of the cremaster spines at the junction with the cremaster (> 0-22 mm, *H. armigera*; <0-20 mm, *H. punctigera*) (CPC, 2002; Deuter *et al.*, 2000; DEFRA, 2001; Zalucki *et al.*, 1986).

1.1.2.4. Morphology of *Helicoverpa armigera* Adult

Stout-bodied moth of typical noctuid appearance, with 3.5-4 cm wing-span; broad across the thorax and then tapering, 14-18 mm long; colour variable, but male usually greenish-grey and female orange-brown. Forewings have a line of seven to eight blackish spots on the margin and a broad, irregular, transverse brown band. Hind wings are pale-straw colour with a broad dark-brown border that contains a paler patch; they have yellowish margins and strongly marked veins and a dark,

comma-shaped marking in the middle. Antennae are covered with fine hairs (Garcia-Tejero, 1957; Hardwick, 1965; Cayrol, 1972; Delatte, 1973).

1.1.3. Destruction Types of *Helicoverpa armigera*

1.1.3.1. Destruction Types of *Helicoverpa armigera* on Cotton

Bore holes are visible at the base of flower buds, the latter being hollowed out. Bracteoles are spread out and curled downwards. Leaves and shoots may also be consumed by larvae. Larger larvae bore into maturing green bolls; young bolls fall after larval damage. Adults lay fewer eggs on smooth-leaved varieties.

1.1.3.2. Destruction Types of *Helicoverpa armigera* on Tomatoes

Young fruits are invaded and fall; larger larvae may bore into older fruits. Secondary infections by other organisms lead to rotting. Eggs are laid on the silks, larvae invade the cobs and developing grain is consumed. Secondary bacterial infections are common.

1.1.3.3. Destruction Types of *Helicoverpa armigera* on Sorghum

Larvae feed on the developing grain, hiding inside the head during the daytime.

1.1.3.4. Destruction Types of *Helicoverpa armigera* on Chickpea

Foliage, sometimes entire small plants are consumed; larger larvae bore into pods and consume developing seed.

1.1.3.5. Destruction Types of *Helicoverpa armigera* on Pigeon pea

Flower buds and flowers bored by small larvae may drop; larger larvae bore into locules of pods and consume developing seed. Short duration and determinate varieties are subject to greater damage.

1.1.3.6. Destruction Types of *Helicoverpa armigera* on Groundnut

Leaves, sometimes flowers attacked by larvae; severe infestations cause defoliation.

1.1.4. Economical Consequences of *Helicoverpa armigera*

As mentioned before, larvae of *H. armigera* mostly prefer to feed with flower buds, flowers, seed, fruits and leaves, this leads to important yield reduction of economically important crops such as corn, cotton, maize, grain legumes and oilseeds. For example, 50% of all insecticides used in India and China are used to control for it. Farmers spend up to 40% of their annual income to buy chemicals to curb *H. armigera* (www.fightthemoth.org/mozilla/global/global.html). In India, losses were estimated to exceed $US 500 million in the late 1980s with an additional $US 127 million spent on insecticides annually (KN Mehrotra, Indian Agricultural Research Institute, New Delhi, unpublished data, 1987/88 in CAB, 2006). *H. armigera* also costs Australian agriculture $AUS225 million per annum. To deal with this species, growers estimated the cost of insect control on cotton at roughly $30/ha in 1966 and this increased rapidly to more than $800/ha in 1998 (Sequeira, 2001). Sekulic *et al.* (2004) reported damage in Europe, mainly on maize, sunflower, soybean, tomato, pepper and beans, in the Voivodina Province of Serbia and Montenegro in the very warm summer of 2003. 93.7% of maize plants were infested, in sunflower crops 80-100% of the plants were damaged and 85.3% of the soybean pods were injured in August. In addition, another serious damage by *H. armigera* in Europe was reported by Horvath *et al.* (2004) in sunflower fields in Kecskemet and Bacsalmas, Hungary; in 2003 (64.4% of the sunflower heads were infested with more than five *H. armigera* larvae per head).

1.2. Resistance History of *Helicoverpa armigera* to Conventional Insecticides

In order to control *H. armigera* firstly organochloride insecticides such as endosulfan and dichloro-diphenyl-trichloroethane (DDT) were used. But, they caused significant toxicity to animals including human and produced persistent organic pollutants. Then, organophosphates such as malathion and parathion become appear as they were rapidly degrading compare to the organochloride. Nonetheless, they have much acute toxicity to anyone else who may expose to it large amounts compare to organochloride. After those carbamates has been used to control *H. armigera*. As carbamates, cholinesterase inhibitor prevents neurotransmitter breakdown, they cause severe poisoning and the victim dies of respiratory failure in mammals. It is highly toxic for birds, as well. Finally, pyrethroids introduced into replace the previously used insecticides. Pyrethroids are partially refined extract of the *Chrysanthemum cinerariaefolium* flowers. They possess high activity and require extremely low doses for control *H. armigera*. Moreover, they were safe to mammals and immobile in the soil. After discovery of photo stable form of pyrethroids "synthetic pyrethroids" in 1977 cause increase in agricultural use of this insecticide (Elliott, 1977; Sattelle and Yamamoto, 1988). The pyrethroids have considered most potent insecticides for control of *H. armigera*. The early reports indicated that cypermethrin and fenvalerate insecticides effectively reduced *H. armigera* population. Continuous usage of these pyrethroids against to this pest causes development of resistance towards these insecticides. The first report related with this pyrethroid resistance in *H. armigera* reported from Australia in 1983 (Gunning *et al.*, 1984). Later, reports came from other countries in the following years. For example, Thailand and Columbia in 1984-85, Indonesia in 1987 and early 1988 (McCaffery *et al.*, 1991), India (Andhra Pradesh state) in 1987, New Zealand in 1992-94 (Cameron *et al.*, 1995; Suckling, 1996), Pakistan from 1991 onwards (Ahmad *et al.*, 1995), in China related with fenvalerate and deltamethrin and other pyrethroids such as cyhalothrin, cypermethrin, esfenvalerate, fenpropathrin and cyfluthrin being used from the mid- to late-1980s (Tan *et al.*, 1987; Shen *et al.*, 1991, 1992, 1993; Wu *et al.*, 1996, 1997). Recent studies also show that *H. armigera* have developed resistance towards synthetic pyrethroids (deltamethrin and cypermethrin, bifenthrin and fenvalerate) in

West Africa (Martin *et al.,* 2002; Djihinto *et al.,* 2009), in France through deltamethrin and methomyl (Bues *et al.,* 2005) and Spain against seven pyrethroids (lambda-cyhalothrin, cypermethrin, fenvalerate, bifenthrin, permethrin, deltamethrin and cyfluthrin) (Torres Vila *et al.,* 2002), India, Pakistan and China through fenvalerate, deltamethrin, cyhalothrin and cypermethrin (Kranthi *et al.,* 2001; Y. Yang *et al.,* 2004; E. Yang *et al.,* 2005). In Central Africa high resistance to cypermethrin was observed among *H. armigera* field populations and laboratory-selected strains **(**Achaleke *et al.,* 2009).

Resistance to synthetic pyrethroids in Turkish populations of *H. armigera* was firstly reported in 1984, after their initial use around 1980 (Anon, 1986). Ernst and Dittrich (1992) reported similar findings, as well. Ugurlu (2001) found that there was resistance development at field populations of *H. armigera* against synthetic pyrethroids lambda-cyhalothrin 20-41 folds and tralomethrin 15-24 folds compared to susceptible population. It was also reported that while resistance ratios for lambda-cyhalothrin were 3 and 98-fold increased in the Adana and Antalya strains of *H. armigera*, respectively, as esfenvalerate ratios were 3.33 and 92.3-fold increased in the Adana and Antalya strains of *H. armigera*, respectively, with respect to the susceptible strain (Ugurlu *et al.,* 2007). Ugurlu S. (unpublished data, sugurlu@hotmail.com) also found that both Adana and Mardin field populations showed higher resistance factors through pyrethroid insecticides compare to other type insecticides. For example, while resistance ratios through selected pyrethroids (esfenvalerate, bifenthrin, beta-cyfluthrin and lambda cyhalothrin) were in the range of 6.0-67.0 fold, carbamate (indoxcarb) and organophosphates (methomyl and azinphosmethyl) were in the range of 0.5-8.6 fold in Adana population. Similar results were detected for Mardin populations, as well. While resistance ratios for these pyrethroids were in range of 7.5-62.6, carbamate and organophosphates were in range of 0.45-2.96 in Mardin population. In addition, Ugurlu S. found that resistance ratio for lambda-cyhalothrin was 14.5 fold in Çanakkale field population compared with the reference susceptible strain.

1.3. Resistance Mechanisms Against to Conventional Insecticides

Insects develop resistance against applied insecticides by following three main mechanisms; (1) by reducing the amount of insecticide entering into the insect body, (2) developing insensitivity of the insecticide effective site and (3) increasing detoxification metabolism of insecticides using major enzyme systems such as, glutathione S-transferases (GST), cytochrome P-450 monooxygenases (CYP450) and esterases (EST), especially for carboxylesterase. Mutation or upregulation of one or more members of these gene families causes increased detoxification metabolism.

1.3.1. Reduced Penetration

Reduced penetration of insecticide amount through the cuticle is one of the important mechanisms to develop resitance against insecticides. One of the possible ways for reducing insecticide penetration is increasing cuticular thickness of insect body. This thicker cuticles cause slower rates of insecticide absorption into the body, likely to increase the efficiency of metabolic detoxification processes. There would be other way(s) except cuticle thickening that cause slower insecticide penetration across the cuticle. This reduction is also associated with insecticide resistance in the cotton bollworm *Helicoverpa armigera* (Ahmad *et al.*, 2006; Gunning *et al.*, 1995). However, this mechanism is not commonly encountered in practice. The other two mechanisms, metabolic detoxification, or decreased sensitivity of the target, are most commonly seen in field populations of insects.

1.3.2. Site Insensitivity

This form of resistance occurs as a consequence of mutations in the amino acid sequence of the voltage-gated sodium channel. Changes have been shown to occur at (or close by) positions 1014 for knockdown resistance (kdr) and 918 (super-kdr) (Williamson *et al.*, 1996). The level of resistance due to the former (kdr mutation) alone is generally similar for all pyrethroid molecules (20–50 fold). In contrast, it is much higher (up to 500 fold) for the super-kdr mutation (methionine to threonine in

houseflies). The importance of a methionine residue at position 918 for sensitivity to pyrethroids has recently been demonstrated by Vais *et al.,* (2000). They observed a significant increase in the sensitivity of mammalian sodium channels to pyrethroids when the isoleucine residue at the position equivalent to 918 in houseflies was replaced with methionine. In contrast to the kdr mutation, the levels of resistance observed for the super kdr mutations are closely linked to the structure of the pyrethroid molecule (Farnham and Khambay, 1995a and 1995b; Beddie *et al.*, 1996). Consequently, there is scope for the identification and development of pyrethroids that retain high efficacy against the pest but exhibit relatively low levels of resistance. The kdr and super kdr mutations can occur singly or in combination. For example, in houseflies and *Plutella xylostella* (diamond-back moth), both mutations are found but in *Aphis gossypii* only the super kdr mutation is found. Recent studies have indicated different combinations of these mutations can confer variable levels of resistance (Vais *et al.*, 2001).

1.3.3. Increased Metabolism

1.3.3.1. Carboxylesterases

Carboxylesterases are hydrolytic enzymes. They are belonging to the α/β-hydrolase fold superfamily. They cleave carboxylic acid esters by a two-step mechanism similar to the action of acetylcholinesterase (AchE). In this process, firstly, oxygen of the serine at the active site makes a nucleophilic attack on the carbonyl carbon of the substrate. Then, an acyl-enzyme linkage is formed. Finally, water molecule makes nucleophilic attack, releasing the acid product and regenerating the free enzyme.

Although insect esterases nomenclature has not standardized yet, Oakeshott *et al.,* (2005) have defined fourteen major clades according to the sequence similarity. It is also indicated that higher expression levels of esterases, involved in organophosphate resistance in Diptera and Hemiptera, are caused by gene amplification. These up-regulated esterases help to protect the insect by sequestering rather than hydrolyzing the insecticide (Oakeshott *et al.,* 2005). For example, while clade E esterase genes

are amplified in Hemiptera, including the brown rice planthopper, *N. lugens*, and the aphid *M. Persicae*, clade C esterase genes are amplified in culicine and anopheline mosquitoes. However, enzyme in housefly *M. Domestica* and sheep blowfly *L. Cuprina*, from clade B, has point mutations that increase its organophosphate hydrolase activity to confer resistance. In addition, there are numerous biochemical studies support esterase involvement in organophosphate and pyrethroid resistance in *H. armigera* (Gunning *et al.*, 1996 and 1999).

1.3.3.2. Glutathione S-Transferases

The glutathione S-transferases (GSTs) (EC.2.5.1.18) are enzymes that participate in cellular detoxification of endogenous as well as foreign electrophilic compounds by catalyzing conjugation of reduced glutathione (GSH) (**Equation 1.1**).

$$GSH + RX \longrightarrow GSR + HX$$

Reduced Glutathione Electrophilic Compound GSH Adduct (X could be Br, Cl, NO2, SO4)

Equation. 1.1. Typical GST Catalyzed Reaction

GSTs as a part of detoxification systems are evolved to protect cells against reactive oxygen metabolites by conjugating the reactive molecules to the nucleophile scavenging tripeptide glutathione (GSH, γ-glu-cys-gly) (Coles and Ketterer, 1990). GSTs serve as transporters of potentially harmful substances out of the cell with different electrophilic species (Jemth and Mannervik., et *al.*, 1999). Following conjugation, these generally harmless GSH adducts, or their mercapturic metabolites, are secreted into the bile or urine.

GSH, first described in 1888 as philothion, is the most ubiquitous and abundant non-protein thiol in mammal cells and serves as a necessary nucleophile in a number of detoxification reactions (Tew, 1994). In addition to its role in intracellular detoxification, it participates in interconversions of arachidonic acid pathway metabolites (prostaglandins and leukotrienes) (Flatgaard *et al*., 1993) and contributes

to regulation of protein and DNA synthesis (Rass, 1988) (**Figure 1.4**). Maintenance of a homeostatic GSH content is achieved by both de novo synthesis and salvage synthesis and a number of interrelated pathways are also involved (**Figure 1.5).**

All GST isozymes used reduced GSH as an acceptor species, but they differ in the specificity with which different substrates are transferred to the thiol group of cysteine of GSH. The GSTs are found in all eukaryotes and prokaryotic systems, in the cytoplasm, in the microsomes, and in the mitochondria.

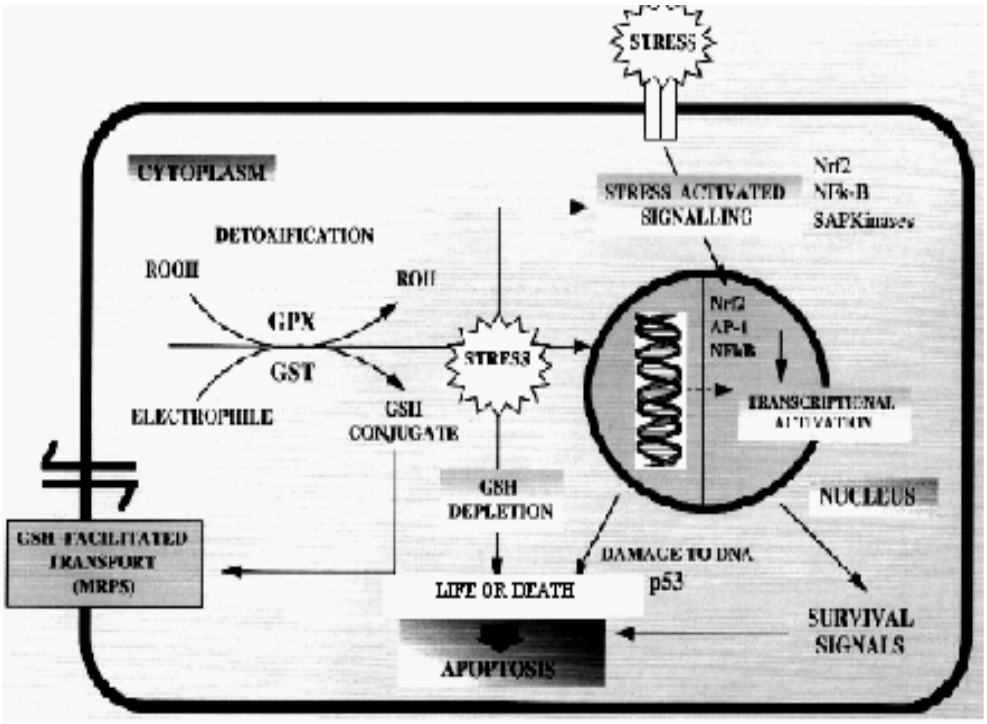


Figure 1.4. Mechanism for Cellular Protection by Glutathione (McLellan, 1999).

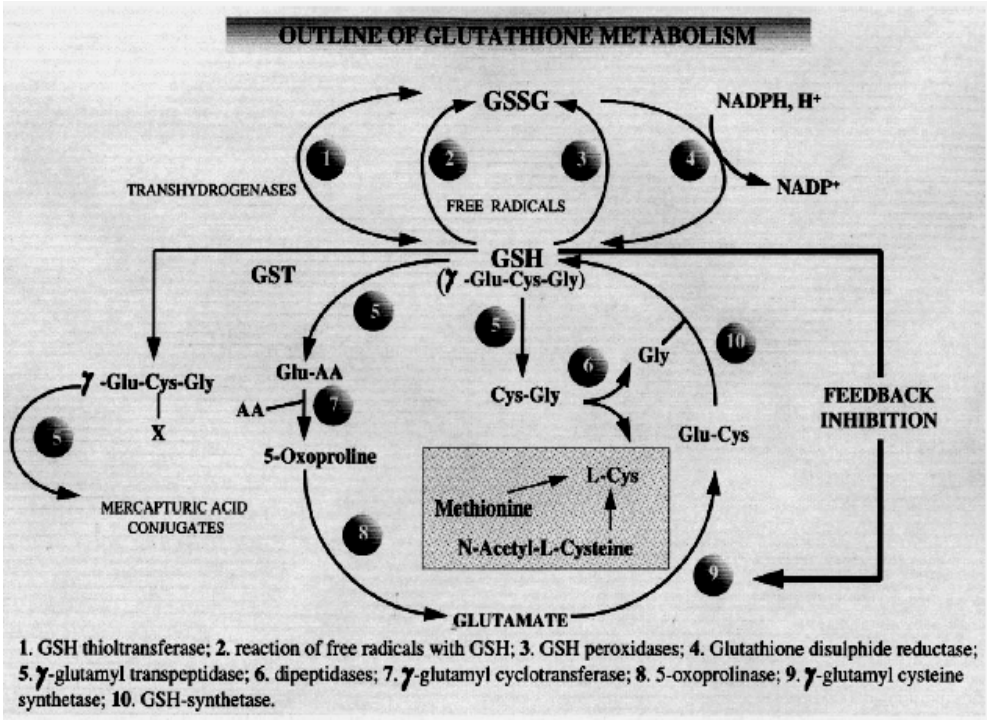


Figure 1.5. Glutathione Metabolism (McLellan, 1999).

In insects, when GSTs are classified according to sequence similarity, there are six GST classes present in insects. These are Delta, Epsilon, Omega, Sigma, Theta and Zeta classes. They may detoxify organophosphate insecticides by O-dealkylation or O-dearylation. They may also sequester pyrethroids and involve in resistance development by detoxifying lipid peroxidation products that are induced by pyrethroids (Vontas *et al.*, 2001).

Nevertheless, there is a number of studies where biochemical evidence implicates GSTs take role in resistance (Ranson *et al.*, 2005; Ugurlu *et al.*, 2007), there is only one case leading to the cloning of a GST in a lepidopteran. In this case, Chiang *et al.*, (1993) isolated three different GST isozymes from *Plutella Xylostella*. Isolated GST-3 isozyme showed lower activity against 1-chloro-2,4-dinitrobenzene (CDNB) but a higher activity against 3,4-dichloronitrobenzene (DCNB) compared to the other two isozymes (Chiang and Sun 1993). Later, fourth isozyme (GST-4) was isolated from *Plutella Xylostella*. GST-4 had a higher specific activity against DCNB and the OP

insecticides (Ku *et al.*, 1994). After that, GST-3 gene was cloned and expressed in *E. coli* (Huang *et al.*, 1998). It was found that mRNA levels of GST-3 were much greater in the resistant strains compared with the reference susceptible strains. However, southern blots analysis showed that higher mRNA levels of GST-3 was not caused by gene amplification. GST-3 gene was also studied by Sonoda and Tsumuki (2005) in Japanese strains, resistant to permethrin and the chitin synthesis inhibitor chlorfluazuron, similarly they found that the reason of higher mRNA levels was not occured with gene amplification. Although, there is more GST-3 present in resistant strains, the other isozymes appear to be present in the analyzed strains. In addition, it was classified as an Epsilon GST by Enayati, Ranson, and Hemingway (2005).

1.3.3.3. Cytochrome P450 Monooxygenase Enzymes

Cytochrome P450 monooxygenases (CYP450) are a large and ubiquitous class of heme-thiolate proteins. They catalyze a lot of reactions, including detoxification of xenobiotics. Likewise, in insects CYP450s play roles in detoxification of xenobiotic compounds such as hostplant toxins and insecticides. In addition, they have role in synthesis of hormones and pheromones, fatty acid metabolism, as well. Most of the CYP450s, involving in detoxification, are induced by xenobiotics; that is, transcription rates of these genes, normally regulated at a low level, are increased in response to the appearance of xenobitic compounds. However, inducers may be another molecule(s) instead of toxins that need to be eliminated.

The first cloned CYP450s implicated in pyrethroid resistance from *H. armigera* belong to the CYP6B subfamily. CYP6B2 (Wang and Hobbs 1995) and CYP6B6 and CYP6B7 (Ranasinghe and Hobbs 1998) were cloned from Australian *H. armigera*. It was found that CYP6B7 mRNA levels were elevated in field strains compared to a susceptible strain (Ranasinghe, Campbell, and Hobbs 1998). Ranasinghe and Hobbs (1999) hypothesized that overexpression of CYP6B7 gene was the main reason for resistance in Australian field populations. Then, Grubor and Heckel (2007) found that the three genes were arranged in a cluster, in the order CYP6B7-CYP6B6-CYP6B2 in AN02 strain of *H. armigera* from Australia, 50 fold

resistant to fenvalerate. They also analysed mRNA levels of these three genes with quantitative real-time -PCR in the AN02 strain. It was found that CYP6B levels did not change with pyrethroid resistance. Finally, Wee *et al.,* (2008) used the cDNA-AFLP technique to search for mRNAs of genes in pyrethroid resistance that were up- or down-regulated in the AN02 strain of *H. armigera*. It was found that CYP337B1 gene showed a significant up-regulation in pyrethroids resistant individuals of *H. armigera*. In addition, Yang *et al.,* (2006) cloned CYP9A12 and CYP9A14 genes from the YGF strain of *H. armigera* from China, which developed 1.690-fold resistance to fenvalerate. Quantitative real-time RT-PCR showed that while CYP9A12 gene expression was increased 19-fold in midgut and 433-fold in fat body, CYP9A14 gene expression was increased 4-fold and 59-fold, respectively, compared to a susceptible strain.

In order to study the role of CYP450s biochemically, it has been used model substrates to monitor the progress of a particular type of reaction such as O-demethylation. Due to their lack of specificity for single enzyme together with the multiplicity of CYP450 genes (e.g., 160 in *A. aegypti*, Strode *et al*,. 2008; 87 in *B. mori*, Kozaki *et al.,* 2008) has made difficult the implication of a specific CYP450 gene. There are a lot studies for lepidopteran species put evidence that CYP450s play imporatant role in metabolic insecticide resistance include *H. armigera* (Forrester *et al.,* 1993; Kranthi *et al.,* 2001; Yang *et al.,* 2004).

Researches have done in the different part of the world related with the relative roles of drug metabolizing enzyme systems in metabolic resistance of *H. armigera*, there are controversy mainly between monooxygenases and esterases. Early studies in 1990s indicated that hydrolysis by esterases in *H. armigera* were probably the main detoxification enzyme action contribute to pyrethroid resistance through esfenvalerate, alpha and zeta cypermethrin, fenvalerate, permethrin, deltamethrin and flucythrinate, (Gunning *et al.,* 1996, 1999) in Australia. However, recent studies in China, India, Pakistan and West Africa have put the evidences that resistant strains showed significantly increase in cytochrome P450 monooxygenases activity compare to the esterase and GSTs activity increment. Therefore, cytochrome P450

monooxygenases are possibly major contributor of this resistance (Martin *et al.,* 2002, Yang, Y. *et al.,* 2004 and Yang, E., 2005). Although GSTs have not yet implicated in the direct metabolism of pyrethroid insecticides, they may play an important role in mediating oxidative stress responses such as conjugating reactive species and activated compounds, detoxifying lipid peroxidation products and oxidized DNA bases (Vontas *et al.,* 2001). Vontas *et al.,* (2002) also proposed that GSTs involved in the prevention or repair of oxidative damage induced by insecticide exposure. In addition, GSTs may have role protecting insects from the toxicity of the pyrethroids by sequestering the insecticide (Kostaropoulos *et al.,* 2001).

As a result of these findings, metabolic resistance studies related with *H. armigera* mainly focused on measuring certain cytochrome P450 monooxygenases activities or mRNA levels of certain genes and also by looking on similar changes on esterases and GSTs, other two main detoxifying enzyme systems.

1.4. Proteomics

Proteomics is the study of proteins expressed in organism or system, referred to as the proteome. Proteomics characterize the proteome in terms of structure, function and expression level under certain conditions of biological stress (James 1997). As expressed proteins are functional molecules in organism, there will be differences on expressed proteins from cell to cell in the same organism according to the physiological needs. This expression will vary with time, stress and requirements of organism. Proteomic studies, especially for expression proteomics, generally provide information understand mechanism of diseases especially for human. Thus, there is a considerable interest for expression proteomics, important contributions into disease diagnostics and treatment (Foster *et al.,* 2005; Mazzanti *et al.,* 2006; Alexander-Kaufman *et al.,* 2006). Expression proteomics analysis include monitoring the changes in protein concentration level. It also attempts to identify certain proteins whose expression level correlates to the certain physiological state or strees of the organism.

As proteome content may be different from cell to cell in same organism according to physiological needs of organism, the methodologies, employed in proteomics analysis, may be variable depending on the aim of the study. The analysis scheme used to study proteome referred to as the proteomics workflow. The proteomics workflow, used to study *Helicoverpa armigera* midgut proteomics is shown at **Figure 1.6**.

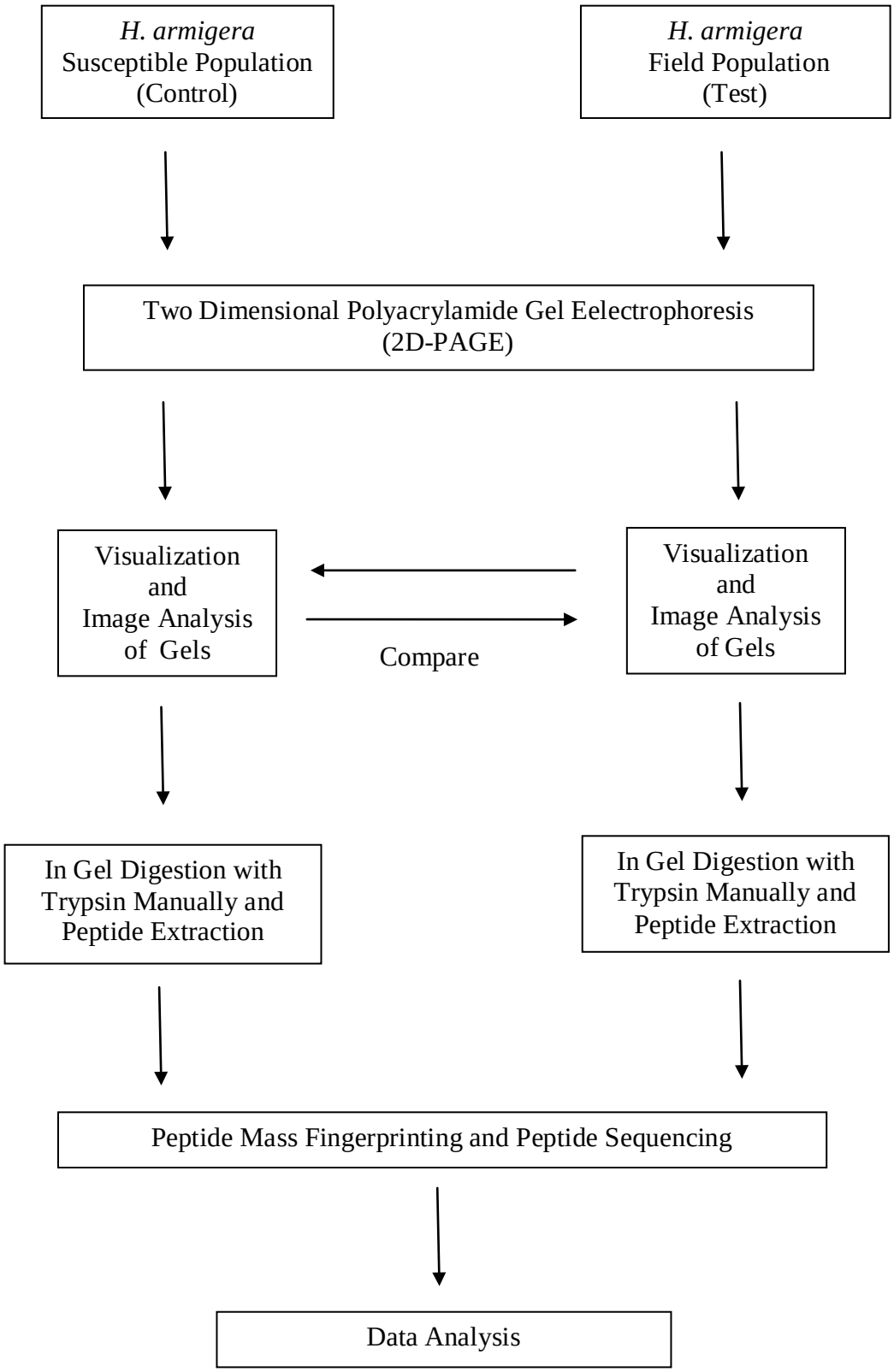


Figure 1.6. The Proteomics Workflow for Comperative Proteomic Analysis of *H. armigera* Midgut

1.4.1. Comparative Proteomic Analysis

Comparative proteomic analyses compare target organism proteome under two different physiological conditions such as control and field populations of *H. armigera*. Thus, it may discover the role of expressed/repressed proteins in biological systems under certain conditions, including insecticide exposure. It is also give important clues about mechanisms evolved to counteract to any conditions causes stress in organism. For example, triazophos, insecticide, causes an increase in the content of brown planthopper (*Nilaparvata lugens*) male accessory gland proteins. As these proteins may be transferred to females via mating, they cause an increase in fecundity of females (Wang *et al.*, 2010). In order to understand changes in the types of accessory gland proteins and reproductive proteins that mated female planthoppers, researchers focused on comparative proteomic analysis of mated *N. lugens* females. It was found that these proteins seemed to participate in the reproductive process of *N. lugens* adult females and males (Ge *et al*., 2011).

In order to study existence of proteins in complex protein extracts with mass spectrophotometer (MS), electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) techniques are used for ionization these biomolecules. As MALDI technique has higher tolerance to sample contaminants and static nature compared to ESI, this technique is commonly selected for mass spectrophotometric identification for protein analysis (Zhang *et al.*, 2001).

Because of large mass range (up to 13 kDa) of time-of-flight mass spectrometer, it is commonly used with MALDI technique (MALDI-TOF-MS) for protein identification processes (**Figure 1.7)**. MALDI-TOF-MS was used in our study for identification proteins in *H. armigera* midgut, as well.

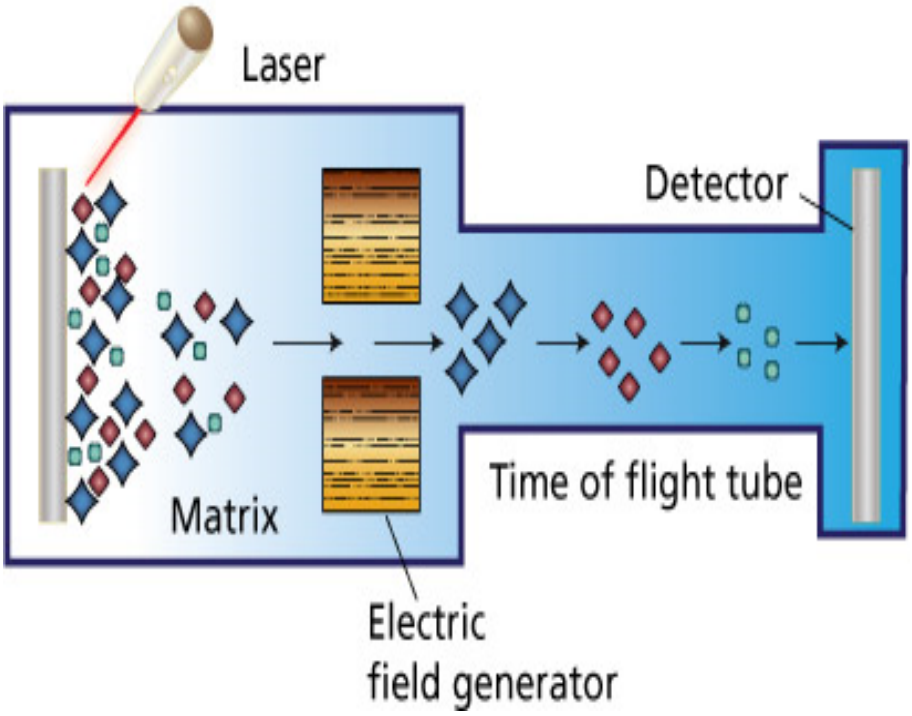


Figure 1.7. MALDI-TOF Mass Spectrometry (www.sigmaaldrich.com)

Proteomic data analyses are done with databases (e.g., Swiss-Prot, PDB) freely available servers such as the Expert Proteomics Analysis System (ExPASy) (www.expasy.org) or the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). With the help of developed searching algorithms, analyses thousands of mass spectrophotometric peptide spectra may be done with popular search engines including SEQUEST (Eng *et al.*, 1994) and MASCOT (Perkins *et al.*, 1999) . Experimentally collected fragmentation spectra is matched to the spectra of potential peptide from proteomics databases by these algorithms. In addition, employed scoring systems helps to maintain the quality of matches for removing false positives matches.

1.4.2. MASCOT Search

Mascot is a search engine that used for analysing mass spectrophotometric peptide spectra to identify proteins from protein databases such as EST, NCBI and SwissProt. It can be accessed in to the MASCOT search program through http://www.matrixscience.com website.

MASCOT search program use following different search methods to reach correct protein identification;

-Peptide Mass Fingerprint (mass values of peptide mixtures, belogs to analyzed protein, searched in database, see an example of Peptide Mass Fingerprint search and its result in Appendix A)

-Sequence Query (one or more peptide mass values with peptide sequence information used to search in database)

-MS/MS Ion Search (one or more peptide MS/MS ions searched in database)

1.5. Aim of the Study

The aim of this study is to analyze the role of detoxification enzyme systems, glutathione S-transferases, esterases, and cytochrome P-450 monooxygenases, in metabolic resistance against synthetic pyrethroid in field populations of *Helicoverpa armigera* in Turkey from Adana and Çanakkale and Mardin provinces. In order to understand role of these enzymes on metabolic resistance development at protein level comparative differential proteomic analysis together with certain enzymatic activity measurements were done in *H. armigera* midgut samples. In addition to this, mRNA level inductions of selected enzymes from those systems were analyzed by real-time polymerase chain reaction (*q*RT-PCR) method compare to susceptible strain. Thus, study the expression of certain genes by determining the mRNA amounts of these genes would expect to give us extra information about relative roles of detoxification enzyme systems in pyrethroid resistance development in field of *H. armigera* from Turkey.

CHAPTER II

MATERIAL AND METHOD

2.1. Materials

1-chloro-2,4-dinitrobenzene (CDNB), 3,4-dichloronitrobenzene (DCNB), p-nitrobenzylchloride (PNBC), 1,2-epoxy-3-(p-Nitrophenoxy)propane (EPNP), reduced glutathione (GSH), phenylmethanesulfonylfluoride (PMSF), p-nitrophenylacetate (PNPA), α-naphthyl acetate (α-NA), bovine serum albumin (BSA), dipotassium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), ammonium persulphate (APS), N,N'-methylene-bisacrylamide (Bis), acrylamide were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. 2-mercaptoethanol, ether, ultra pure methanol, potassium dihydrogen phosphate, potassium sodium tartrate tetrahydrate and Ciocalteu's Folin phenol reagent were from Merck, Darmstadt, Germany. Copper (II) sulphate 5-hydrate extra pure and sodium carbonate were from Riedel de-Haen. All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.2. Method

2.2.1. Insects and Tissue Preparation

Samples of *H. armigera* larvae were obtained from cotton fields in Adana and Çanakkale and Mardin provinces in 2008. The susceptible strain of *H. armigera* was obtained from Bayer CropScience, in Germany. Larvae were fed on artificial diet, allowed to pupate. Emerging adults were allowed to breed and the resulting first and second generation of sixth instar larvae used for the experiments.

In order to remove midguts of *H. armigera*, larvae were paralyzed by keeping on ice. 2-days-old sixth instars larvae were cut along their length by razor blade on ice midguts were removed. Midguts were immediately cleaned in 1.15 M KCl in order to remove fats or other unwanted substances like Malpighi tubes. Then, they were individually dried on filter paper and their weights were recorded. Finally, they were placed into eppendorf tube and stored in deep freezer at -80^{o}C until they were homogenized (Figure 2.1.).

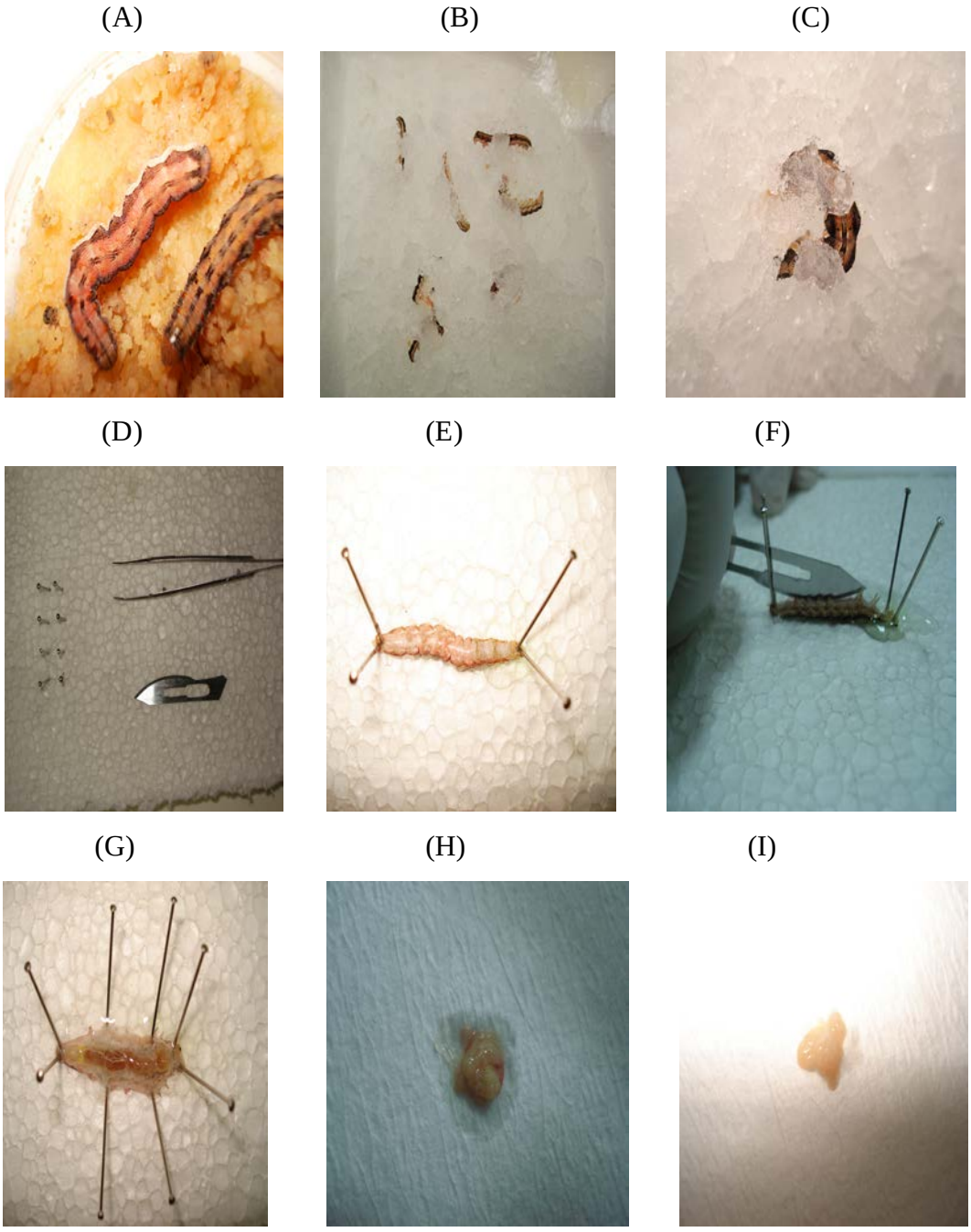


Figure 2.1. Preparation Steps of *Helicoverpa armigera* Midguts from Larvae

A-) 6th instar larvae on diet, B-) 6th instar larvae on ice, C-) Individual larvae on ice, D-) Equipments need for midgut removal, E-) Larva fixed on plate at reverse position with pins, F-) Larva skin cut horizontally with lancet G-) Larvae skin opened with help of pins, H-) Midgut removed from larva body and wash with 1.15% KCl, I-) Midgut dried on filter paper

2.2.2. Preparation of Cytosols from *H. armigera* Midguts for GST and EST Assays

Each midgut was homogenized (5 times for 30 seconds with 60 seconds intervals) in 1 ml of 100 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA and 1 mM PMSF, with an ultraturrax homogenizer on ice. The homogenate was centrifuged at 10.000 x g for 30 min in a Sigma Hettich centrifuge. The supernatant (S9 fraction) was used as the enzyme source for GST and EST assays, respectively. The protein concentrations in the prepared crude extracts were determined by the method of Lowry *et al.*, (1951).

2.2.3. Protein Determinations with Lowry Method

The protein concentrations in the prepared crude extracts were determined by the method of Lowry *et al.*, (1951) with crystalline bovine serum albumin (BSA) as a standard. Aliquots of 0.1 ml of 1:10 diluted midgut cytosol were taken into test tubes and were completed to a final volume of 0.25 ml with distilled water. Then, alkaline copper reagent was prepared by mixing 2% copper sulfate, 2% sodium potassium tartrate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100, respectively. Afterwards, 1.25 ml of the alkaline copper reagent was added to each tube, mixed by vortex and allowed to stand undisturbed for 10 minutes at room temperature. Finally, 0.125 ml of 1 N Folin Phenol reagent was added to each test tube, mixed immediately within 8 seconds by vortex and incubated 30 minutes at room temperature. The intensity of colour developed in each tube was measured at 660 nm.

The protein concentrations in the crude extracts were calculated from a standard calibration curve that was constructed from the corresponding $O.D_{660nm}$ values of BSA standards 0 to 200 μg (**Table 2.1**).

Table 2.1. Components for the Preparation of the Standard Curve in Lowry Method

BSA Concentration	Volume of BSA Standards	Alkaline Copper Reagent	Volume of Folin Phenol Reagent	Total
0	250 µl	1250 µl	125 µl	1625 µl
0.02 mg/ml	250 µl	1250 µl	125 µl	1625 µl
0.05 mg/ml	250 µl	1250 µl	125 µl	1625 µl
0.1 mg/ml	250 µl	1250 µl	125 µl	1625 µl
0.15 mg/ml	250 µl	1250 µl	125 µl	1625 µl
0.2 mg/ml	250 µl	1250 µl	125 µl	1625 µl

2.2.4. Determination of Cytosolic GST Activity with CDNB

Glutathione S-transferase activity measurements were done by a modified Habig *et al.*, (1974) method using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate at 340 nm wavelength in a microplate reader. Each reaction mixture contained 100 mM potassium phosphate buffer, pH: 7.5, 1 mM GSH, 1 mM CDNB and ~2 µg cytosolic proteins in a final volume of 250 µl in 96 well plates (**Table 2.2**). The reactions were started by the addition of enzyme-containing protein extracts into each well. GST-CDNB activity measurements were done automatically every 20 seconds for 10 minutes at 25^{o}C. Slopes of the best lines drown for each well separately by the software of the instrument (ELX 808 Absorbance Microplate Reader) was used as the rate of reaction. Finally, the rate of reaction (dA/dt) in each well was determined separately. (dA/dt) and the further calculations were completed as below equation;

$$\frac{dA/dt}{\varepsilon\ (\mu M^{-1})} \times \frac{250\ \mu l\ (\text{Total volume})}{15\ \mu l\ (\text{Protein extract volume})} \times \text{Dilution Factor}^{*} \times \frac{1}{\text{Concentration (mg/ml)}^{**}}$$

* Dilution factor was determined previously to adjust ~2 µg cytosolic proteins in a final volume of 250 µl in 96 well plates for each sample.

** Protein concentration of the individual whose reaction rate (dA/dt) was determined with ELX 808 absorbance microplate reader.

The actual extinction coefficient (ε) for CDNB at 340 nm is 0.0096 $\mu M^{-1}cm^{-1}$. This value was adjusted as 0.00629 $\mu M^{-1}cm^{-1}$ for the path length (0.65 cm) of the solution in the well and used for calculation of GST-CDNB activities. The GST activities were expressed as nmole/min/mg protein.

2.2.5. Determination of Cytosolic GST Activity with DCNB

GST-DCNB enzyme activity was determined with a microplate reader by monitoring the thioether formation at 340 nm using 3,4-dichloronitrobenzene (DCNB) as substrate according to the method of Habig *et al.*, (1974). Each reaction mixture contained 100 mM potassium phosphate buffer, pH: 7.5, 5.0 mM GSH, 1.0 mM DCNB in a final volume of 250 µl (**Table 2.2**). Likewise GST-CDNB activity calculations, slopes of the best lines drown for each well separately by the software of the instrument was used as the rate of reaction. (dA/dt) and the further calculations were completed as below;

$$\frac{dA/dt}{\varepsilon\ (\mu M^{-1})} \times \frac{250\ \mu l\ (\text{Total volume})}{15\ \mu l\ (\text{Protein extract volume})} \times \frac{1}{\text{Concentration (mg/ml)}^{**}}$$

** Protein concentration of the individual whose reaction rate (dA/dt) was determined with ELX 808 Absorbance microplate reader.

The actual extinction coefficient (ε) for DCNB at 340 nm is 0.0085 $\mu M^{-1}cm^{-1}$. This value was adjusted as 0.00557 $\mu M^{-1}cm^{-1}$ for the path length (0.65 cm) of the solution in the well. The GST activities were expressed as nmole/min/mg protein.

2.2.6. Determination of Cytosolic GST Activity with PNBC

GST-PNBC enzyme activity was determined in a shimadzu UV-Visible spectrophotometer by monitoring the thioether formation at 310 nm using p-nitrobenzylchloride (PNBC) as a substrate according to the method of Habig *et al.*, (1974). In the PNBC-GST assay, each reaction mixture contained 100 mM potassium phosphate buffer, pH: 6.5, 5.0 mM GSH, 1.0 mM PNBC in a final volume of 1 ml (Table 2.2.).

The actual extinction coefficient (ε) for PNBC at 310 nm is 0.0019 $\mu M^{-1}cm^{-1}$. The GST-PNBC activities were expressed as nmole/min/mg protein. Similarly in CDNB and DCNB activity calculations, dA/dt and the further calculations were completed as below;

$$\frac{dA/dt}{\varepsilon\ (\mu M^{-1})} \times \frac{1000\ \mu l\ (\text{Total volume})}{50\ \mu l\ (\text{Protein extract volume})} \times \frac{1}{\text{Concentration (mg/ml)}^{**}}$$

** Protein concentration of the individual whose reaction rate (dA/dt) was determined with UV-Visible spectrophotometer.

2.2.7. Determination of Cytosolic GST Activity with EPNP

GST-EPNP activity was measured according to the method of Habig *et al.*, (1974). 1,2-epoxy-3-(p-Nitrophenoxy)propane (EPNP) is a specific substrate for theta isozyme of Glutathione S-transferases. Determination of GST-EPNP activity in the presence of the cofactor reduced glutathione (GSH) was performed to monitor the thioether formation at 360 nm in a 1 ml cuvette. Each reaction mixture contained 100 mM potassium phosphate buffer, pH: 6.5, 5.0 mM GSH, 0.25 mM EPNP in a final volume of 1 ml (**Table 2.2**). Then product formation was followed for 2 minutes at 360 nm at spectrophotometer. Finally, the enzyme activity was calculated by using 0.5 $mM^{-1}cm^{-1}$ as an extinction coefficient at 360 nm.

Table 2.2. Constituents of Biochemical Assay Mixtures for Glutathione S-Transferases

Assay	Phosphate Buffer (pH and Concentration)	GSH (mM)	Substrate (mM)	Protein Concentration
GST-CDNB	pH: 7.5-100 mM	1	1[a]	~2.0 µg
GST-DCNB	pH: 7.5-100 mM	5	1[b]	10.2-57.3 µg
GST-PNBC	pH: 6.5-100 mM	5	1[c]	34-191 µg
GST-EPNP	pH: 6.5-100 mM	5	0.25[d]	34-191 µg

GSH:Reduced Glutathione
[a] CDNB: 1-chloro-2,4-dinitrobenzene
[b] DCNB: 3,4-dichloronitrobenzene
[c] PNBC: p-nitrobenzylchloride
[d] EPNP: 1,2-epoxy-3-(p-Nitrophenoxy)propane

2.2.8. Determination of EST Activity towards p-nitrophenyl acetate (PNPA)

EST-PNPA enzyme activity was determined spectrophotometrically in 96 well micro plates by monitoring p-nitrophenol formation in a final volume of 200 µl at 405 nm using p-nitrophenylacetate (PNPA) as a substrate at 37°C, with 30 second intervals for 10 minutes according to the method of van Asperen (1962). In the EST-PNPA assay, each reaction mixture contained 100 mM potassium phosphate buffer, pH: 7.0, containing 0.05 % Triton X-100, 3.8 mM PNPA and 0.7-3.8 µg proteins in a final volume of 200 µl (**Table 2.3**). In order to determine EST-PNPA activity, molar extinction coefficient for p-nitrophenol at 405 nm= 0.0181 $nmole^{-1}$ at 200 µl (Ugurlu S., 2001) was used to calculating activities of *H. armigera* samples.

The EST activities of all individuals were measured triplicate and expressed as nmole/min/mg protein. (dA/dt) and the further calculations were completed as below;

$$\frac{dA/dt}{\varepsilon\ (\mu M^{-1})} \times \frac{200\ \mu l}{10\ \mu l} \times \text{Dilution Factor*} \times \frac{1}{\text{Concentration(mg/ml)**}}$$

* All of the samples were diluted as 10 times before adding in to the well.

** Protein concentration of the individual whose reaction rate (dA/dt) was determined with ELX 808 Absorbance microplate reader.

2.2.9. Determination of EST Activity towards α-naphthylacetate (α-NA)

EST- α-NA enzyme activity was determined in 96 well micro plates by monitoring α-naphthol formation in a final volume of 250 µl at 450 nm using α-naphthyl acetate (α-NA) as a substrate at 15 second intervals for 10 minutes according to the method of van Asperen (1962). Each reaction mixture contained 200 mM potassium phosphate buffer, pH: 6.0, containing 6 mg fast blue RR salt, 1 mM α-NA and 0.7-3.8 µg protein in a final volume of 250 µl (**Table 2.3**). The molar extinction

coefficient for α-naphthol at 450 nm is 0.00925 μM^{-1}at 250 µl (Grant *et al.,* 1989) and was used to calculate activities as nmole/min/mg protein.

EST- α-NA activities of all individuals were measured triplicate and expressed as nmole/min/mg protein. (dA/dt) and the further calculations were completed as below;

$$\frac{dA/dt}{\varepsilon\ (\mu M^{-1})} \times \frac{250\ \mu l}{10\ \mu l} \times \text{Dilution Factor*} \times \frac{1}{\text{Concentration(mg/ml)**}}$$

* All of the samples were diluted as 10 times before adding in to the well.

** Protein concentration of the individual whose reaction rate (dA/dt) was determined with ELX 808 Absorbance microplate reader.

2.2.10. Statistical Analysis of Enzyme Activity Results

Differences in measured GST and EST activities between susceptible, Adana, Çanakkale and Mardin populations of *Helicoverpa armigera* were assessed by student t-test MINITAB 15.0 statistics software.

Table 2.3. Constituents of Biochemical Assay Mixtures for Esterases

Assay	Phosphate Buffer (pH and Concentration)	Substrate (mM)	Protein Amount
EST-PNPA	pH: 7.0-100 mM (contain 0.05% Triton X-100)	3.8[a]	0.7-3.8 µg
EST-α-NA	pH: 6.0-200 mM (contain 6mg Fast Blue RR Salt)	1[b]	0.7-3.8 µg

[a] PNPA: p-nitrophenyl acetate
[b] α-NA: α-naphthyl acetate

2.2.11. Protein Extract Preparation from H. armigera Midguts for Proteomic Analysis

Protein extraction was performed according to Just *et al.*, (2006) and Zhao *et al.*, (2006), respectively, with modifications. Pooling ~10 midguts (each midgut weight ~25-30 mg) resulted in 250 mg samples were homogenized on ice in lysis buffer containing 1 ml mixture consisting of 40 mM Tris, 3 mM EDTA and 1 mM PMSF, with an ultraturrax homogenizer. The homogenate was centrifuged at 10.000 x g at +4 ºC for 30 min. After that, supernatant was aliquoted into 300 µl aliquots and lyophilized by freeze drying. It was resolved in 150 µl lysis buffer containing 2 M thiourea, 7 M urea, 4% CHAPS, 0.5% (v/v) 3-10 IPG buffer and 70 mM DTT. These resolved mixtures were filtered with Microcon 10 filters (nominal molecular weight limit 10 Dalton) by collecting proteins in modified rehydration buffer containing 8 M urea, 2% CHAPS and 16mM DTT. It was used as a protein source for isoelectric focussing and two-dimensional gel electrophoresis (**Table 2.4**). The protein concentrations of filtrated samples were determined by the Bradford method (1976).

As the lyophilisation process generally causes some protein losses in the samples, fresh-frozen tissue samples of susceptible strain were included into the study as the fourth sample source to reach accurate results for differential analysis of lyophilized samples. Fresh midgut tissue homogenates without lyophilisation were done in mixtures of lysis buffer (2 M thiourea, 7 M urea, 4% CHAPS, 0.5% (v/v) 3-10 IPG buffer and 70 mM DTT) and inhibitors (1 mM PMSF and 1 tablet Complete^{TM} (Roche Diagnostics, Mannheim, Germany) containing 1mM EDTA) by mortar and pestle according to the procedures from the Proteome Center Rostock (Just *et al.*, 2006). These homogenates were also filtered with Microcon 10 filters and proteins collected in a modified rehydration buffer as a protein source.

Table 2.4. General information about samples from *H. armigera* populations used for 2D –PAGE gel electrophoresis

STRAIN	Source	Animal No.	Fresh Weight of Tissue	Protein Extract Volume	Protein Concentration before lyophilization	Protein Concentration after lyophilization
CF	Monheim [a)]	40	165 mg [c)]	2 ml	ca. 9,8 mg/ml	-
CL	Monheim [b)]	50	250 mg [d)]	1 ml	ca. 13,7 mg/ml	ca. 10,9 mg/ml [e)]
A	Adana [b)]	50	250 mg [d)]	1 ml	ca. 17,7 mg/ml	ca. 13,2 mg/ml [e)]
M	Mardin [b)]	50	250 mg [d)]	1 ml	ca. 14,7 mg/ml	ca. 13,9 mg/ml [e)]

a) CF: control, fresh frozen midgut tissue from Monheim.
b) CL: freeze dryed protein extracts from midgut tissue from Monheim. Adana population (A), and Mardin population (M).
c) Extraction solution contains 2 M thiourea, 7 M urea, 4% CHAPS, 0.5% (v/v) IPG buffer pH 3-10, 70 mM DTT and protease inhibitors (1mM PMSF, 1.4 µM pepstatin A, and Complete^{TM} containing EDTA according to the manufacturer´s protocol).
d) Extraction solution contains 40 mM Tris, 3 mM EDTA and 1mM PMSF.
e) filtered after lyophilization

2.2.12. Bradford Method

The protein extract concentrations of *H. armigera* samples for proteome analysis were determined by the method of Bradford (1976). Aliquots of 10 and 20 µl of 1:20 diluted protein extracts were taken into eppendorf tubes and were completed to a final volume of 800 µl with distilled water. Then, 200 µl dye reagent were added into these tubes. Finally, these mixture vortexed and incubated at least 5 minutes at room temperature before measuring absorbencies at 595 nm wavelength.

The protein concentrations in the extracts were calculated from a standard calibration curve that was constructed from the corresponding $O.D_{595nm}$ values of BSA standards 0 to 18 µg/µl (**Table 2.5**).

Table 2.5. Components for the Preparation of the Standard Curve in Bradford Method

	BSA (0.1 mg/ml)	**Final Amount of BSA**	**Distilled Water**	**Volume of Dye Reagent**	**Total**
1	0 µl	0 µg	800 µl	200 µl	1000 µl
2	10 µl	1 µg	790 µl	200 µl	1000 µl
3	30 µl	3 µg	770 µl	200 µl	1000 µl
4	60 µl	6 µg	740 µl	200 µl	1000 µl
5	90 µl	9 µg	710 µl	200 µl	1000 µl
6	120 µl	12 µg	680 µl	200 µl	1000 µl
7	150 µl	15 µg	650 µl	200 µl	1000 µl
8	180 µl	18 µg	620 µl	200 µl	1000 µl

2.2.13. 2D-PAGE and Image Analysis

Samples were mixed with rehydration solution containing 8 M urea, 2% CHAPS, 0.0002% Bromophenol blue, 2% (v/v) 4-7 IPG buffer and 18 mM DTT according to Standard Operating Procedures from the Proteome Center Rostock (Madi *et al.*, 2003). For isoelectric focussing, 400 and 800 µg proteins from lyophilized and non-lyophilized samples, respectively, were loaded on an rehydrated IPG strips (18 cm, pH 4-7; GE Healthcare), (Heitner *et al.*, 2006; Zhao *et al.*, 2006). Focusing were performed at 500 V for 0.5 h (gradual), 500 V for 0.5 h (hold), 2000 V for 0.5 h (gradual) and 2000 V for 0.5 h (hold), 8000 V for 1 h (gradual) and 8000 V for 4 h (hold). Second-dimensional electrophoresis were carried out on SDS 15% polyacrylamide gels at constant 100 V for 16-18 h (Madi *et al.*, 2003 and Heitner *et al.*, 2006). The gels were stained with colloidal Coomassie Brillant Blue G-250 as described (Madi *et al.*, 2003; Aminin *et al.*, 2009) and scanned as 16 bit grey Tif-images using a Mirage II scanner (Umax Data Systems, Willich, Germany). Image analysis and expression data generation were performed applying the software package Phoretix 2D Advanced Version 5.1 (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK). Gels of field and susceptible population larvae were matched to each other; only spots present in all gels of at least one population belonging to the respective subsets were considered to be detected.

2.2.14. Mass Spectrometric Analysis of Protein Spots

Protein spots were excised from the gels with a spot picker (Flexys Proteomics picker, Genomic Solutions, Ann Arbor, MI, USA) or manually, and in-gel digestion with trypsin (Promega, Madison, WI, USA; 10 ng/µL in 3 mM Tris-HCl, pH 8.5) (Just *et al.*, 2006). In-gel digestion of protein spots, firstly, excised gel plugs were washed two times with 30% acetonitrile in 25 mM ammonium bicarbonate (100 µl) and 50% acetonitrile in 25 mM ammonium bicarbonate (100 µl). Then, gel plugs were shrunk in pure acetonitrile (100 µl). After that, dried gel plugs were swollen in trypsin solution at room temperature with overnight incubation. In order to extract digested peptides, extraction solution (0.3% trifluoroacetic acid and 50% acetonitrile)

was added and mixed 30-60 min. Finally, peptide extracts were transferred into 96 well collection plates by centrifugation.

MALDI–MS and MS/MS analyses were performed on a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) and on a Reflex III mass spectrometer (Bruker Daltonik, Bremen, Germany).

For measurement with the 4700 Proteomics Analyzer 0.6 µl of the tryptic digest and 0.45 µl of matrix solution consisting of 9 mg/ml α-cyano-4-hydroxy-cinnamic acid (CHCA) in 50% acetonitrile, 0.1% trifluoroacetic acid (TFA) were spotted on standard stainless steel plates. Analyses were performed in a two-step process in batch mode after external calibration was done. At first, an MS spectrum was recorded from each sample position. By means of an interpretation method, the three most intense ion signals from each sample were then selected for MS/MS measurements using air as collision gas (**Figure 2.2)**. All acquired spectra were processed using 4700 Explore™ software (Applied Biosystems) and MASCOT Distiller software (Matrix Science, London, UK).For measurements with the Reflex III mass spectrometer, AnchorChip targets (Bruker Daltonik) were applied as described (Nordhoff et al. 2003, Mikkat et al. 2010). Mass spectra were acquired and analyzed automatically using Bruker software (flexControl 2.4 and flexAnalysis 2.4), but, if necessary, peak picking and calibration were corrected manually (Sinz et al., 2002).

For protein identification, MS and MS/MS spectra were submitted to MASCOT (V2.2, Matrix Science, London, UK) via MASCOT Deamon or BioTools 3.0 software (Bruker Daltonik). Searches were performed against the insecta and helicoverpa subsets of the SWALL (Swiss-Prot and TrEMBL) protein sequence database (SWALL 2011_02; 603096 sequences from insecta, 795 sequences from helicoverpa). A mass tolerance of 70 ppm and 1 missing cleavage site were allowed, oxidation of methionine residues was considered as variable modification, and carbamidomethylation of cysteines as fixed modification. Peptide masses of trypsin autoproteolysis products were excluded. MS/MS fragment mass tolerance was set

between 0.5 and 0.9 Dalton. MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 75 were considered statistically significant ($p \leq 0.05$).

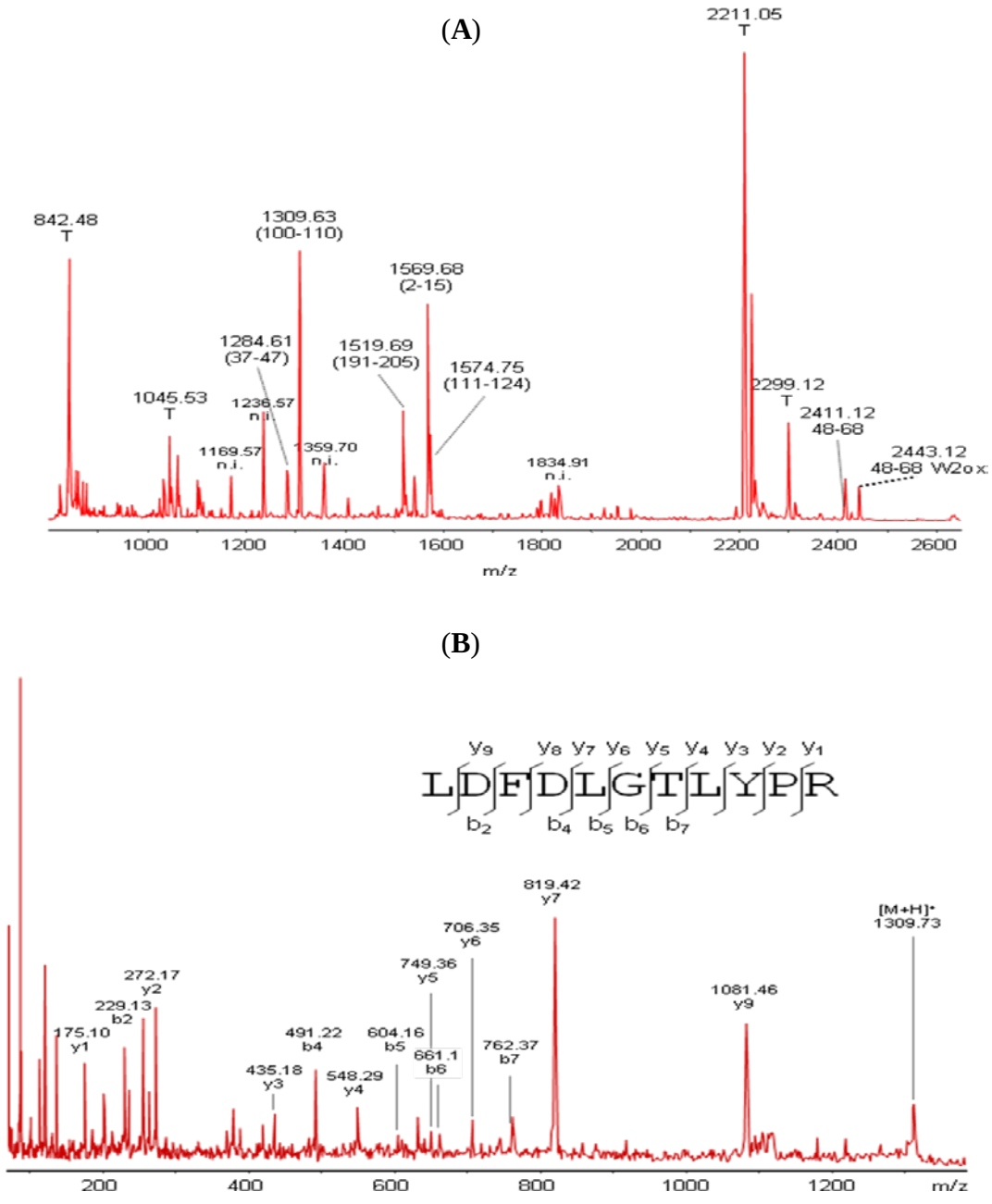


Figure 2.2. (A) MALDI Mass Spectrum of the Tryptic Digest of Spot 401.(identified as glutathione S-transferase) (B) MALDI-PSD (post source decay) Spectra of the Ion Signal of at m/z 1309.6 and Results of MS/MS Ion Searches. (The fragment ions of the y- and b-type series are indicated and the determined amino acid sequences are depicted at the top.)

2.2.15. Comparative Proteomic Analysis

For better understanding the roles of metabolizing enzyme systems in pyrethroid resistant *Helicoverpa armigera* samples, it has been planned to conduct comparative proteomic studies. By this way, it will be possible to detect proteins which are more expressed in resistant insects as compared to susceptible control samples. Ideally, the results also should supply additional data for confirming other experiments. In addition, it might be possible to find proteins that play a hitherto unknown role in these insecticide metabolizing enzyme systems.

Because of *H. armigera* sample limitation from Çanakkale population, it would not be possible to do comparative proteomic analysis for this population. On the other hand, protein expression differences in Adana and Mardin field populations of *H. armigera* compared to susceptible population, three replicas of 2D-PAGE gels were prepared from these three populations to ensure reproducibility of protein spots. In order to do this, firstly, spot area of each protein spot was defined according to the gel thickness with this program (**Figure 2.3**). It was called as a "volume". Then, darkness of each volume was determined in a 16 bit grey scale. In general, such as technical uses (e.g. in medical imaging or remote sensing applications) often require more levels, to make full use of the sensor accuracy (typically 10 or 12 bits per sample) and to guard against round off errors in computations. Sixteen bits per sample (65,536 levels) is a convenient choice for such uses, as computers manage 16-bit words efficiently. The TIF and PNG image file formats supports 16-bit grayscale natively. No matter what pixel depth is used, the binary representations assume that 0 is black and the maximum value (255 at 8 bpp, 65,535 at 16 bpp, etc.) is white. After that, numerical value of each defined spot area was normalized by reducing the darkness of that gel. Thus, it was obtained normalized volume for each spot. In addition, each normalized value converted to percentages, so the scale is then from 0% to 100% (**Figure 2.4**).

By this way, each spot normalized volume in 9 gels (lyophilized samples came from Ankara) was determined for individually. Then, mean values of that spot was

calculated and used to comparison. If ratio (compared to counterpart spot in susceptible population gels) more than 2≥, it was assumed that this protein up-regulated. However, ratio less than ≤0.5, it was assumed as down-regulated. In addition, if there was ratio between these two values, it was assumed as a not changed.

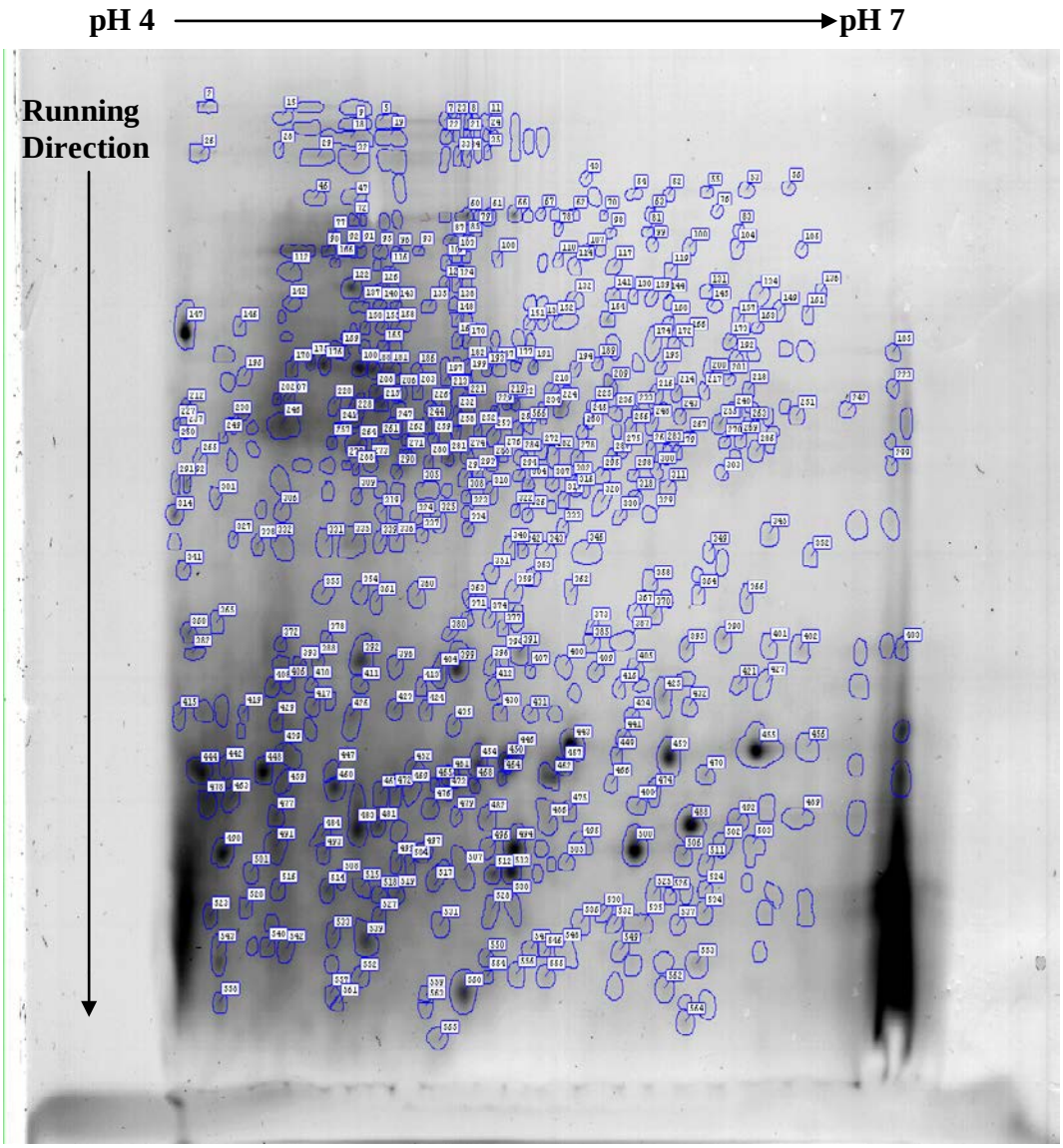


Figure 2.3. Image of 2D-PAGE Gel from susceptible population samples processed with progenesis software. (Each spot area was determined by drawing blue circles and labeled numerically starting from the upper left corner of the gel. Nonreproduced protein spots were not labeled with numbers.)

c_1 Spot	c_1 Norm_Vol	c_2 Spot	c_2 Norm_Vol	c_3 Spot	c_3 Norm_Vol	a_1 Spot	a_1 Norm_Vol	a_2 Spot	a_2 Norm_Vol	a_3 Spot	a_3 Norm_Vol	m_1 Spot	m_1 Norm_Vol	m_2 Spot	m_2 Norm_Vol	m_3 Spot	m_3 Norm_Vol
105	0.038767407	105	0.129725087	105	0.044678458	105	0.045527749			105	0.059516781	105	0.066403378			105	0.248763844
				127	0.019657281					127	0.016703097	127	0.036420524	127	0.037016076	127	0.066721921
134	0.064135594	134	0.047562567	134	0.055158207	134	0.124163496	134	0.13119311	134	0.130840924	134	0.268474698	134	0.217521438	134	0.325901765
136	0.066694996	136	0.116003847	136	0.058134704	136	0.191264184	136	0.067052507	136	0.300751735	136	0.040652696	136	0.012596161	136	0.189452163
147	3.663632897	147	3.545917997	147	3.505972217	147	2.467651649	147	2.504238002	147	2.596083604	147	1.894344119	147	2.072573494	147	2.112602914
154	0.300428587	154	0.222079586	154	0.231856702	154	0.249415846	154	0.255539308	154	0.299791787	154	0.318694488	154	0.33641256	154	0.325128005
161	0.063947403	161	0.152648793	161	0.058599781	161	0.042703532	161	0.032109651	161	0.077179826	161	0.064436312			161	0.187339203

Figure 2.4. Protein Spots Normalized Volumes in Adana, Mardin and Susceptible Populations Gels.

c_1, c_2 and c_3 indicate susceptible population gels. Similarly, while a_1, a_2 and a_3 indicate Adana population gels, m_1, m_2 and m_3 indicate Mardin population gels.

Spot term point out number of labeled protein spots in that gel.

Norm Vol term point out protein spot normalized volume.

For example, spot 105 in susceptible population gel 1 has a 0.038767407 normalized volume.

The Empty parts in the **figure 2.4** means that these spots were not defined in those gels, so normalized volume of them could not be calculated.

2.2.16. Total RNA isolation, quantity and quality check and cDNA preparation

Isolation of total ribonucleic acids (RNA) from single midgut was done by the method of Chomczynski and Sacchi (1987). This method is based on differential extraction of RNAs by organic solvents. Proteins are denaturized by strong denaturizing agent, Guanidine isothocyanide, and DNAs are selectively fractionated from RNAs by phenol at acidic pH. In order to decrease the possibility of RNA degradation during the procedure, all glassware and plastics were treated by 0.1% DEPC (Diethyl Pyrocarbonate) solution overnight and then autoclaved and dried in oven which converts DEPC into CO_2 and ethanol. Furthermore, all solutions, used in RNA isolation experiments, were also DEPC treated or prepared by 0.1% DEPC treated water.

About 25-30 mg midgut tissues of *H. armigera* were minced on ice and homogenized (at room temperature) with 1 ml of GTC solution, containing 4M Guanidine isothiocyanide, 25mM Sodium Citrate(pH:7.0), 0.5% (w/v) L-Lauryl Sarcosine and 0.1 M 2-mercaptoethanol, in ultraturrax homogenizer and subsequently transferred to a 15 ml polypropylene tube. Sequentially, 0.1 ml of 2M sodium acetate pH 4.0, 1 ml of phenol (water saturated, acidic pH) 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. Then, final suspension was shaken vigorously for 10 sec and cooled on ice for 15 min. Then, samples were centrifuged at 10,000 x g for 20 min at 4°C, and after that, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase, reciprocally. After that, aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, and then placed at -20°C for at least 1 h to precipitate RNA. Sedimentation at 10.000 x g for 20 min was again performed and the resulting RNA pellet was redissolved in 0.3 ml of GTC solution, transferred into a 1.5 ml Eppendorf tube, and precipitated with 1 ml isopropanol at -20°C for 1 h. After centrifugation in an Eppendorf centrifuge for 10 min at 4°C, the RNA pellet was resuspended in 75% ethanol, sedimented, dried in oven (60-65^{o}C, 15 min), and

dissolved in 50µL DEPC treated water or 0.5% SDS at 65^{o}C for 10min. Finally, dissolved RNA was stored in -80°C deep freeze until use.

2.2.17. Spectrophotometric Analysis of Isolated Total RNAs

Seven micro liters of total RNA isolate were diluted with 693 µl of pH: 8.0TE buffer, containing 100 mM Tris Base and 10 mM EDTA, in a quartz cuvette. Then, the absorbance of the solution was measured at 260 and 280 nm using TE buffer as blank. The purity of the isolated RNA was determined by taking the ratio of A_{260} and A_{280} readings. The optimal value for RNA purity is accepted to be between 1.9-2.2 (MacDonald *et al.*, 1987). As 40µg/ml solution of single stranded RNA gives absorbance of 1.000, concentrations of the RNA in samples were calculated as below;

RNA concentration (µg/ml) = (OD_{260}) x (dilution factor) x (40 µg RNA/µl)

2.2.18. Electrophoretic Analysis of Isolated Total RNAs

In order to reduce RNase activity when running agarose gels, electrophoresis apparatus should never be directly exposed to DEPC because acrylic is not resistant to DEPC. Hence, electrophoresis apparatus must be first cleaned with detergent solution, then rinsed with distilled water and dried with ethanol. After that, gel apparatus were filled with 3% hydrogen peroxide and soaked for 10 min. Finally, they were rinsed thoroughly with DEPC-treated water.

One percent of agarose gel was prepared in pH: 8.3TBE buffer, containing 0.89 M Tris Base, 0.89 M Boric acid, 20 mM EDTA and 1 µg ethidium bromide. Ten micro liters of total RNA were mixed with 2 µl of loading dye solution containing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 0.25% (v/v) glycerol and 1 mM EDTA and loaded onto the gel. Electrophoreses were carried out at 1hour 70Vof distance between electrodes until bromophenol blue has traveled at least 80% of the

way through the gel. Completing the electrophoresis, RNA bands were visualized under UV transilluminator and were photographed. Typical markers of RNA quality are fairly sharp and intense bands of 18S (~1900bases) and 28S (~4800 bases) rRNA subunits. If DNA contamination of the RNA occurs migrate as high molecular weight ethidium-bromide staining material. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band (**Figure 2.5**, Intact). This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit a 2:1 ratio. If there is a smear of the ribosomal RNA bands, it is evidence of degradation of the RNA Completely degraded RNA will appear as a very low molecular weight smear (**Figure 2.5**, Degraded).

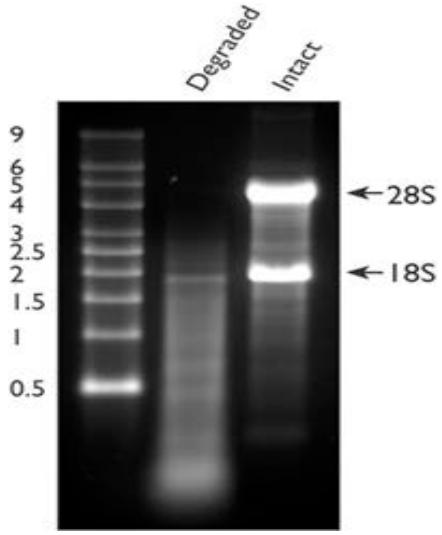


Figure 2.5. Intact vs. Degraded RNA. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The degraded RNA appears as a lower molecular weight smear (Ambion, the RNA company).

2.2.19. cDNA Synthesis from Isolated Total RNA Samples

cDNAs were prepared from isolated total RNA samples according to the manual of Fermentas RevertAid™ 1st strand cDNA Synthesis Kit. 1.0 µg of total RNA from samples and 1 µL of oligo(dT)18 primers (0.5µg/µL) were put into DEPC-treated PCR tubes, and total volume was filled up to 12 µL by addition of DEPC-treated

water. Then, the compounds were mixed gently and PCR tubes were incubated at 70ºC for 5 minutes, chilled on ice and drops were collected with brief centrifugation. After that, tube was placed on ice and the following components were added in indicated order: 4 µL of 5X reaction buffer, 1 µL RiboLock™ Ribonuclease Inhibitor (20U/ µL) and 2 µL of 10mM dNTP mixture and they were mixed gently, centrifuged for 3-4 seconds and the tubes were incubated at 37 ºC for 5 minutes. After incubation, 1 µL of RevertAid™ M-MuLV Reverse Transcriptase (200U/ µL) was added to reach 20 µL final volume and the tubes were incubated at 42 ºC for 60 minutes. Lastly, the tubes were incubated at 70 ºC for 10 minutes to stop the reaction and chilled on ice.

2.2.20. Real Time –PCR (*q*RT-PCR) Analysis

The real-time PCR system is related with detection and quantization of a fluorescent reporter (Lee, 1993; Livak, 1995). Fluorescent signal increases in directly amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of analysed gene product, the sooner a significant increase in fluorescence the use of a non-sequence specific fluorescent intercalating agent (SYBR-green I is observed).

In order to determine C_T values for all genes, one of the samples was chosen from susceptible population as a reference sample. From this reference sample cDNA 10-fold serial dilutions were prepared as; no dilution, 1/10, 1/100 and 1/1000. Then, concentrations of these dilutions were assigned arbitrarily as; 100000, 10000, 1000 and 100, reciprocally. After that, it was tried to obtained C_T versus Log concentration curves for all genes with its slopes in a range of $-3.6 > \text{slope} > -3.1$. The slope of that curve reflects the amplification efficiency. Efficiency was calculated by the formula:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

The efficiency of the PCR should be 0.9-1.0 (– 3.6 > slope > – 3.1). If efficiency is 1.0, there would be a doubling of the amount of DNA at each cycle.

Standard curves of all genes were determined with a similar efficiency (0.95-1.0). Subsequently, C_T values of all genes, including internal standard gene, were determined according to its standard curve (**Figure 2.6)** for susceptible and field populations of *H. armigera*.

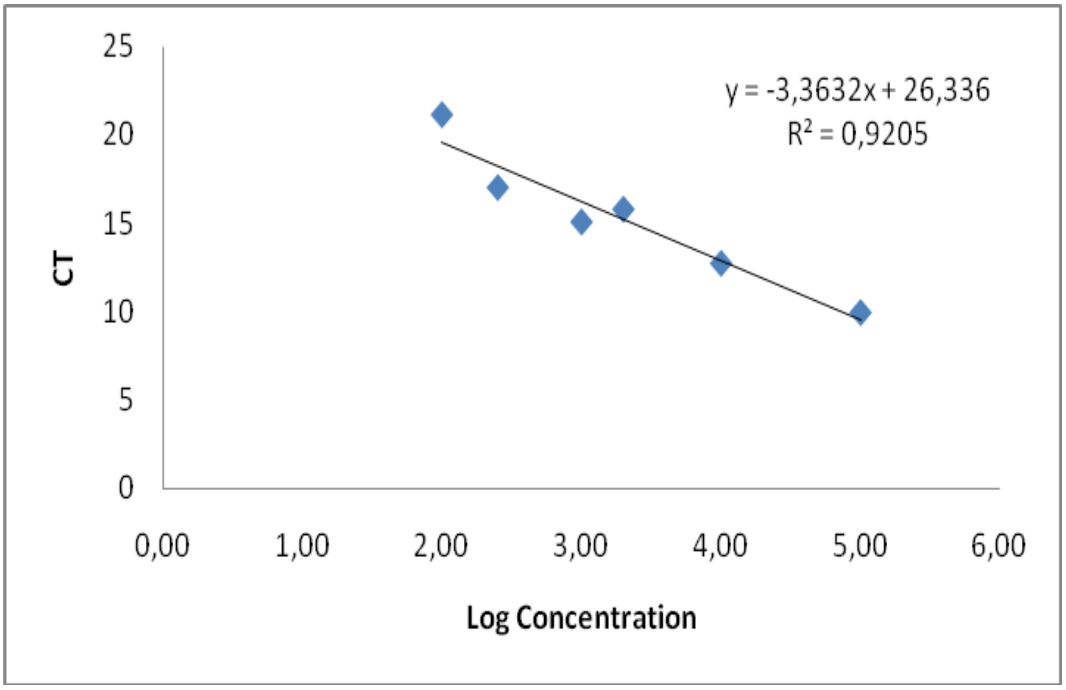


Figure 2.6. EF-1Alfa Gene Standard Curve

As CYP450s role was not analyzed individually by enzyme activity measurements, it was planned to analyze individual expression levels of five suspected CYP450 genes from 3 main CYP450 gene families (CYP4, CYP6 and CYP9) for better understanding this system in resistant *H. armigera* samples. In addition, two different GST gene expression and one esterase gene expression levels were analyzed, too. FastStart SYBR Green Master kit (Roche Diagnostics, Mannheim, Germany) was used in *q*RT-PCR analysis. Final concentrations of components of reaction mixture described at **Table 2.6**. Analyzed genes primer couples, forward and reverse primers, were purchased from Iontek Company (İstanbul, Türkiye) as a lyophilized form. They were dissolved with distilled water before usage. Elongation factor-1α gene

was selected as an internal standard in these experiments for normalization of *q*RT-PCR data. The forward and reverse primer sequences of analyzed genes were indicated at **Table 2.7** with annealing temperature and amplicon size of that gene, used in *q*RT-PCR experiments. Each sample was measured triplicate. Melting curve analysis was done for checking reaction specificity **(Figure 2.7)**.

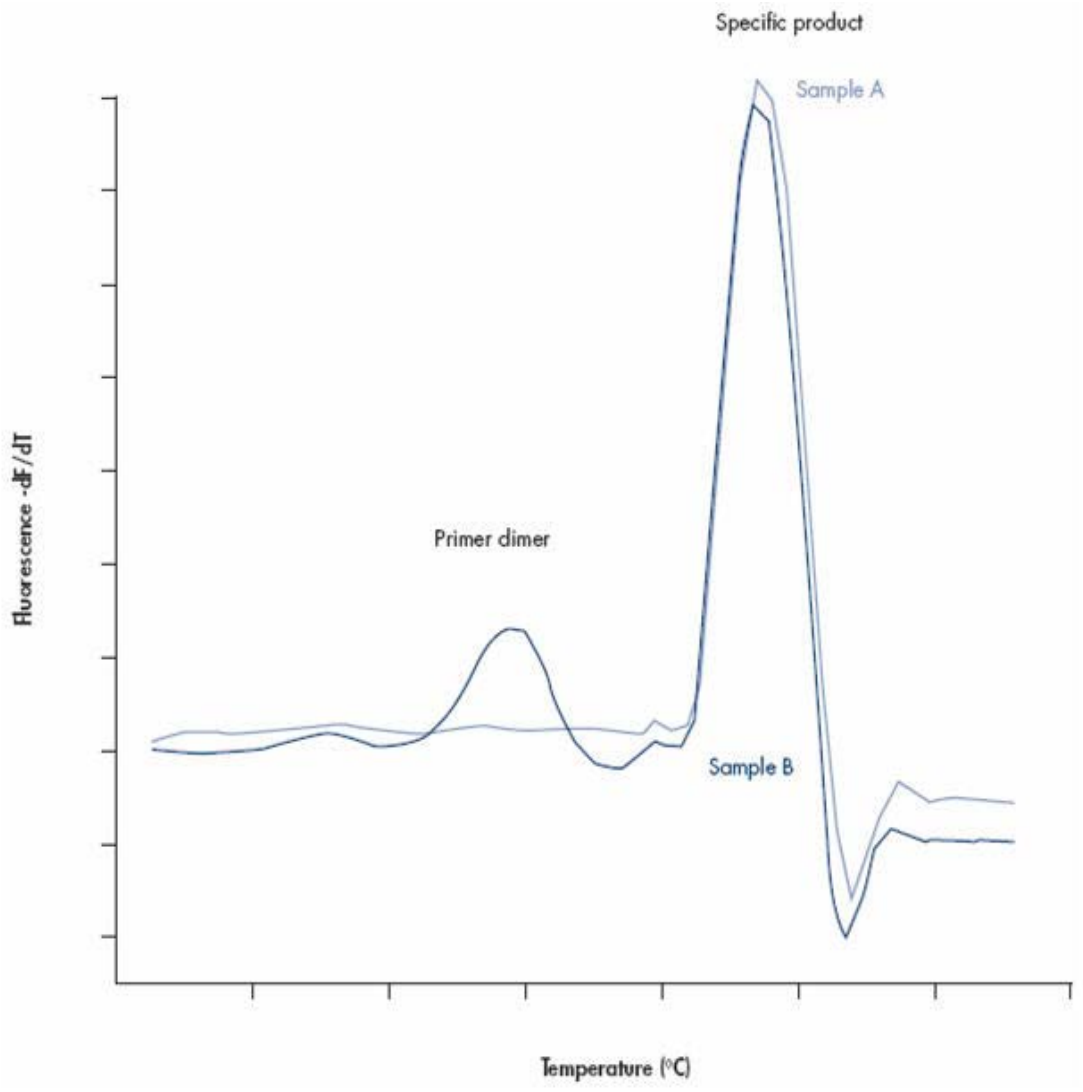


Figure 2.7. Melting curve analysis (from www.qiagen.com)

Melting curve analysis of two samples (A and B). Sample A yields only 1 peak resulting from the specific amplification product (primer-dimers not coamplified). Sample B shows a peak from the specific product and a peak at a lower temperature from amplification of primer-dimers.

Table 2.6. *q*RT-PCR Analysis Reaction Mixture Ingredients

Ingredient	**Concentrations**	**Added Volume**	**Final Concentration of Ingredient**
SYBR Green Master Mix*	2X	5 µl	1X
Forward Primer***	2 µM	2 µl	400 nm
Reverse Primer***	2 µM	2 µl	400 nm
cDNA**	---	1 µl	---
Total Volume		10 µl	

***** SYBR master mixes that contains FastStart Taq DNA Polymerase, Reaction Buffer, Nucleotides (dATP, dCTP, dGTP, dUTP), SYBR Green I, and a reference dye (www.roche-applied-science.com).

****** cDNA was synthesized from 1 µg isolated total RNA.

Table 2.7. Primer Sequences and Annealing Temperature for Real-Time PCR Experiments

Gene	Forward and Reverse Primer Sequences	Amplicon Size (basepair)	Annealing Temperature (^{o}C)	PCR[e] Efficiency
EF-1α[a]	F 5''-GACAAACGTACCATCGAGAAG-3" R 5''-GATACCAGCCTCGAACTCAC-3"	279[a]	58	-3.319
CYP4S1[b]	F 5''-AGCGTGCCTTTTATTGCGAGAG-3" R 5''-CGGCGGTGCAGGTCATAGAT-3"	100-120[b]	60	-3.287
CYP6B6[c]	F 5''-TTGAAGAAAGGCGTATGAAA-3" R 5''-ACACGCAAGATACACAAAGG-3"	60-150[c]	60	-----
CYP6B7[a]	F 5''-TCTTGTGGACAACATTATTAGC-3" R 5''-AAGTGATGTTACTTCATCAAGA-3"	130[a]	52	-3.248
CYP9A12[a]	F 5''-ATCACCTCATAGAAGATATCC-3" R 5''-CATGTCTTTCCATTCTTGACC -3"	234[a]	55	-3.242
CYP9A14[a]	F 5''-ACCCTGAGGTACAGGAGA-3" R 5''-TAGACCACACCGGGATCA-3"	258[a]	58	-3.265
GSTX01[b]	F 5''-TAAACAGTCTTCGCGTATATAGC-3" R 5''-ATCAGATAGTTGACTTGATTGATG-3"	100-120[b]	60	-3.317
GST[d]	F 5''- CTGTGCTAGAGGATGGGGA-3" R 5''-AGCGATGTAGGTGGTGCGA -3"	289[d]	55	-3.249
ESTX018[b]	F 5''-TCCCATATGAACATCCCAAACAG-3" R 5''-TTGAGATCCTCATTGTTGGGTAG -3"	100-120[b]	60	-3.484

[a] Primer sequences were obtained Yang *et al.*, 2006.
[b] Primer sequences were obtained Wee *et al.*, 2008.
[c] Primer sequences were obtained Grubor *et al.*, 2007.
[d] Primer sequences were obtained Tang *et al.*, 2005.
[e] PCR efficiency was indicated as slope of log-linear phase of a set of serials dilutions (up to 1000X).

2.2.21. Statistical Analysis of *q*RT-PCR Results

As finding relative gene expression of analysed genes "comparative C_T method (Delta Delta C_T)" was used at the beginning. It compares the relative amount of the target sequence to any of the reference values chosen. Hence, result is given as relative to the selected reference value. In order to C_T calculation to be valid, the efficiency of the target amplification and reference amplification must be approximately equal. Serial dilutions of the target and normalizer genes are prepared and real-time PCR is run in separate tubes. It is expected that the normalizer will have a higher expression level than the target (so, a smaller C_T value). The calculations for the quantization start with getting the difference (Delta C_T) between the C_T values of the target and the normalizer:

$$\text{Delta } C_T = C_T \text{ (target)} - C_T \text{ (normalizer)}$$

This value is calculated for each sample to be quantities. One of these samples should be chosen as the reference for each comparison to be made. The comparative Delta Delta C_T calculation involves finding the difference between each sample's Delta C_T and the reference Delta C_T. If the reference value is representing the minimum level of expression, the Delta Delta C_T values are expected to be negative (because the Delta C_T for the baseline sample will be the largest as it will have the greatest C_T value). The last step in quantization is to transform these values to absolute values. The formula for this is:

$$\text{Comparative expression level} = 2^{-\text{Delta Delta Ct}}$$

Comparative C_T method is useful for estimation of gene expression ratios of individual samples. However, ratio distributions do not have a standard deviation; it is not simple to perform traditional statistical analysis for comparing field and susceptible populations of *H. armigera* samples. Relative Expression Software Tool overcomes these problems by using simple statistical randomization tests. Thus, it was decided to use REST 2008 for analyzing gene expression. REST 2008 (Pfaffl *et*

al., 2002) (http://www.genequantification.de/rest-2008.html) is a standalone software package for analyzing gene expression using real-time PCR amplification data. The software addresses issues surrounding the measurement of uncertainty in expression ratios by introducing randomization and bootstrapping techniques. New confidence intervals for expression levels also allow measurement of not only the statistical significance of deviations but also their likely magnitude, even in the presence of outliers. Whisker box plots provide a visual representation of variation for each gene, highlighting potential issues such as distribution skew.

The hypothesis test P (H1) indicated in the results table represents the probability of the alternate hypothesis that the difference between sample and control groups is due only to chance. The hypothesis test performs a large number of random reallocations of samples and controls between the groups. It then counts the number of times the relative expression of the randomly assigned group is greater than the sample data.

CHAPTER III

RESULTS

3.1. *Helicoverpa armigera* Cytosolic GST and EST Activities

Glutathione S-transferases (GSTs) activities in the cytosolic fractions, prepared from *H. armigera* midguts, were determined spectrophotometrically by using 1-chloro-2,4-dinitrobenzene (CDNB), 3,4-dichloronitrobenzene (DCNB), p-nitrobenzylchloride (PNBC), 1,2-epoxy-3-(p-Nitrophenoxy)propane (EPNP) as substrates by monitoring the thioether formation as described by Habig and co-workers (Habig *et al.*, 1974). The reaction conditions for these substrates were described in chapter II (see details in **Table 2.2** at chapter II).

Non specific esterase activities (EST) in *H. armigera* midgut samples were also determined spectrophotometrically in 96 well microplates using p-nitrophenylacetate (PNPA) and α- naphthyl acetate (α-NA) substrates by monitoring p-nitrophenol and α-naphthol products formation, reciprocally, according to the method of van Asperen (1962) (see details in **Table 2.3** at chapter II).

In order to measure GST and EST activities, 30 individual *H. armigera* samples were used from all populations, Adana, Çanakkale, Mardin and susceptible populations. After preparation of each individual cytosolic fraction (~900 µl), it was aliquoted into 100 µl fractions for further protein determinations, GST and EST assays.

3.1.1. GST-CDNB Enzyme Activities of *Helicoverpa armigera* Populations

GST-CDNB activity measurements were done with microplate reader by modified Habig *et al.,* (1974), method of using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate and in the presence of cofactor reduced Glutathione (GSH) (see details in **Table 2.2** at chapter II). GST-CDNB activities were determined spectrophotometrically by monitoring the formation of the conjugation product at 340 nm wavelength. After that, GST-CDNB activities for 30 individual samples were calculated individually as explained in chapter II, part 2.2.4. For all populations, means and standard deviations of 30 samples were calculated with the help of student t-test by MINITAB 15.0 statistics software and shown at **table 3.1**. In addition, p values were calculated with student t-test for analyzing similarity or difference between mean GST-CDNB activities of the field and susceptible populations.

Table 3.1. GST-CDNB Activities of *Helicoverpa armigera* Populations

Population	**Sample Size (N)**	**Mean GST-CDNB Activity[a] (nmole/min/mg)**
Susceptible	30	208.5± 18
Adana	30	381*±45
Çanakkale	30	166.3± 15
Mardin	30	314*± 36

[a]nmole min^{-1} mg $protein^{-1}$ ±Standard Error of Mean.

*Value significantly different from the susceptible strain ($p<0.05$).

3.1.2. GST-DCNB Enzyme Activities of *Helicoverpa armigera* Populations

GST-DCNB activities were determined with a microplate reader by monitoring the thioether formation at 340 nm using 3,4-dichloronitrobenzene (DCNB) as a substrate according to the method of Habig *et al.*, (1974) at 340 nm wavelength (see details in **Table 2.2** at chapter II). GST-DCNB activities for 30 individual samples were calculated individually as explained in chapter II, part 2.2.5. Similarly in GST-CDNB activities, means and standard deviations of 30 samples from each population were calculated with the help of student t-test by MINITAB 15.0 statistics software and shown at **table 3.2**. Furthermore, similarity or difference between mean GST-DCNB activities of the field and susceptible populations were analyzed by calculating p values with student t-test.

Table 3.2. GST-DCNB Activities of *Helicoverpa armigera* Populations

Population	**Sample Size (N)**	**Mean GST-DCNB Activity[a] (nmole/min/mg)**
Susceptible	30	9.83 ± 0.93
Adana	30	42.2*±3.5
Çanakkale	30	19.4*± 4.2
Mardin	30	47.2*± 18

[a]nmole min^{-1} mg $protein^{-1}$ ±Standard Error of Mean.

*Value significantly different from the susceptible strain ($p<0.05$).

3.1.3. GST-PNBC Enzyme Activities of *Helicoverpa armigera* Populations

GST enzyme activity was determined spectrophotometrically by monitoring the thio-ether formation at 310 nm in a 1 ml cuvette using p-nitrobenzylchloride (PNBC) as a substrate according to the method of Habig *et al.*, (1974) (see details in **Table 2.2** at chapter II). GST-PNBC activities for 30 individual samples were calculated individually as explained in chapter II, part 2.2.6. Likewise, GST-CDNB and GST-DCNB activities, means and standard deviations of 30 samples from each population were calculated with the help of student t-test by MINITAB 15.0 statistics software and shown at **table 3.3**. Finally, similarity or difference between mean GST-PNBC activities of the field and susceptible populations were analyzed by calculating p values with student t-test.

Table 3.3. GST-PNBC Activities of *Helicoverpa armigera* Populations

Population	**Sample Size (N)**	**Mean GST-PNBC Activity[a] (nmole/min/mg)**
Susceptible	30	37.2 ± 3.0
Adana	30	135.6*±12
Çanakkale	30	80.2*± 6.7
Mardin	30	177.5*± 15

[a]nmole min^{-1} mg $protein^{-1}$ ±Standard Error of Mean.

*Value significantly different from the susceptible strain ($p<0.05$).

3.1.4. GST-EPNP Enzyme Activities of *Helicoverpa armigera* Populations

GST-EPNP activity was measured according to the method of Habig *et al*., (1974). 1,2-epoxy-3-(p-Nitrophenoxy)propane (EPNP) is a specific substrate for theta isozyme of glutathione S-transferases. Determination of GST-EPNP activity in the presence of the cofactor reduced glutathione (GSH) was performed to monitor the thioether formation at 360 nm in a 1 ml cuvette.

Although GST-CDNB, GST-DCNB and GST-PNBC activities were measured in *H. armigera* midgut samples, GST-EPNP activities were not determined in any of the populations. Therefore, it would not be possible to make any statistical analysis for GST-EPNP activities in susceptible and field populations.

Interestingly, except GST-CDNB activity in Çanakkale population, all of the measured GST activities displayed similar activity patterns in Adana, Çanakkale and Mardin field populations. The GST-CDNB, GST-DCNB and GST-PNBC activities showed statistically significant increases in Adana (A) and Mardin (M) field populations as compared to those of the susceptible population (CL). GST-CDNB, GST-DCNB and GST-PNBC activities in the Adana field population showed 1.83, 4.30 and 3.64-fold higher activity as compared to those of the susceptible (CL) population. Furthermore, GST-CDNB, GST-DCNB and GST-PNBC activities in the Mardin (M) field population displayed a 1.51, 4.80 and 4.77-fold more activity as compared to the susceptible (CL) population. Although, GST-DCNB and GST-PNBC activities in Çanakkale (Ç) field population displayed a 1.97 and 2.16-fold more activity as compared to the susceptible (CL) population, GST-CDNB activity did not showed statistically significant increase in Çanakkale field population compared to the susceptible (CL) population (**Table 3.4**). In addition, GST-EPNP activities were not determined in all analyzed populations, so it was indicated as "N.D." in **table 3.4**.

Table 3.4. Summary of GST Activity Results of Field Populations of *H. armigera*

Substrate	Adana	Çanakkale	Mardin
CDNB	1.83 fold ↑	-----	1.51 fold ↑
DCNB	4.30 fold ↑	1.97 fold ↑	4.80 fold ↑
PNBC	3.64 fold ↑	2.16 fold ↑	4.77 fold ↑
EPNP	N.D.	N.D.	N.D.

N.D: Not determined

3.1.5. EST-PNPA Enzyme Activities of *Helicoverpa armigera* Populations

EST-PNPA enzyme activity was determined spectrophotometrically in 96 well microplates by monitoring p-nitrophenol formation according to the method of van Asperen (1962) (see details in **Table 2.3** at chapter II). EST-PNPA activities for 30 individual samples from all populations were calculated individually as explained in chapter II, part 2.2.8. Means and standard deviations of EST-PNPA activities of 30 samples from each population were calculated with the help of student t-test by MINITAB 15.0 statistics software and shown at **table 3.5**. Similarity or difference between mean EST-PNPA activities of the field and susceptible populations were analyzed by calculating p values with student t-test.

Table 3.5. EST-PNPA Activities of *Helicoverpa armigera* Populations

Population	**Sample Size (N)**	**Mean EST-PNPA Activity[a] (nmole/min/mg)**
Susceptible	30	411 ± 44
Adana	30	653*± 57
Çanakkale	30	375 ± 30
Mardin	30	461 ± 29

[a]nmole min^{-1} mg $protein^{-1}$ ±Standard Error of Mean.

*Value significantly different from the susceptible strain ($p<0.05$).

3.1.6. EST-α-NA Enzyme Activities of *Helicoverpa armigera* Populations

EST-α-NA enzyme activity was determined in 96 well microplates by monitoring α-naphthol formation according to the method of van Asperen (1962) (see details in **table 2.3** at chapter II). EST- α-NA activities for 30 individual samples from all populations were calculated individually as explained in chapter II, part 2.2.9. Means and standard deviations of EST-α-NPA activities of 30 samples from each population were calculated with the help of student t-test by MINITAB 15.0 statistics software and shown at **table 3.6**. Similarity or difference between mean EST-α NA activity of the field and susceptible populations were analyzed by calculating p values with student t-test.

Table 3.6. EST-α-NA Activities of *Helicoverpa armigera* Populations

Population	**Sample Size (N)**	**Mean EST-α-NA Activity[a] (nmole/min/mg)**
Susceptible	30	414.5 ± 26
Adana	30	875.5*±50
Çanakkale	30	462.8± 36
Mardin	30	639.8*± 29

[a]nmole min^{-1} mg $protein^{-1}$ ±Standard Error of Mean.

*Value significantly different from the susceptible strain ($p<0.05$).

Enzymatic activity analysis of midgut tissue esterases from *H. armigera* were done with two different substrates, PNPA and α –NA. It was found that increases in EST-PNPA and EST-α-NA activities were statistically significant in the Adana population compared to the susceptible (CL) population (**Table 3.5-3.6**). The fold-changes of EST-PNPA and EST-α-NA activities were 1.59 and 2.11, respectively (**Table 3.7**).

However, while EST-α-NA activity showed statistically significant increase in Mardin population (1.54 fold, **Table 3.7**), EST-PNPA activity was not statistically significantly increased in the Mardin population compared to the susceptible (CL) population (**Table 3.5**).

In addition, it was found that both EST-PNPA and EST-α-NA activities were not statistically significant increased in the Çanakkale population compared to the susceptible (CL) population (**Table 3.5-3.7**).

Table 3.7. Summary of EST Activity Results of Field Populations of *H. armigera*

Substrate	**Adana**	**Çanakkale**	**Mardin**
PNPA	1.59 fold ↑	-----	-----
α -NA	2.11 fold ↑	-----	1.54 fold ↑

3.2. Global Proteome Analysis

In order to obtain a general picture about protein abundances in *H. armigera* midgut tissue, two-dimensional gel electrophoresis experiments with midgut proteins from a non-lyophilized susceptible population (CF) were performed. The resulting reference gel of midgut tissue proteins of *H. armigera* showed on average ca. 800 spots (**Figure 3.1**). From them, 571 clearly distinguishable spots were selected for mass spectrometric identification by peptide mass fingerprinting and MS/MS analysis. In the end, proteins were identified from 220 spots (**Table 3.8**) using this approach. Additionally, protein spots from lyophilized samples were analyzed in the same fashion and confirmed that spots of the same location in the gel resulted in identical identifications. In addition, 23 spots (numbers 1000-1022) that were not previously identified in the CF sample, were added to the list of identified proteins (**Table 3.8**) and are included with their corresponding positions in the reference gel (**Figure 3.1**). Most of the identified proteins were cytoskeletal system related proteins such as actins, tropomyosins, tubulins and myosins. In 61 spots actin was identified (e.g. spots 44, 45, 48, 49 and so on in **Table 3.8**). There were also clearly identified tropomyosins (spots 141, 228, 374, 492 and 642), tubulins (spots 118, 121, 289, 379, 383, 428, 484 and 709), and myosin heavy or light chains (spots 9, 11, 22, 23, 337, 375 and 588).

Another group of identified proteins contained energy metabolism related proteins, for example ATP synthases (e.g. spots 61, 293, 311, 315), V ATPases A and B (e.g. spots 339, 445, 125, 1007 and 1008), putative enolase (e.g. spots 137, 1005), triosephosphate isomerase (spots 342 and 343 and 1019), fatty acid binding proteins

(e.g. spots 636, 673 and 712), and arginine kinases (e.g. spots 598, 1016 and 1017). Interestingly, arginine kinase is known to have an important role in the energy metabolism of insects (Werr *et al.*, 2009). In addition, enoyl-CoA hydratase 3 (spot 426) and putative mitochondrial cytochrome c oxidase polypeptide Va (spot 566) proteins were identified.

Noteworthy, we also identified proteins that are involved in protein metabolism and detoxification of cellular toxins. Protein metabolism related proteins encompassed heat shock proteins (e.g. spots 79, 92, 321, 334), eukaryotic translation initiation factor 4A (spot 475), 60S acidic ribosomal protein P2 (spot 606) and alanyl-tRNA synthetase (spot 1011). Furthermore, certain detoxification proteins were also identified such as NADPH cytochrome P450 reductase (spots 155 and 231), aldehyde dehydrogenase (spots 393 and 427), thioredoxin peroxidase (spots 413 and 1012) and glutathione S-transferases (spots 401 and 623).

Our reference gel (**Figure 3.1**) from fresh midgut tissue served as control for the subsequent differential proteome analyses that were performed using lyophilized protein extracts. Obviously, the necessity of using two different sample preparation protocols in this work presented an additional challenge. The samples from Turkey needed to be lyophilized in order to transfer them from Turkey to Germany. As the lyophilisation process may cause changes in ionic strength and pH, this generally may be regarded as a source for losses of proteins. In fact, 2D-PAGE gels with lyophilized samples appeared fainter as compared to fresh-frozen tissue samples. Furthermore, there were less protein spots detected in these gels stained with

Coomassie Brilliant Blue G250 (CBB G250). Nevertheless, visual inspection of the gels that were obtained with and without lyophilization, respectively, showed that most of the spots that were identified in the reference gel were also present in the gels used for differential proteome analysis.

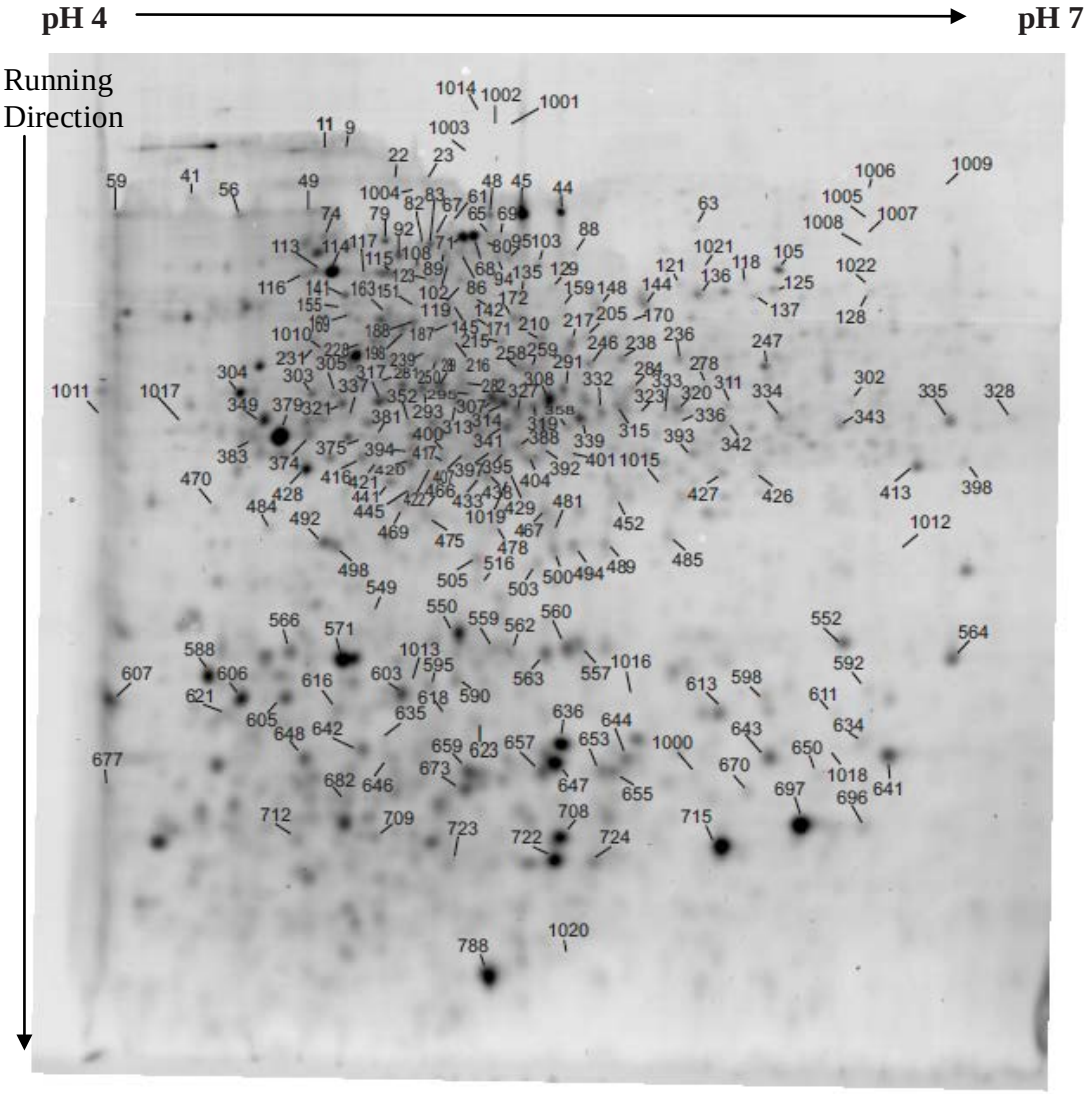


Figure 3.1. Image of a 2-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) reference Gel Prepared from *H. armigera* midgut tissue from susceptible population (CF, fresh midgut tissues, non-lyophilized). 400 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension. Gel was stained with Coomassie brilliant blue (CBB). In addition, selected 60 proteins spots from *H. armigera* field samples gels were manually picked and subjected to in-gel tryptic digestion.

Table 3.8. List of Identified Proteins from 2D Polyacrylamide Gel Electrophoresis of Protein Extracts

Spot no.	Access. no.	Organism [a)]	Protein name	Score	Sequence coverage (%)	Match. / search. signals	RMS error (ppm)	MW (kDa)
9 [d)]	B5M9A2	B.m.	Myosin heavy chain (Fragment)	97	29	25/120	15	96131
11 [d)]	C4P7G5	B.ma.	Myosin heavy chain (Fragment)	74	26	21/113	16	96071
22 [d)]	C4P7H1	B.ma.	Myosin heavy chain variant C (Fragment)	72	22	20/103	15	105088
23 [d)]	Q179E8	A.a.	Myosin heavy chain, nonmuscle or smooth muscle	76	16	30/102	14	222353
41 [d)]	018437	H.a.	Chymotrypsin-like protease (Fragment)	52	29	7/79	4	31178
44 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	77	32	10/69	4	42148
45 [d,f)]	E2IV57	H.a.	Actin	124	36	15/62	6	42177
48 [d,f)]	E2IV57	H.a.	Actin	170	63	19/82	6	42177
49 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	85	53	13/112	12	42148
56 [d)]	E2IV57	H.a.	Actin	81	43	10/93	10	42177
59 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	127	49	14/77	3	42148
61 [d,f)]	D9ILX6	H.z.	ATP synthase	155	42	21/83	5	55252
63 [d)]	E2IV57	H.a.	Actin	98	37	11/48	6	42177
65 [d,f)]	E2IV56	H.a.	Actin	131	50	13/96	5	42239
67 [d,f)]	E2IV57	H.a.	Actin	119	46	13/83	4	42177
68 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	200	64	27/114	10	42148
69 [d,f)]	E2IV57	H.a.	Actin	155	56	15/73	4	42177
71 [d,f)]	E2IV57	H.a.	Actin	216	67	27/118	6	42177
74 [d)]	Q0MUU6	T.n.	Heat shock cognate 70 protein	73	31	19/145	14	73207
79 [d,f)]	C8CCR4	H.z.	Heat shock protein 70 cognate	175	32	25/124	6	70397
80 [d)]	E2IV58	H.a.	Actin	116	51	13/115	10	42193
82 [d,f)]	E2IV57	H.a.	Actin	159	57	19/112	5	42177
83 [d f)]	Q25010	H.a.	Actin, cytoplasmic A3a	146	61	20/124	7	42148
86	E2IV57 [d,f)] D9ILX6 [d)]	H.a. H.z.	Actin ATP synthase	96 85	46 35	14/128 17/128	5 7	42177 55252

Table 3.8. (continued)

88 [d)]	E2IV57	H.a.	Actin	120	61	15/90	7	42177
89 [d)]	C0KJJ4	S.e.	Heat shock protein 70	82	26	17/111	10	75041
92 [d,f)]	C7SIR9	H.a.	Heat shock protein 70	85	35	23/146	12	71801
94 [d)]	Q6XIP2	D.y.	Similar to Drosophila melanogester alphaTub84B(fragment)	144	57	11/106	9	28810
95	E2IV54 [d)] D9ILX6 [d,f)] E2IV57 [d,f)]	H.a. H.z. H.a.	V ATPase A (Fragment) ATP synthase Actin	87 52 48	60 28 37	13/127 14/127 11/127	8 8 12	33379 55252 42177
102 [d)]	ACT3A	H.a.	Actin	74	55	13/153	10	42148
103 [d,f)]	Q6XIP2	D.y.	Similar to Drosophila melanogester alphaTub84B(fragment)	120	44	10/77	3	28810
105 [b,d,f)]	E2IV54	H.a.	V ATPase A (Fragment)	164	60	17/100	11	33379
108	C8CCR4 [d,f)] ACT3A [d)]	H.z. H.a.	Heat shock protein 70 cognate Actin, cytoplasmic A3a	131 48	33 31	24/117 10/117	13 8	70397 42148
113 [d,f)]	Q0MUU6	T.n.	Heat shock cognate 70 protein	133	38	23/129	14	73207
114 [d,f)]	Q0MUU6	T.n.	Heat shock cognate 70 protein	183	40	28/128	10	73207
115	C7SIR9 [d)] ACT3A [d,f)]	H.a. H.a.	Heat shock protein 70 Actin, cytoplasmic A3a	105 84	31 46	23/137 15/137	6 6	71801 42148
116 [d,f)]	Q0MUU6	T.n.	Heat shock cognate 70 protein	65	32	17/138	10	73207
117 [d)]	ACT3A	H.a.	Actin, cytoplasmic A3a	58	41	11/151	10	42148
118 [d)]	TBB1	M.s.	Tubulin beta-1 chain	99	32	19/103	5	50654

Table 3.8. (continued)

119 [d,f]	C0KJJ4	S. e.	Heat shock protein 70	79	32	19/121	14	75041
	E2IV57	H.a.	Actin	76	44	12/121	10	42177
121 [d,f]	TBB1	M.s.	Tubulin beta-1 chain	124	31	18/78	2	50654
123	C8CCR4 [d,f]	H.z.	Heat shock protein 70 cognate	130	32	23/119	14	70397
	E2IV56 [d]	H.a.	Actin	53	25	9/119	12	42239
125 [b,d,f]	E2IV54	H.a.	V ATPase A (Fragment)	131	58	17/87	5	33379
128 [d]	Q68YP2	H.a.	Hexamerine	49	14	9/59	7	82043
129 [d,f]	E2IV57	H.a.	Actin	146	57	17/92	6	42177
135 [d,f]	A2I3X7	M.h.	Putative tubulin beta-1 chain	125	39	22/107	7	50628
	ACT	M.s.	Actin, muscle	91	54	16/107	10	42149
136 [d,f]	ACT3A	H.a.	Actin, cytoplasmic A3a	187	49	16/68	8	42148
137 [d]	D2WJC4	S.p.	Putative enolase (Fragment)	114	39	12/48	11	40944
141 [d,f]	TPM1	B.m.	Tropomyosin-1	131	52	16/93	7	32603
142 [d]	ACT3A	H.a.	Actin, cytoplasmic A3a	67	39	11/117	11	42148
144 [d,f]	E2IV56	H.a.	Actin	117	47	15/90	4	42239
145 [d]	C4P7G5	B.ma.	Myosin heavy chain (Fragment)	85	32	27/180	11	96071
	C7SIR9	H.a.	Heat shock protein 70	81	36	23/180	20	71801
148 [d,f]	E2IV56	H.a.	Actin	106	50	12/85	4	42239
151 [d,f]	ACT3A	H.a.	Actin, cytoplasmic A3a	77	45	12/130	15	42148
155 [d]	E0A3A7	H.a.	NADPH cytochrome P450 reductase	101	28	17/90	9	78029
159 [d,f]	ACT1	D.m.	Actin-5C	94	40	13/99	6	42194
	Q0PHP1	A.a.	Beta-4 tubulin	88	25	13/99	11	50591

Table 3.8. (continued)

163 [d,f)]	E2IV57	H.a.	Actin	95	48	13/112	14	42177
169 [d)]	E0A3A7	H.a.	NADPH cytochrome P450 reductase	133	27	17/91	5	78029
170 [h)]	E5EVW5	B.m.	Inorganic pyrophosphatase	N.A.	N.A.	N.A.	N.A.	N.A.
171	ACT3A [d,f)]	H.a.	Actin, cytoplasmic A3a	81	39	12/118	5	42148
	C8CCR4 [d)]	H.z.	Heat shock protein 70 cognate	70	30	17/118	14	70397
172	001676 [d,f)]	B.m.	Beta-tubulin	100	31	17/111	3	50675
	E2IV56 [d)]	H.a.	Actin	80	49	12/111	15	42239
187	D3TQ00 [d,f)]	G.m.	Myosin heavy chain	77	32	22/129	10	87494
	B2Z6M7 [d,f)]	A.i.	Cytoplasmic actin	70	46	13/129	12	42147
	D9ILX6 [d)]	H.z.	ATP synthase	62	38	13/129	15	55252
188 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	150	52	15/88	9	42148
198 [d,f)]	E2IV58	H.a.	Actin	128	48	17/106	13	42193
205 [d,f)]	Q25010	H.a.	Actin, cytoplasmic A3a	183	47	17/68	3	42148
210 [d,f)]	D9ILX6	H.z.	ATP synthase	165	46	23/107	7	55252
	E2IV56	H.a.	Actin	105	45	13/107	4	42239
215 [d)]	E2IV57	H.a.	Actin	70	38	10/103	4	42177
	D9ILX6	H.z.	ATP synthase	59	27	11/103	5	55252
216	E2IV57 [d,f)]	H.a.	Actin	64	34	10/91	7	42177
	D9ILX6 [d)]	H.z.	ATP synthase	47	27	11/91	9	55252

Table 3.8. (continued)

217	E2IV56 [d,f)] E2IV54 [d)]	H.a. H.a.	Actin V ATPase A (Fragment)	115 46	42 23	13/93 7/93	4 12	42239 33379
228 [d,h)]	TPM1	B.m.	Tropomyosin-1	88	47	16/105	13	32603
231 [d)]	E0A3A7	H.a.	NADPH cytochrome P450 reductase	86	21	12/110	21	78029
236 [d)]	E2IV54 Q8WQJ2	H.a. S.f	V ATPase A (Fragment) 60S acidic ribosomal protein P0	109 79	47 46	13/96 9/96	2 9	33379 33993
238 [d,f)]	Q8WQJ2	S.f.	60S acidic ribosomal protein P0	107	39	10/70	3	33993
239 [d,f)]	E2IV57	H.a.	Actin	111	47	14/106	7	42177
246 [d)]	D3Y4D1	H.a.	Arginine Kinase	144	47	21/149	11	40203
247 [d)]	Q2F5R4	B.m	Glutamate dehydrogenase	66	19	10/82	3	61929
250 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	94	43	13/102	10	42148
258	E2IV54 [d)] E2IV57 [d,f)]	H.a. H.a.	V ATPase A (Fragment) Actin	64 53	37 34	10/105 10/105	12 11	33379 42177
259 [d,f)]	E2IV54	H.a.	V ATPase A (Fragment)	133	48	16/91	3	33379
278 [d)]	B1NLE2	H.a.	Arginine Kinase	75	30	11/73	12	40323
281 [d)]	D9ILX6 E0XJK9	H.z. H.as.	ATP synthase Heat shock protein 90	59 43	25 16	11/107 11/107	13 6	55252 83103
282	E2IV56 [d,f)] D9ILX6 [d)]	H.a. H.z.	Actin ATP synthase	120 56	43 26	13/107 10/107	8 7	42239 55252
284 [b,d,f)]	B1NLE2	H.a.	Arginine kinase	144	32	14/77	11	40323

Table 3.8. (continued)

289 [d)]	B0WK65	C.q.	Tubulin alpha-1 chain	25 [g)] 84 [g)]	2 3	N.A.. N.A..	24 18	50604 50604
291 [h)]	----	B.m.	Acyl-Coenzyme A Dehydrogenase	N.A.	N.A.	N.A.	N.A.	N.A.
293 [d,f)]	D9ILX6	H.z.	ATP synthase	111	30	14/56	22	55252
295 [d)]	D9ILX6	H.z.	ATP synthase	62	32	12/113	34	55252
302 [d)]	E2IV54	H.a.	V ATPase A (Fragment)	127	52	12/53	21	33379
303 [d,f)]	A3RIW4	H.a.	Midgut aminopeptidase N5	90	23	15/79	18	97899
304 [h)]	Q9GPH2	B.m.	Protein disulfide isomerase	N.A.	N.A.	N.A.	N.A.	N.A.
305 [d)]	Q0MUU6	T.n.	Heat shock cognate 70 protein	103	29	17/91	27	73207
307 [d,f)]	ACT3A P06603	H.a. D.m.	Actin, cytoplasmic A3a Tubulin alpha-1 chain	82 74	37 14	11/63 6/25	27 19	42148 50561
308 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	101	42	12/52	32	42148
311 [d,f)]	D9ILX6	H.z.	ATP synthase	164	37	17/61	36	55252
313 [d,f)]	E2IV57	H.a.	Actin	147	55	14/47	33	42177
314 [d,f)]	E2IV56	H.a.	Actin	135	39	12/35	36	42239
315 [d,f)]	D9ILX6	H.z.	ATP synthase	72	31	12/108	32	55252
317 [d,f)]	C8CCR4 C4P7G5	H.z. B.ma.	Heat shock protein 70 cognate Myosin heavy chain (Fragment)	92 91	32 24	21/103 20/103	21 25	70397 96071
319 [d,f)]	E2IV57	H.a.	Actin	107	39	13/64	24	42177
320 [d)]	E2IV54	H.a.	V ATPase A (Fragment)	90	47	12/98	28	33379
321 [d)]	C8CCR4	H.a.	Heat shock protein 70 cognate	77	16	10/60	23	70397
323 [d)]	E2IV54 D9ILX6	H.a. H.z.	V ATPase A (Fragment) ATP synthase	50 49	37 26	8/101 10/101	41 40	33379 55252

Table 3.8. (continued)

327 [d,f)]	E2IV57	H.a.	Actin	146	45	15/64	45	42177
328 [b,d,f)]	D3Y4D1	H.a.	Arginine kinase	165	53	20/88	30	40203
332	C7SIR9 [d,f)] E2IV54 [d)]	H.a. H.a.	Heat shock protein 70 V ATPase A (Fragment)	111 53	28 35	20/87 9/87	24 21	71801 33379
333 [d,f)]	E2IV54	H.a.	V ATPase A (Fragment)	100	40	11/65	24	33379
334 [d,f)]	A7KCX9	H.e.	Heat shock protein hsp21.4	106	53	10/48	30	21377
335 [d,f)]	E2IV54	H.a.	V ATPase A (Fragment)	144	47	12/39	37	33379
336 [d)]	E2IV54	H.a.	V ATPase A (Fragment)	70	37	10/84	46	33379
337 [d,f,h)]	C4P7G5	B.ma.	Myosin heavy chain (Fragment)	86	27	21/113	41	96071
339 [d,f)]	E2IV54	H.a.	V ATPase A (Fragment)	174	54	17/45	44	33379
341	D9ILX6 [d,f)] E2IV54 [d)]	H.z. H.a.	ATP synthase V ATPase A (Fragment)	65 48	35 31	15/105 8/100	34 37	55252 33379
342 [d)]	Q5XUN5	H.a.	Triosephosphate isomerase	53	39	8/85	23	26547
343 [b,d,f)]	Q5XUN5	H.a.	Triosephosphate isomerase	172	62	16/57	8	26547
349 [h)]	D3TSE2	G.m.m.	FKBP-type peptidyl-prolyl cis-trans isomerase	N.A.	N.A.	N.A.	N.A.	N.A.
352 [d)]	B8RJ87	C.t.	78 kDa glucose-regulated protein (Fragment)	94	35	10/71	12	33019
358 [d,f)]	E2IV54	H.a.	V ATPase A (Fragment)	165	45	14/54	11	33379
374 [d)]	TPM1	B.m.	Tropomyosin-1	88	45	12/122	21	32603
375 [d,f)]	Q26433	D.m.	Myosin heavy chain (Fragment)	81	24	10/67	14	45050
379 [d,f)]	A2I3X7	M.h.	Puatative Tubulin beta-1 chain	153	38	15/41	13	50628
381 [e)]	Q1HPT0	B.m.	ATP synthase subunit beta	60	15	5/10	31	54859
383 [d)]	TBB1	M.s.	Tubulin beta-1 chain	66	23	11/77	27	50654
388 [d,f)]	E2IV57	H.a.	Actin	83	36	11/81	24	42177
392 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	93	29	9/41	45	42148
393 [d,f)]	D2SNS6	H.v.	Aldehyde dehydrogenase (Fragment)	83	56	6/52	44	17718

Table 3.8. (continued)

394 [d)]	D3Y4D1	H.a.	Arginine kinase	79	29	11/85	54	40203
395	E2IV57 [d,f)]	H.a.	Actin	128	50	16/68	39	42177
	A0MSN0 [d)]	H.a.	Glutathione S-transferase	46	32	6/68	40	24459
397 [d,f)]	E2IV57	H.a.	Actin	177	61	17/61	19	42177
398 [d)]	B2LRS8	H.a.	Aminopeptidase N	56	9	9/54	32	111268
400 [d,f)]	E2IV57	H.a.	Actin	72	32	10/82	27	42177
401 [d)]	A0MSN0	H.a.	Glutathione S-transferase	54	29	5/78	35	24459
404 [d)]	A1E4A8	B.m.	Vacuolar ATP synthase subunit B	78	31	12/66	40	54667
407 [d)]	D9ILX6	H.z.	ATP synthase	53	26	9/91	47	55252
	E2IV56	H.a.	Actin	45	32	6/91	47	42239
413 [d,f)]	B2KSE9	H.a.	Thioredoxin peroxidase	164	56	13/50	16	22122
416 [d)]	B1NLE0	H.a.	Putative uncharacterized protein	91	57	12/112	37	26176
	E2IV58	H.a	Actin	28	18	5/112	35	42193
417 [d)]	C8CCR4	H.z.	Heat shock protein 70 cognate	76	21	14/91	42	70397
420 [d)]	ACT3A	H.a.	Actin, cytoplasmic A3a	73	37	8/53	48	42148
421 [d)]	B4Z1D7	H.a.	Alkaline phosphatase 2	47	20	8/74	43	59223
422 [h)]	BOW8G6	C.q.	Vacuolar proton translocating ATPase 116kDa subunit a1	N.A.	N.A.	N.A.	N.A.	N.A.
426 [d)]	Q2F6A8	B.m.	Enoyl-CoA hydratase 3	17 [g)]	3	N.A.	7	30393
				25 [g)]	6	N.A.	9	30393
427 [d)]	B0FBK1	E.o.	Aldehyde dehydrogenase (Fragment)	78	28	7/57	25	21963
428 [h)]	TBB1	N.c.	Tubulin beta-1 chain	N.A.	N.A.	N.A.	N.A.	N.A.
429 [d,f)]	E2IV60	H.a.	Actin	123	28	10/35	10	42269

Table 3.8. (continued)

433 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	119	55	17/117	15	42148
438 [d,f)]	E2IV57	H.a.	Actin	141	39	14/52	4	42177
441 [h)]	Q1HPK5	B.m.	Thymosin isoform 1	N.A.	N.A.	N.A.	N.A.	N.A.
445 [d)]	E2IV52	H.a.	V ATPase B (Fragment)	48	22	5/37	14	23589
452	E2IV56 [d,f)] E2IV54 [d)]	H.a. H.a.	Actin V ATPase A (Fragment)	56 52	14 30	7/41 6/41	32 29	42239 33379
466 [e,h)]	P31400	M.s.	V-type proton ATPase catalytic subunit A	71	11	6/13	41	68408
467 [d,f)]	E2IV56	H.a.	Actin	83	27	8/51	15	42239
469 [h)]	Q2F5J2	B.m.	Prohibitin protein WPH	N.A.	N.A.	N.A.	N.A.	N.A.
470 [d,f)]	D9ILX6	H.z.	ATP synthase	57	23	7/37	34	55252
475 [d,f)]	Q285R3	B.m.	Eukaryotic translation initiation factor 4A	93	34	13/80	46	47837
478 [d,f,h)]	Q5XUN5 Q285R3	H.a. B.m.	Triosephosphate isomerase Translation initiation factor 4A	83 76	29 31	8/71 12/63	42 42	26547 47837
481 [d,f)]	E2IV57	H.a.	Actin	102	41	13/70	19	42177
484 [e)]	O76149	B.m.	Beta-tubulin	74	12	6/12	22	50582
485 [d,f)]	D9ILX6	H.z.	ATP synthase	99	23	9/30	18	55252
489 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	129	29	11/35	31	42148
492 [d,f)]	TPM1	B.m.	Tropomyosin-1	66	34	12/66	33	32603
494 [d,f)]	E2IV60	H.a.	Actin	72	30	8/44	30	42269
498 [d,h)]	D5LTC5	H.a.	Translational controlled tumor protein	107	72	12/73	18	19806
500 [d)]	ACT3A	H.a.	Actin, cytoplasmic A3a	93	30	10/49	21	42148
503 [d,f)]	E2IV56	H.a.	Actin	147	30	13/35	25	42239
505 [d)]	E2IV57	H.a.	Actin	87	28	12/68	28	42177
516 [d,f)]	E2IV58	H.a.	Actin	94	26	10/42	8	42193
549 [d)]	E2IV56	H.a.	Actin	66	27	8/56	29	42239

Table 3.8. (continued)

550 [b,d,f)]	VATB	H.v.	V-type proton ATPase subunit B	97	25	13/50	8	55144
552 [d,f)]	Q1HPP5	B.m.	Actin-depolymerizing factor 1	152	64	13/53	14	17227
557 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	154	42	16/57	13	42148
559	ACT3A [d,f)] C8CCR4 [d)]	H.a. H.z.	Actin, cytoplasmic A3a Heat shock protein 70 cognate	82 74	38 20	11/62 15/62	13 17	42148 70397
560 [d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	74	24	8/33	25	42148
562 [d,f)]	E2IV57	H.a.	Actin	161	40	17/50	28	42177
563 [e)]	Q25010	H.a.	Actin, cytoplasmic A3a	82	18	6/13	15	42148
564 [d)]	B6A8J6	H.a.	Apolipophorin-III (Fragment)	68	71	5/66	14	5233
566 [d,h)]	Q0PXX1	D.c.	Putative mitochondrial cytochrome c oxidase polypeptide Va	40 [g)] 45 [g)]	5 6	N.A.. N.A..	7 13	17702 17702
571 [h)]	E3UKK4	Miss	Farnesoic acid O-methyl transferase(fragment)	N.A.	N.A.	N.A.	N.A.	N.A.
588 [h)]	Q5MGI8	L.o.	Myosin 1 light chain	N.A.	N.A.	N.A.	N.A.	N.A.
590 [d)]	E2IV60	H.a.	Actin	58	24	8/49	37	42269
592 [h)]	Q2F6C1	B.m.	Cytidine deaminase	N.A.	N.A.	N.A.	N.A.	N.A.
595 [d)]	ACT3A	H.a.	Actin, cytoplasmic A3a	72	18	8/67	33	42148
598 [d,f)]	B1NLE2	H.a.	Arginine kinase	83	27	12/57	29	40323
603 [d,f)]	D9ILX6	H.z.	ATP synthase	112	20	10/21	7	55252
605 [h)]	----	B.m.	Putative farnesoic acid O-methyl transferase	N.A.	N.A.	N.A.	N.A.	N.A.
606 [d,f)]	Q8WQJ0	S.f.	60S acidic ribosomal protein P2	114	71	9/58	25	11518
607 [h)]	Q1HPK1	B.m.	Troponin C	N.A.	N.A.	N.A.	N.A.	N.A.
611 [h)]	B6CMF8	H.a.	Fatty acid-binding protein 1	N.A.	N.A.	N.A.	N.A.	N.A.
613 [d)]	E2IV54	H.a.	V ATPase A (Fragment)	101	35	10/59	33	33379
616 [d,f)]	D9ILX6	H.z.	ATP synthase	119	23	10/46	29	55252
618 [e)]	C6YYU1	N.l.	Sodium potassium adenosine triphosphatase fragm.	91	20	6/8	26	37433

Table 3.8. (continued)

621 [d)]	B9UCR0	H.a.	Cuticle protein 4	76	69	5/69	36	12377
623 [d)]	A0MSN0	H.a.	Glutathione S-transferase	44	27	5/100	41	24459
634 [d)]	D9ILX6	H.z.	ATP synthase	74	22	10/36	10	55252
635 [e)]	Q1HPP7	B.m.	Tropomyosin isoform 4 or	78	26	6/11	19	29623
	Q1HPP8	B.m.	Tropomyosin isoform 3	77	26	6/11	19	29485
636 [d)]	B6CMF8	H.a.	Fatty acid-binding protein 1	81	55	7/34	12	15023
641 [d)]	B6CMF9	H.a.	Fatty acid-binding protein 2	114	60	9/45	8	14901
642 [d,f)]	B2DBI0	P.x.	Tropomyosin 2	148	52	16/71	21	32603
643 [d,f)]	B6CMG0	H.a.	Fatty acid-binding protein 3	165	71	14/38	6	14848
644 [d)]	076515	H.z.	Fatty acid-binding protein	68	56	6/57	24	15082
	B6CMF9	H.a.	Fatty acid-binding protein 2	64	55	6/57	6	14901
646 [d)]	D5LTC5	H.a.	Translational controlled tumor protein	131	74	13/60	7	19806
	A0MSN0	H.a.	Glutathione S-transferase	61	40	7/60	24	24459
647 [d,h)]	B6CMF8	H.a.	Fatty acid-binding protein 1	144	86	11/35	8	15023
648 [d)]	EOVJX6	P.h.	Vacuolar ATP synthase subunit B, putative	96	26	12/54	24	55503
650 [d)]	B6CMF9	H.a.	Fatty acid-binding protein 2	104	58	8/38	10	14901
653 [d,f)]	B6CMG0	H.a.	Fatty acid-binding protein 3	199	85	20/88	36	14848
655 [d,f)]	B6CMF9	H.a.	Fatty acid-binding protein 2	123	68	10/66	14	14901
	B6CMG0	H.a.	Fatty acid-binding protein 3	117	71	11/66	17	14848
657 [d,f)]	B6CMF8	H.a.	Fatty acid-binding protein 1	165	84	15/60	17	15023

Table 3.8. (continued)

659	B6CMF8 [d,h)] B6CMG0 [d)] B6CMF9 [d)]	H.a. H.a. H.a.	Fatty acid-binding protein 1 Fatty acid-binding protein 3 Fatty acid-binding protein 2	62 57 56	50 44 48	7/61 7/61 6/61	28 23 21	15023 14848 14901
670 [d)]	B6CMG0	H.a.	Fatty acid-binding protein 3	121	67	11/68	58	14848
673 [d,h)]	B6CMF8	H.a.	Fatty acid-binding protein 1	71	55	7/57	39	15023
677 [d)]	CALM	D.m.	Calmodulin	74	41	5/34	25	16800
682 [e)]	A1E9B3	B.m.	Vacuolar ATP synthase catalytic subunit A	65	10	6/8	16	68558
696 [d)]	VATB	H.v.	V-type proton ATPase subunit B	71 [g)] 73 [g)]	2 2	N.A.. N.A..	31 38	54800 54800
697 [d)]	VATB	H.v.	V-type proton ATPase subunit B	82	23	11/60	31	55144
708 [d)]	B6CMG0	H.a.	Fatty acid-binding protein 3	94	59	11/64	27	14848
709 [d,f)]	Q9BMF4	G.m.	Alpha-tubulin (Fragment)	74	20	5/47	31	31064
712 [d)]	O76515	H.z.	Fatty acid-binding protein	83	41	6/42	29	15082
715 [d,f)]	B6CMG0	H.a.	Fatty acid-binding protein 3	165	64	15/48	20	14848
722 [d,f)]	B6CMG0	H.a.	Fatty acid-binding protein 3	198	83	17/65	27	14848
723 [d)]	B6CMG0	H.a.	Fatty acid-binding protein 3	101	59	10/60	26	14848
724 [e)]	Q1HPS9	B.m.	Vacuolar ATP synthase subunit E	54	12	5/12	15	26160
788 [d)]	B6A8H5	H.a.	Acyl-CoA binding protein	43	50	3/43	15	9465
1000 [c,d,f)]	B6CMF8	H.a.	Fatty acid-binding protein 1	107	79	11/86	24	15023
1001 [c,d,f)]	C7ED93	C.s.	Heat shock protein 60	159	44	23/71	26	61147
1002 [c,d,f)]	C7ED93	C.s.	Heat shock protein 60	93	35	16/85	29	61147
1003 [c,d,f)]	ACT3A	H.a.	Actin, cytoplasmic A3a	208	53	16/35	25	42148
1004 [c,d,f)]	D9ILX6	H.z.	ATP synthase	212	48	25/87	11	55252
1005 [c,d,f)]	D2WJB6	H.s.	Putative enolase (Fragment)	121	51	13/55	22	40918

Table 3.8. (continued)

1006 [c,d,f]	A1YQ87	B.m.	Enolase	188	54	21/83	25	47164
1007 [c,d,f]	E2IV54	H.a.	V ATPase A (Fragment)	99	40	9/43	23	33379
1008 [c,d,f]	E2IV54	H.a.	V ATPase A (Fragment)	110	33	8/37	8	33379
1009 [c,d,f]	D2WJ88	H.f.	Putative enolase (Fragment)	228	61	22/67	16	40761
1010 [b,c,d,f]	Q8I866	S.f.	Heat shock cognate 70 protein	124	29	21/79	25	73178
1011 [c,e]	C9D996	M.p.	Alanyl-tRNA synthetase (Fragment)	90	30	7/22	43	23171
1012 [c,d,f]	B2KSE9	H.a.	Thioredoxin peroxidase	114	44	8/29	11	22122
1013 [c,d,f]	D9ILX6	H.z.	ATP synthase	124	22	11/31	21	55252
1014 [c,d,f]	C0KJJ4	S.e.	Heat shock protein 70	93	32	18/118	17	75041
1015 [c,d,f]	VATB	H.v.	V-type proton ATPase subunit B	125	31	16/65	14	55144
1016 [c,d,f]	B1NLE2	H.a.	Arginine kinase	60	24	11/87	25	40323
1017 [c,d,f]	B1NLE2	H.a.	Arginine kinase	106	52	21/161	24	40323
1018 [c,d]	B6CMF9	H.a.	Fatty acid-binding protein 2	105	71	10/92	20	14901
1019 [c,d]	Q5XUN5	H.a.	Triosephosphate isomerase	60	39	8/70	18	26547
1020 [c,d]	Q6SKX8	H.a.	Ubiquitin(Fragment)	113	72	7/31	19	8560
1021 [c,d]	C7SIR9	H.a.	Heat shock protein 70	86	32	20/182	31	71801
1022 [c,d]	Q8T7V0	H.z.	Cytoplasmic actin A3a2	74	35	10/69	6	42186

a) A.a.: Aedes aegypti
A.i.:Agrotis ipsilon
B.m.: Bombyx mori
B.ma.: Bombyx mandarina
C.f.: Camponotus floridanus
C.n.: Culex nigripalpus
C.q.: Culex quinquefasciatus
C.s.: Chilo suppressalis
C.t.: Culex tarsalis
D.c.: Diaphorina citri
D.m.: Drosophila melanogaster
D.y.: Drosophila yakuba
E.o.: Ectropis obliqua
G.m.m.: Glossina morsitans morsitans
H.a.: Helicoverpa armigera
H.as.:Helicoverpa assulta
H.e.: Heliconius erato
L.o.: Lonomia obliqua
H.f.: Hyblaea firmamentum
H.s.: Hypena scabra
H.v.: Heliothis virescens
H.z.: Heliothis zea
M.h.: Maconellicoccus hirsutus
M.p.: Microchorista philpotti
M.s.: Manduca sexta
N.c.: Notothenia coriiceps
N.l.: Neffapis longilingua
P.h.: Pediculus humanus
P.v.: Polypedilum vanderplanki
P.x.: Papilio xuthus
S.e.: Spodoptera exigua
S.f.: Spodoptera frugiperda
S.p.:Synemon plana
T.n.: Trichoplusia ni
T.s.: Thelaxes suberi

Table 3.8. (continued)

b)Doubly picked spot.
c)Manually picked spot.
d)From MS plus MS/MS identifications (ABI 4700).
e)From Maldi TOF analysis.
f)Confirmed by independent Maldi TOF analysis.
g)Score from MS/MS experiment.
h)From EST analysis.
N.A..: Not applicable.

3.2.1. Differential Proteome Analysis

Isoelectric focusing and %15 SDS-PAGE of Adana, Mardin and susceptible population (lyophilized) samples were done as three replicates from each population in same apparatus together at described conditions at chapter II , part 2.2.13 with 800 µg proteins (**Figure 3.2-3.10**).

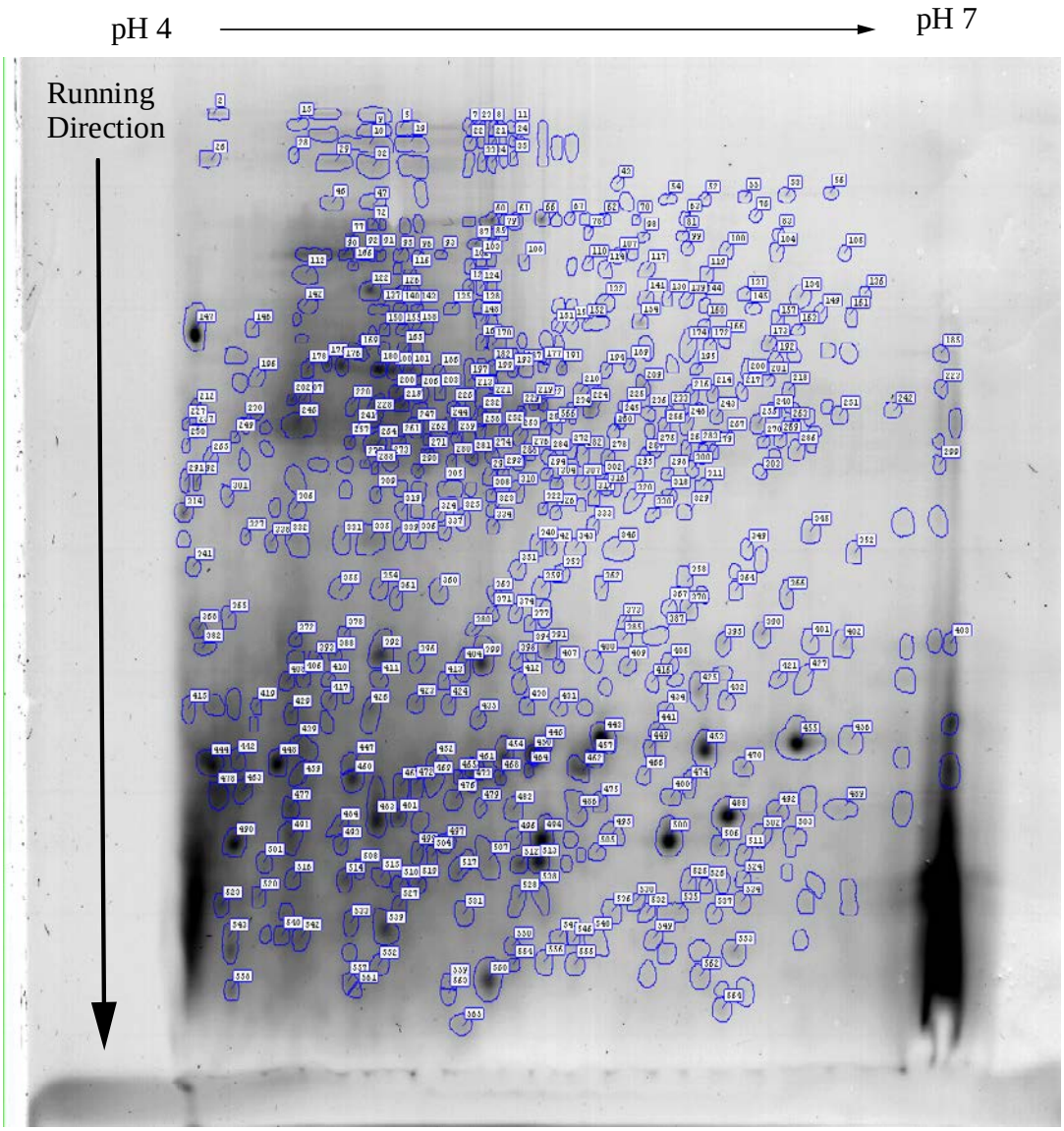


Figure 3.2. Image of a First 2D-PAGE Gel Prepared from *H. armigera* midgut tissue from susceptible population (CL, lyophilized). 800 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension.

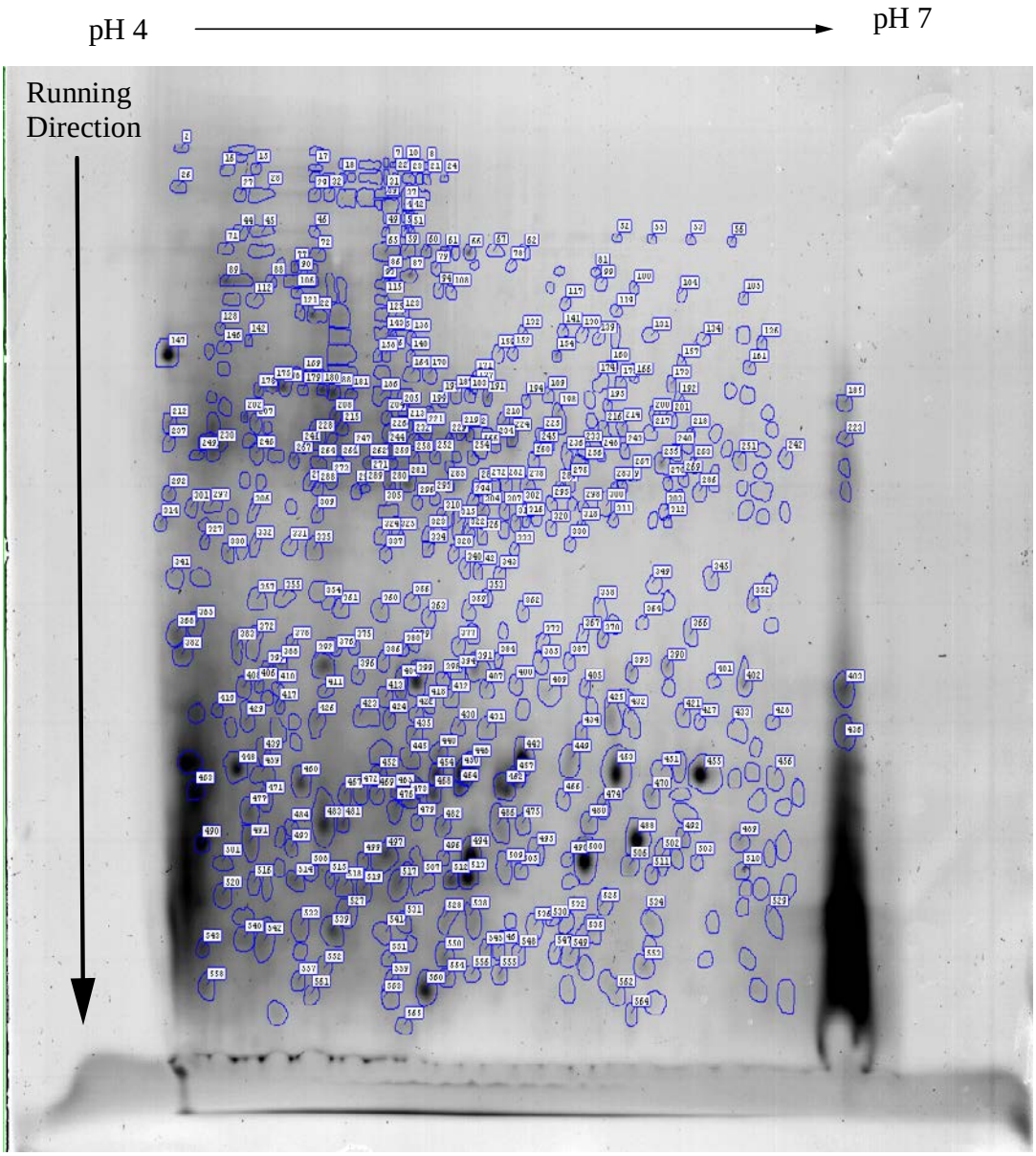


Figure 3.3. Image of a Second 2D-PAGE Gel Prepared from *H. armigera* midgut tissue from susceptible population (CL, lyophilized). 800 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension.

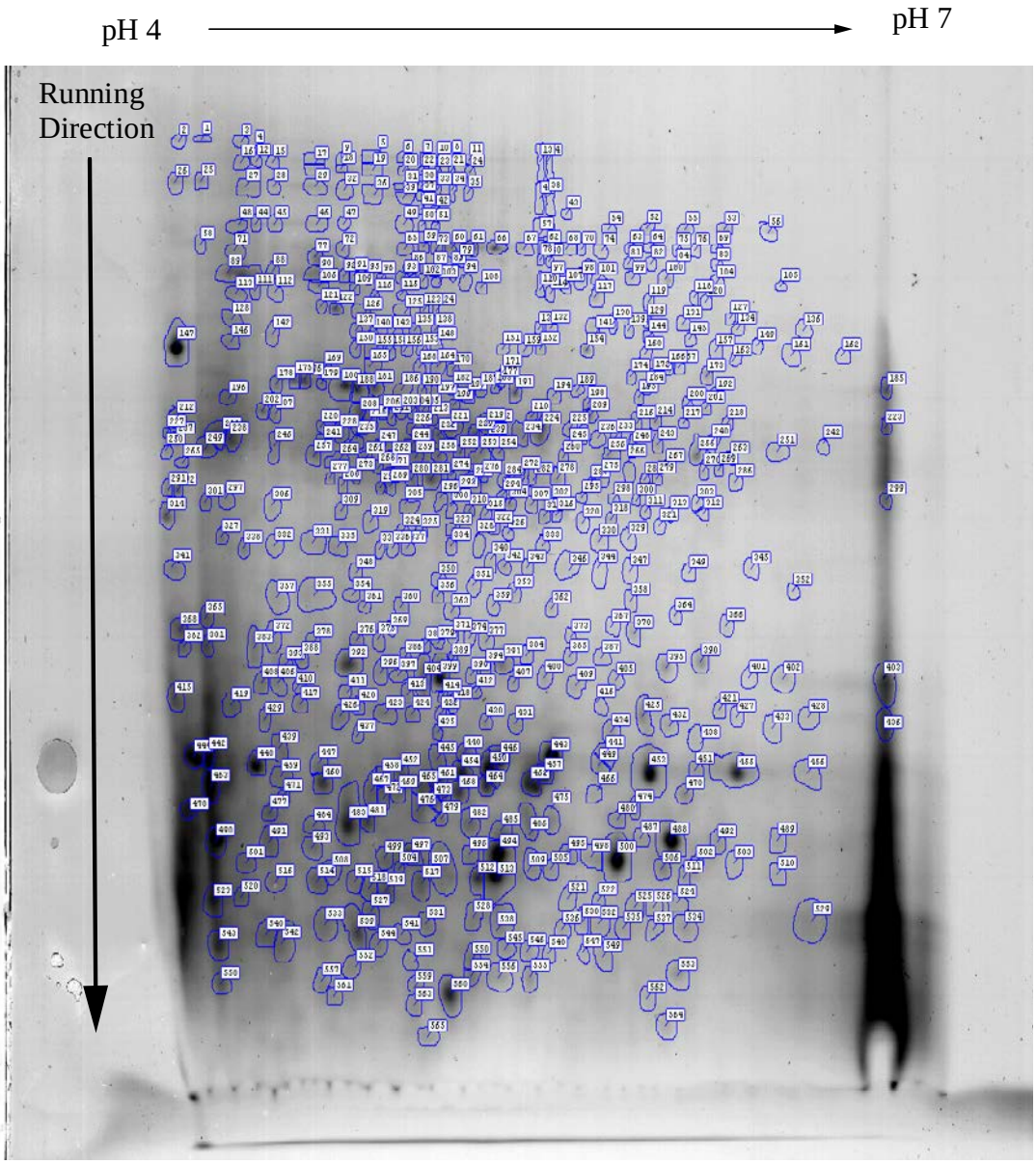


Figure 3.4. Image of a Third 2D-PAGE Gel Prepared from *H. armigera* midgut tissue from susceptible population (CL, lyophilized). 800 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension.

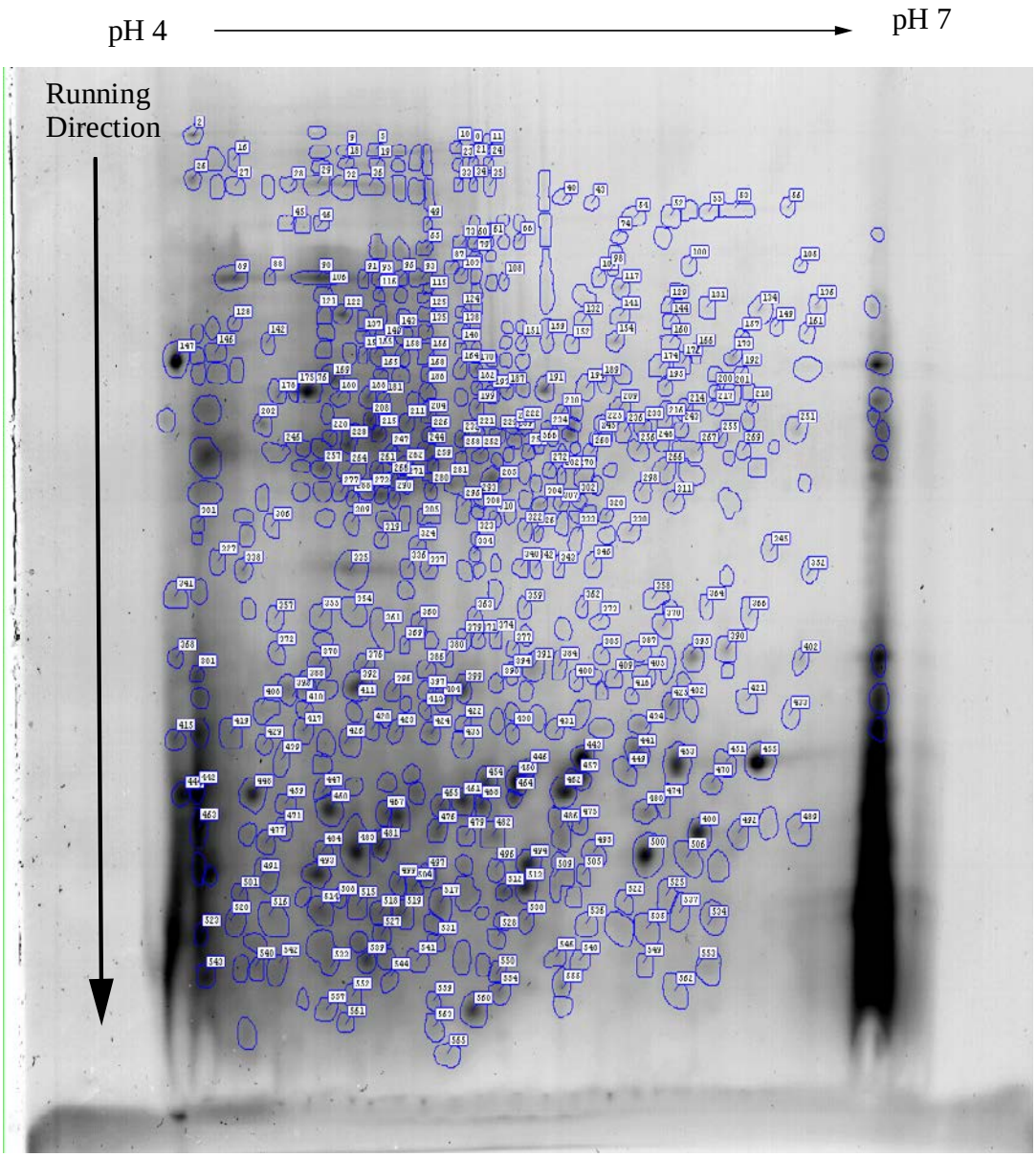


Figure 3.5. Image of a First 2D-PAGE Gel Prepared from *H. armigera* midgut tissue from Adana population (A, lyophilized). 800 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension.

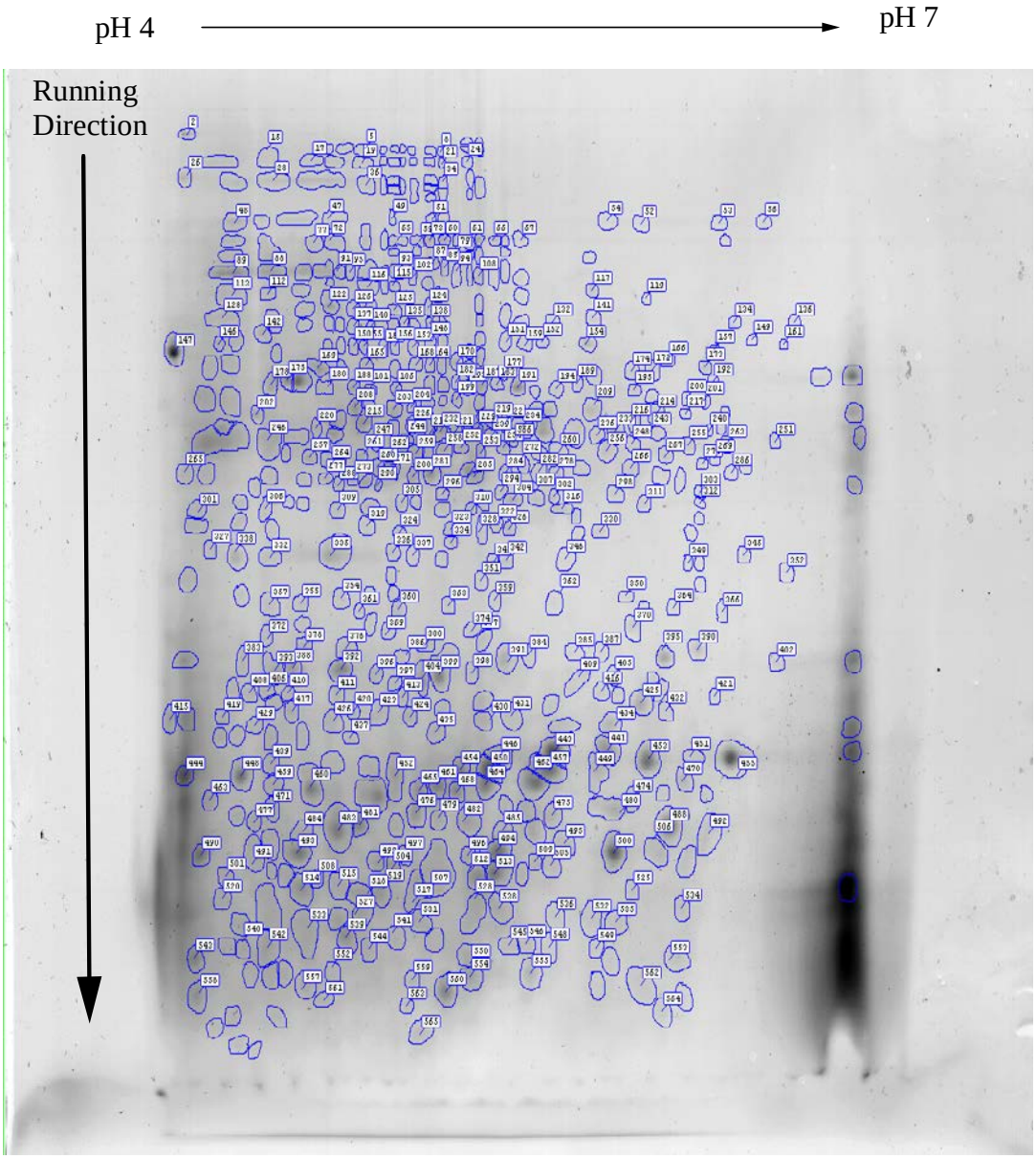


Figure 3.6. Image of a Second 2D-PAGE Gel Prepared from *H. armigera* midgut tissue from Adana population (A, lyophilized). 800 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension.

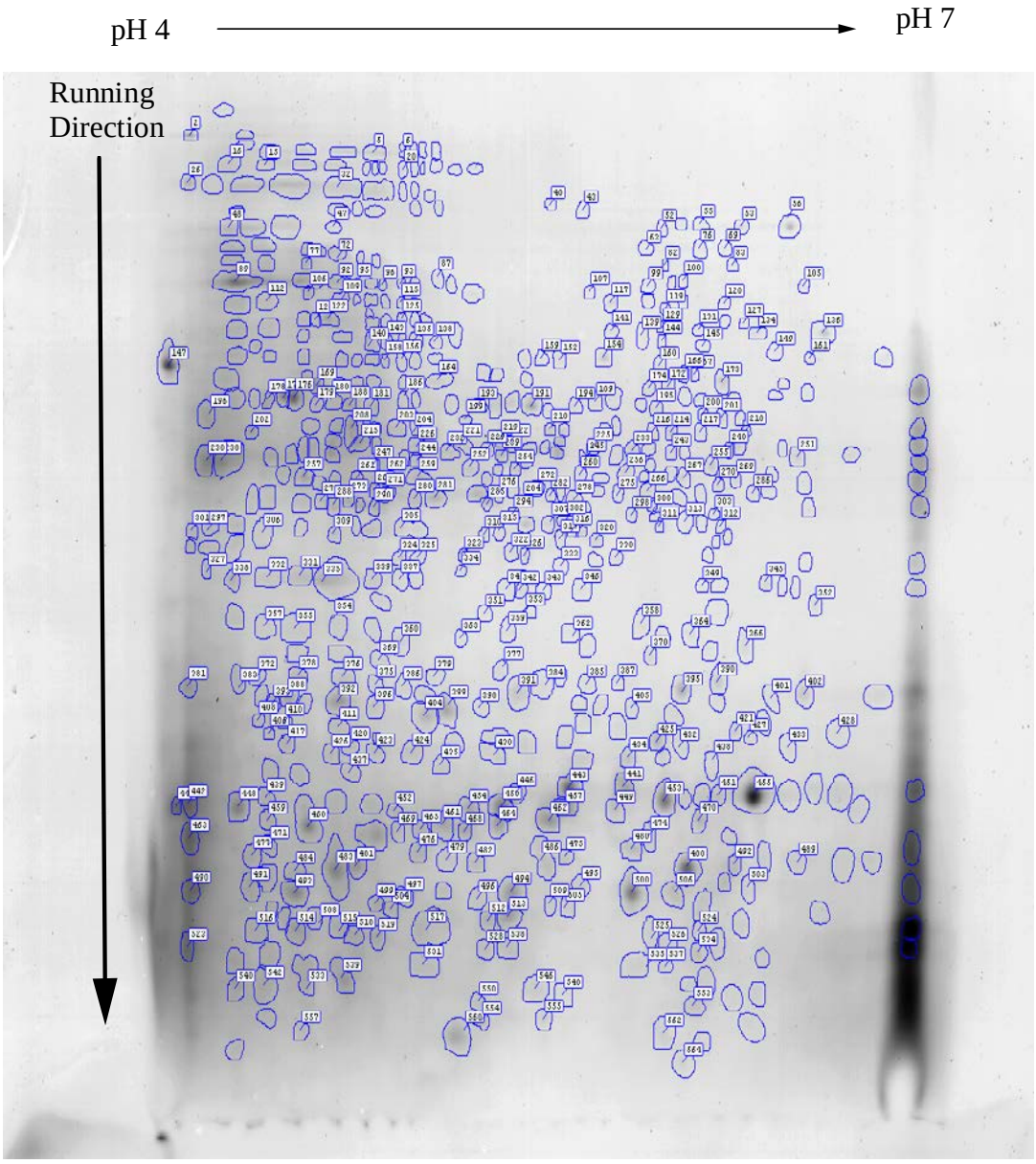


Figure 3.7. Image of a Third 2D-PAGE Gel Prepared from *H. armigera* midgut tissue from Adana population (A, lyophilized). 800 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension.

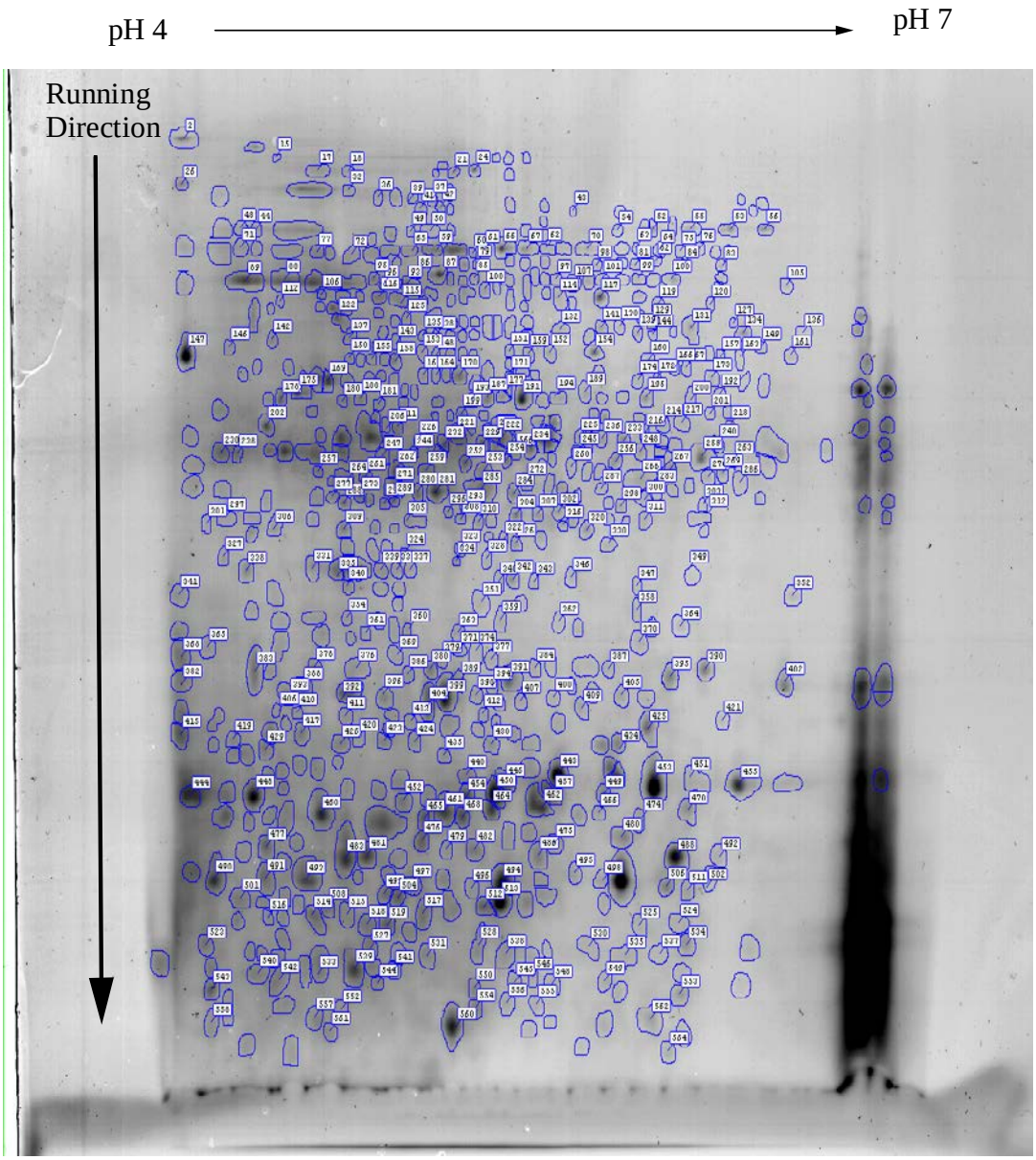


Figure 3.8. Image of a First 2D-PAGE Gel Prepared from *H. armigera* midgut tissue from Mardin population (M, lyophilized). 800 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension.

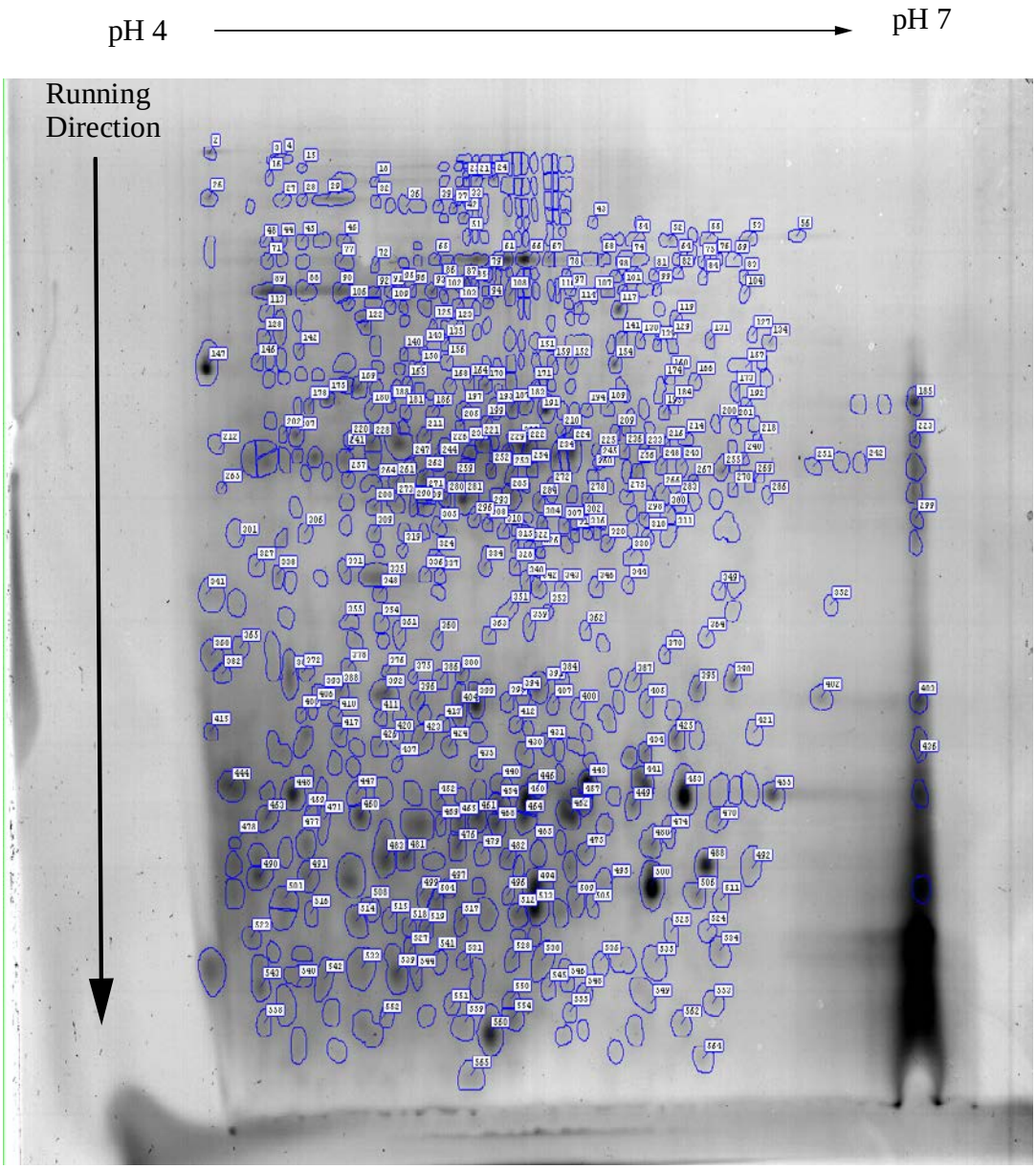


Figure 3.9. Image of a Second 2D-PAGE Gel Prepared from *H. armigera* midgut tissue from Mardin population (M, lyophilized). 800 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension.

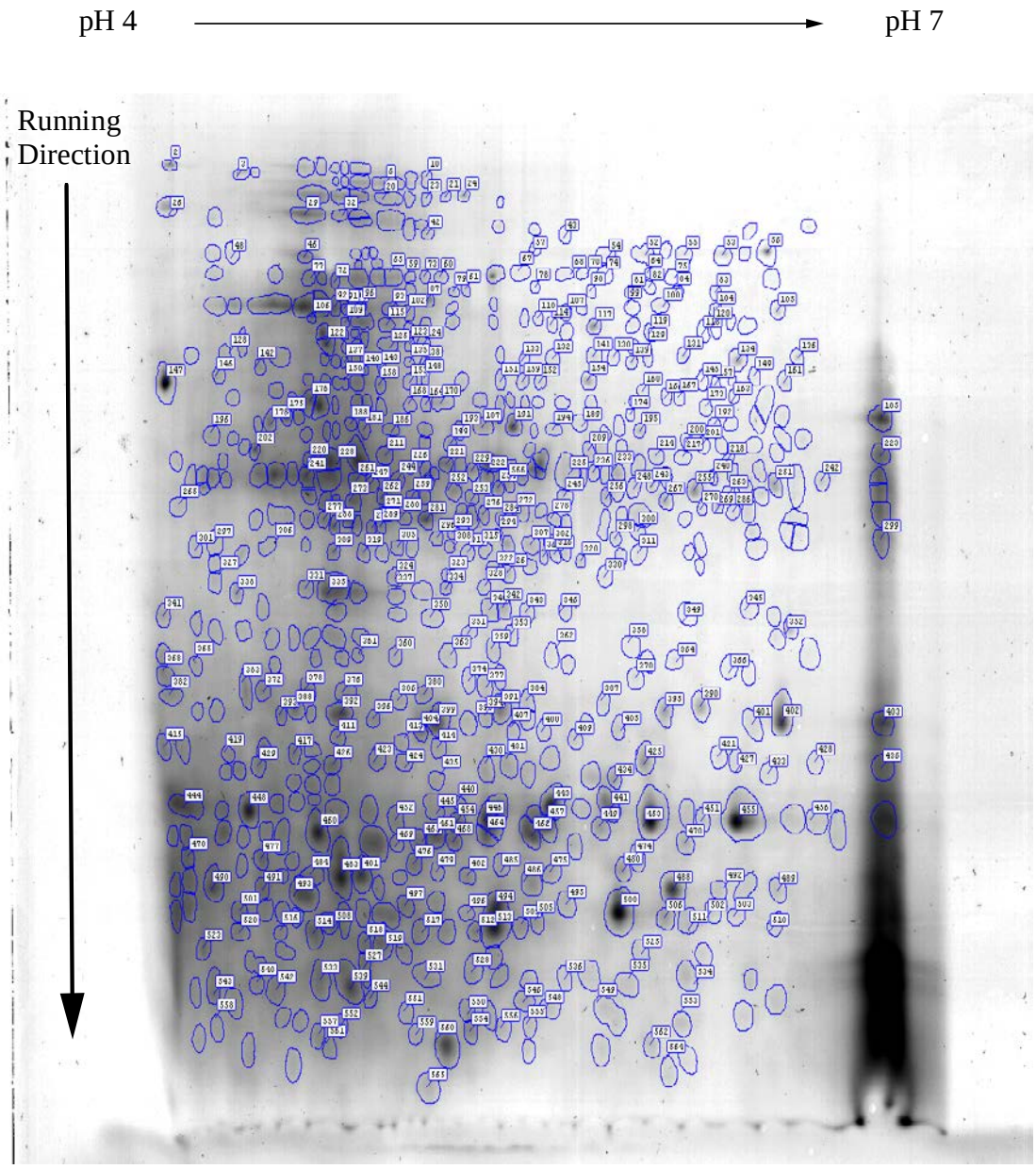


Figure 3.10. Image of a Third 2D-PAGE Gel Prepared from *H. armigera* midgut tissue from Mardin population (M, lyophilized). 800 µg proteins were loaded on immobiline strips (pH 4–7) that were used for the first dimension. SDS-PAGE (15%T) was applied for the second dimension.

In order to do comparative proteomic analysis, 2D-Gel images of Adana, Mardin and susceptible populations were compared by overlapping gels into one of the susceptible gel that had a maximum spot number (**Figure 3.4**). Afterwards, overlapped gels were stretched from edges to adjust optimum orientation according

to the clearly visible dense spots. Then, stretching continued with other protein spots, locate approximate same places and same orientation, by matching them each other. Thereafter, a hypothetical gel image was constructed on computer with matched protein spots together with unmatched protein spots (**Figure 3.11).** Finally, density and differential expression analysis were done for all spots in that gel.

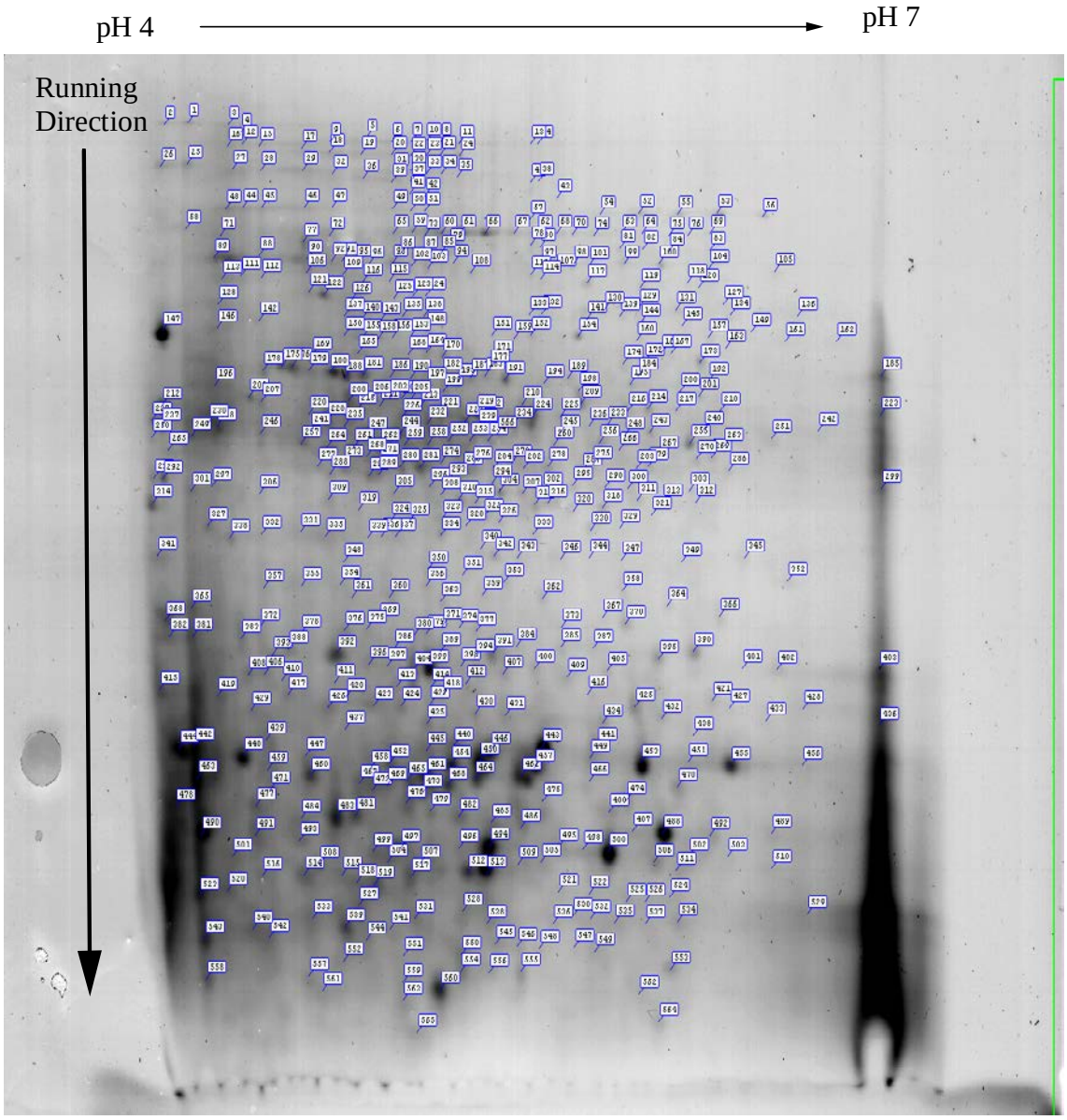


Figure 3.11. Overlap of 9 Gels by Using Phoretix 2D Software Program.

In addition, these constructed gels image called as a “master gel” including all proteins (565 protein spots (matched and unmatched)) in 9 gels**.** This master gel was also used for comparison with reference gel image for determining proteins without making mass spectrophotometric analysis again for identified proteins.

For checking consistency of these gels and analyzing differentially expressed unknown proteins in lyophilized samples 2-DE gels, total 60 protein spots from these manually picked and analyzed mass spectrophotometrically. There were 6 protein spots in total 60 picked spots that were selected to analyze consistency of reference and master gels. These proteins showed good consistency with reference gel by giving same protein identifications results such as spot 105 and 125, were labelled with superscript b in **table 3.8**. There were also 23 spots (numbers 1000-1022) that were not identified in the reference gel, were added to the list of identified proteins (**Table 3.8**).

3.2.2. Differentially Expressed Proteins in Adana Population

The differential protein expression analysis of midgut proteins derived from the Adana (A) population showed that in comparison to the control (CL) most of the up-regulated proteins belonged to energy system related proteins (**Table 3.9**). These up-regulated proteins consisted of mitochondrial proteins (e.g. ATP synthase and V-type proton ATPase subunit B, see in **Table 3.9**), cytosolic proteins (enolase and fatty acid binding protein) and kinase/phosphatase proteins (e.g. arginine kinase, alkaline phosphatase 2 and sodium potassium adenosine triphosphatase). Furthermore, protein metabolism proteins (e.g. eukaryotic translation initiation factor 4A, heat shock cognate 70 protein), cytoskeleton proteins (e.g. tubulin beta-1 chain, thymosin isoform 1) and a translationally controlled tumor protein (spot 498) were up-regulated in the Adana field population. In addition, most of the identified down-regulated proteins belonged to the protein metabolism category (e.g. heat shock proteins and alanyl-tRNA synthetase) and to cytosolic energy associated proteins (putative enolase (fragment) and fatty acid binding proteins). Aldehyde

dehydrogenase (spot 393) and putative tubulin beta-1 chain (spot 379) were also down-regulated in the Adana population.

As actin proteins are common and excess cytoskeleton proteins in insects, these proteins were eliminated from **table 3.9**. Therefore, any up or down-regulated actin proteins in Adana population were not present in **table 3.9**. Nonetheless, there were some identified protein spots in **table 3.8** containing two or more proteins, these spots were not used for differential analysis in Adana population, as well.

Notably, only one differentially expressed detoxification protein, aldehyde dehydrogenase (D2SNS6 from *Heliothis virescens*), was found as down-regulated with high reproducibility (**Figure 3.12**). This protein was detected in 3 gels of the susceptible population (CL), labelled as a spot 393. By contrast, it was never detected in none of the 3 gels of the Adana (A) population. However, the gels of both population displayed one spot containing another form of aldehyde dehydrogenase. This spot (spot 427) was located in the vicinity of spot 393 and was identified as aldehyde dehydrogenase (B0FBK1) from *Ectropis obliqua*.

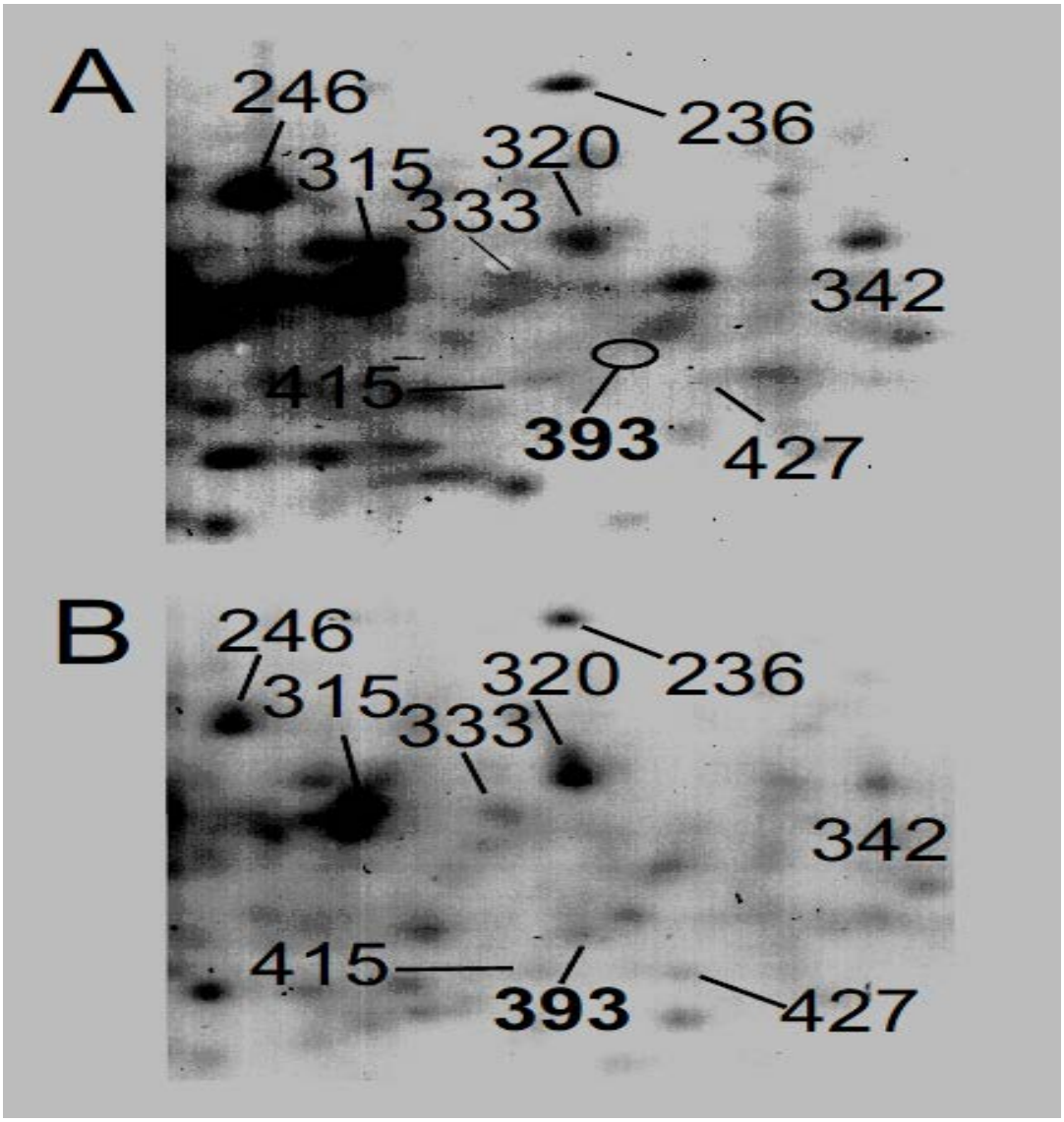


Figure 3.12. Differential protein expression analysis with zoomed view of a gel region showing a down-regulated spot of aldehyde dehydrogenase; **A:** Adana; **B:** Control. Down-regulated aldehyde dehydrogenase protein is found in spot 393 (see **Table 3.9**).

Table 3.9. List of differentially expressed proteins in Adana population

Spot no. [c)]	Access. no.	Organism [a)]	Protein name	Group	Regulation in n	Fold Change [d)]	No. of Spots in Mardin [e)]	No. of Spots in Control [e)]
293	D9ILX6	H.z.	ATP synthase	Energy (mitochondrial)	Up	∞	2	0
311	D9ILX6	H.z.	ATP synthase		Up	∞	3	0
470	D9ILX6	H.z.	ATP synthase		Up	2.62	3	3
1013	D9ILX6	H.z.	ATP synthase		Up	∞	3	0
404	A1E4A8	B.m.	Vacuolar ATP synthase subunit B		Up	2.43	3	3
445	E2IV52	H.a.	V ATPase B (Fragment)		Up	4.75	2	1
339	E2IV54	H.a.	V ATPase A (Fragment)		Up	2.48	3	3
1015	VATB	H.v.	V-type proton ATPase subunit B		Up	2.50	3	3
550	VATB	H.v.	V-type proton ATPase subunit B		Up	30.2	2	2
566	Q0PXX1	D.c	Putative mitochondrial cytochrome c oxidase polypeptide Va		Up	3.05	3	3
598	B1NLE2	H.a.	Arginine kinase	Kinase/ Phosphatase	Up	2.07	3	3
1016	B1NLE2	H.a.	Arginine kinase		Up	∞	3	0
1017	B1NLE2	H.a.	Arginine kinase		Up	2.29	1	3
421	B4Z1D7	H.a.	Alkaline phosphatase 2		Up	2.46	3	2
618	C6YYU1	N.l.	Sodium potassium adenosine triphosphatase		Up	2.83	3	3
349 [b)]	D3TSE2	G.m.m.	FKBP-type peptidyl-prolyl cis-trans isomerase	Protein Metabolism	Up	5.09	3	2
475	Q285R3	B.m.	Eukaryotic translation initiation factor 4A		Up	2.50	3	3
1010	Q8I866	S.f.	Heat shock cognate 70 protein		Up	2.92	3	3
1020	Q6SKX8	H.a.	Ubiquitin(Fragment)		Up	2.70	1	3
383	TBB1	M.s.	Tubulin beta-1 chain	Cytoskeleton	Up	6.60	3	2

Table 3.9. (continued)

441 [b)]	Q1HPK5	B.m.	Thymosin isoform 1		Up	∞	3	0
492	TPM1	B.m.	Tropomyosin-1		Up	3.49	3	3
498	D5LTC5	H.a.	Translational controlled tumor protein	Unknown	Up	4.28	3	3
1005	D2WJB6	H.s.	Putative enolase (Fragment)	Energy (cytosolic)	Up	3.35	1	3
1009	D2WJ88	H.f.	Putative enolase (Fragment)		Up	2.22	3	3
712	O76515	H.z.	Fatty acid-binding protein		Up	3.95	3	3
1011	C9D996	M.p.	Alanyl-tRNA synthetase (Fragment)	Protein Metabolism	Down	0	0	3
334	A7KCX9	H.e.	Heat shock protein hsp21.4		Down	0	0	3
79	C8CCR4	H.z.	Heat shock protein 70 cognate		Down	0	0	2
1002	C7ED93	C.s.	Heat shock protein 60		Down	0	0	3
379	A2I3X7	M.h.	Puatative Tubulin beta-1 chain	Cytoskeleton	Down	0.14	3	3
137	D2WJC4	S.p.	Putative enolase (Fragment)	Energy (cytosolic)	Down	0	0	2
426	Q2F6A8	B.m.	Enoyl-CoA hydratase 3		Down	0	0	2
673	B6CMF8	H.a.	Fatty acid-binding protein 1		Down	0	0	3
723	B6CMG0	H.a.	Fatty acid-binding protein 3		Down	0.47	3	3
393	D2SNS6	H.v.	Aldehyde dehydrogenase (Fragment)	Detoxification	Down	0	0	3

a) B.m.: Bombyx mori, C.s.: Chilo suppressalis, D.c.: Diaphorina citri, G.m.m.: Glossina morsitans morsitans, H.a.: Helicoverpa armigera, H.e.: Heliconius erato, H.f.: Hyblaea firmamentum, H.s.: Hypena scabra, H.v.: Heliothis virescens, H.z.: Heliothis zea, M.h.: Maconellicoccus hirsutus, M.p.: Microchorista philpotti, M.s.: Manduca sexta, N.l.: Neffapis longilingua, S.f.: Spodoptera frugiperda, S.p.: Synemon plana

b) From EST analysis with WU-BLAST.

Table 3.9. (continued)

c) Spot numbers as in 2D reference gel (see **Figure 3.1**).

d) Fold change was the ratio between average normalized volumes of each protein in Adana population that of the corresponding spot in control gel. If that ratio higher than 2 fold, it was accepted as an up-regulation. But, if it was lower than 0.5 fold, it was accepted as a down-regulation. In addition, if differentially expressed spot contained more than one protein, it was not included in **table 3.9**.

e) Number of spot is indicating that number of presence of that protein in three replica gels for that population.

3.2.3. Differentially Expressed Proteins in Mardin Population

Differential proteome results with Mardin field populations (M) gave similar protein expression pattern as were obtained with the Adana field population (A), especially for energy- and protein metabolism-related as well as cytoskeleton-related proteins (**Table 3.10**). Likewise, up-regulated energy metabolism-related proteins consisted of mitochondrial proteins (e.g. ATP synthase and V ATPase A/B, see in **Table 3.10**), cytosolic proteins (e.g. enolase, fatty acid binding proteins and triosephosphate isomerase), and kinase/phosphatase proteins (e.g. arginine kinase). There were also up-regulated protein spots that contained protein metabolism (e.g. heat shock proteins, glutamate dehydrogenase and eukaryotic translation initiation factor 4A) and cytoskeletal system (e.g. tubulin and tropomyosin) proteins in the Mardin population.

Table 3.10. List of Differentially Expressed Proteins in Mardin Population

Spot no. [c)]	Access. no.	Organism [a)]	Protein name	Group	Regulation in Mardin	Fold Change [d)]	No. of Spots in Mardin [e)]	No. of Spots in Control [e)]
61	D9ILX6	H.z.	ATP synthase	Energy (mitochondrial)	Up	∞	3	0
293	D9ILX6	H.z.	ATP synthase		Up	∞	3	0
311	D9ILX6	H.z.	ATP synthase		Up	∞	3	0
470	D9ILX6	H.z.	ATP synthase		Up	2.79	3	3
1004	D9ILX6	H.z.	ATP synthase		Up	∞	3	0
1013	D9ILX6	H.z.	ATP synthase		Up	∞	3	0
445	E2IV52	H.a.	V ATPase B (Fragment)		Up	2.34	3	1
125	E2IV54	H.a.	V ATPase A (Fragment)		Up	2.93	3	2
339	E2IV54	H.a.	V ATPase A (Fragment)		Up	8.35	3	3
1007	E2IV54	H.a.	V ATPase A (Fragment)		Up	∞	3	0
1008	E2IV54	H.a.	V ATPase A (Fragment)		Up	∞	3	0
550	VATB	H.v.	V-type proton ATPase subunit B		Up	21.12	3	2
1005	D2WJB6	H.s.	Putative enolase (Fragment)	Energy (cytosolic)	Up	2.84	3	3
1006	A1YQ87	B.m.	Enolase		Up	∞	3	0
1019	Q5XUN5	H.a.	Triosephosphate isomerase		Up	2.51	3	1
712	O76515	H.z.	Fatty acid-binding protein		Up	3.49	3	3
1000	B6CMF8	H.a.	Fatty acid-binding protein 1		Up	2,87	3	3
246	D3Y4D1	H.a.	Arginine Kinase	Kinase/ Phosphatase	Up	2.42	3	3
278	B1NLE2	H.a.	Arginine Kinase		Up	∞	3	0
394	D3Y4D1	H.a.	Arginine kinase		Up	2.07	3	3
598	B1NLE2	H.a.	Arginine kinase		Up	2.30	3	3
1016	B1NLE2	H.a.	Arginine kinase		Up	∞	3	0
247	Q2F5R4	B.m	Glutamate dehydrogenase	Protein Metabolism	Up	2.66	3	3
349 [b)]	D3TSE2	G.m.m.	FKBP-type peptidyl-prolyl cis-trans isomerase		Up	8.50	3	2

Table 3.10. (continued)

475	Q285R3	B.m.	Eukaryotic translation initiation factor 4A		Up	2.98	3	3
606	Q8WQJ0	S.f.	60S acidic ribosomal protein P2		Up	∞	3	0
92	C7SIR9	H.a.	Heat shock protein 70		Up	∞	3	0
321	C8CCR4	H.a.	Heat shock protein 70 cognate		Up	2.79	3	3
1014	C0KJJ4	S.e.	Heat shock protein 70		Up	∞	2	0
1001	C7ED93	C.s.	Heat shock protein 60		Up	2.95	3	3
118	TBB1	M.s.	Tubulin beta-1 chain	Cytoskeleton	Up	∞	3	0
379	A2I3X7	M.h.	Puatative Tubulin beta-1 chain		Up	2.34	3	3
383	TBB1	M.s.	Tubulin beta-1 chain		Up	3.95	2	2
441 [b)]	Q1HPK5	B.m.	Thymosin isoform 1		Up	∞	3	0
492	TPM1	B.m.	Tropomyosin-1		Up	2.06	3	3
498	D5LTC5	H.a.	Translational controlled tumor protein	Unknown	Up	3.51	3	3
231	E0A3A7	H.a.	NADPH cytochrome P450 reductase	Detoxification	Up	2.52	3	3
413	B2KSE9	H.a.	Thioredoxin peroxidase		Up	∞	2	0
616	D9ILX6	H.z.	ATP synthase	Energy (mitochondrial)	Down	0.46	3	3
592 [b)]	Q2F6C1	B.m.	Cytidine deaminase	Nucleotide Metabolsim	Down	0.40	3	3
426	Q2F6A8	B.m.	Enoyl-CoA hydratase 3	Energy (cytosolic)	Down	0	0	2
636	B6CMF8	H.a.	Fatty acid-binding protein 1		Down	0.41	3	3
673	B6CMF8	H.a.	Fatty acid-binding protein 1		Down	0	0	3
1018	B6CMF9	H.a.	Fatty acid-binding protein 2		Down	0	0	2
1011	C9D996	M.p.	Alanyl-tRNA synthetase (Fragment)	Protein Metabolism	Down	0	0	3

Table 3.10. (continued)

334	A7KCX9	H.e.	Heat shock protein hsp21.4		Down	0	0	3
1010	Q8I866	S.f.	Heat shock cognate 70 protein		Down	0.28	3	3
337	C4P7G5	B.ma.	Myosin heavy chain (Fragment)	Cytoskeleton	Down	0.39	3	3
393	D2SNS6	H.v.	Aldehyde dehydrogenase (Fragment)	Detoxification	Down	0	0	3
623	A0MSN0	H.a.	Glutathione S-transferase		Down	0,27	3	3
1012	B2KSE9	H.a.	Thioredoxin peroxidase		Down	0	0	3

a) B.m.: Bombyx mori B.ma.: Bombyx mandarina C.s.: Chilo suppressalis G.m.m.: Glossina morsitans morsitans
H.a.: Helicoverpa armigera H.e.: Heliconius erato H.f.: Hyblaea firmamentum H.s.: Hypena scabra
H.v.: Heliothis virescens H.z.: Heliothis zea M.h.: Maconellicoccus hirsutus M.p.: Microchorista philpotti
M.s.: Manduca sexta S.e.: Spodoptera exigua S.f.: Spodoptera frugiperda

b) From EST analysis with WU-BLAST.

c) Spot numbers as in 2D reference gel (see **Figure 3.1**).

d) Fold change was the ratio between average normalized volumes of each protein in Adana population that of the corresponding spot in control gel. If that ratio higher than 2 fold, it was accepted as an up-regulation. But, if it was lower than 0.5 fold, it was accepted as a down-regulation. In addition, if differentially expressed spot contained more than one protein, it was not included in **table 3.10**.

e) Number of spot is indicating that number of presence of that protein in three replica gels for that population.

In contrast to the differential protein expression analysis with the Adana field population (A), in the Mardin population (M) we found five differentially expressed detoxification proteins. While aldehyde dehydrogenase, glutathione S-transferases and one spot of thioredoxin peroxidase (spot 1012, probably incomplete form) were down-regulated, another spot of thioredoxin peroxidase (spot 413) and NADPH cytochrome P450 reductase were up-regulated. For example, up-regulated NADPH cytochrome P450 reductase, labelled as a spot 231, were detected in all gels of the Mardin population (**Figure 3.13**). In addition, this protein was also detected in all gels of the susceptible population (CL). In addition, translationally controlled tumor protein (spot 498) was up-regulated, as well.

In the Mardin population (M) most of the identified down regulated proteins were related to protein metabolism (e.g. heat shock proteins and alanyl-tRNA synthetase) and to cytosolic energy proteins (e.g. enoyl CoA-hydratase 3 and fatty acid binding proteins). Cytidine deaminase (spot 592), ATP synthase (spot 616) and myosin heavy chain (spot 337) proteins were down-regulated, as well. In addition, there were also down-regulated detoxification system associated proteins such as aldehyde dehydrogenase (spot 393), glutathione S-transferases (spot 623), and thioredoxin peroxidase, incomplete form, (spot 1012) in the Mardin population (M).

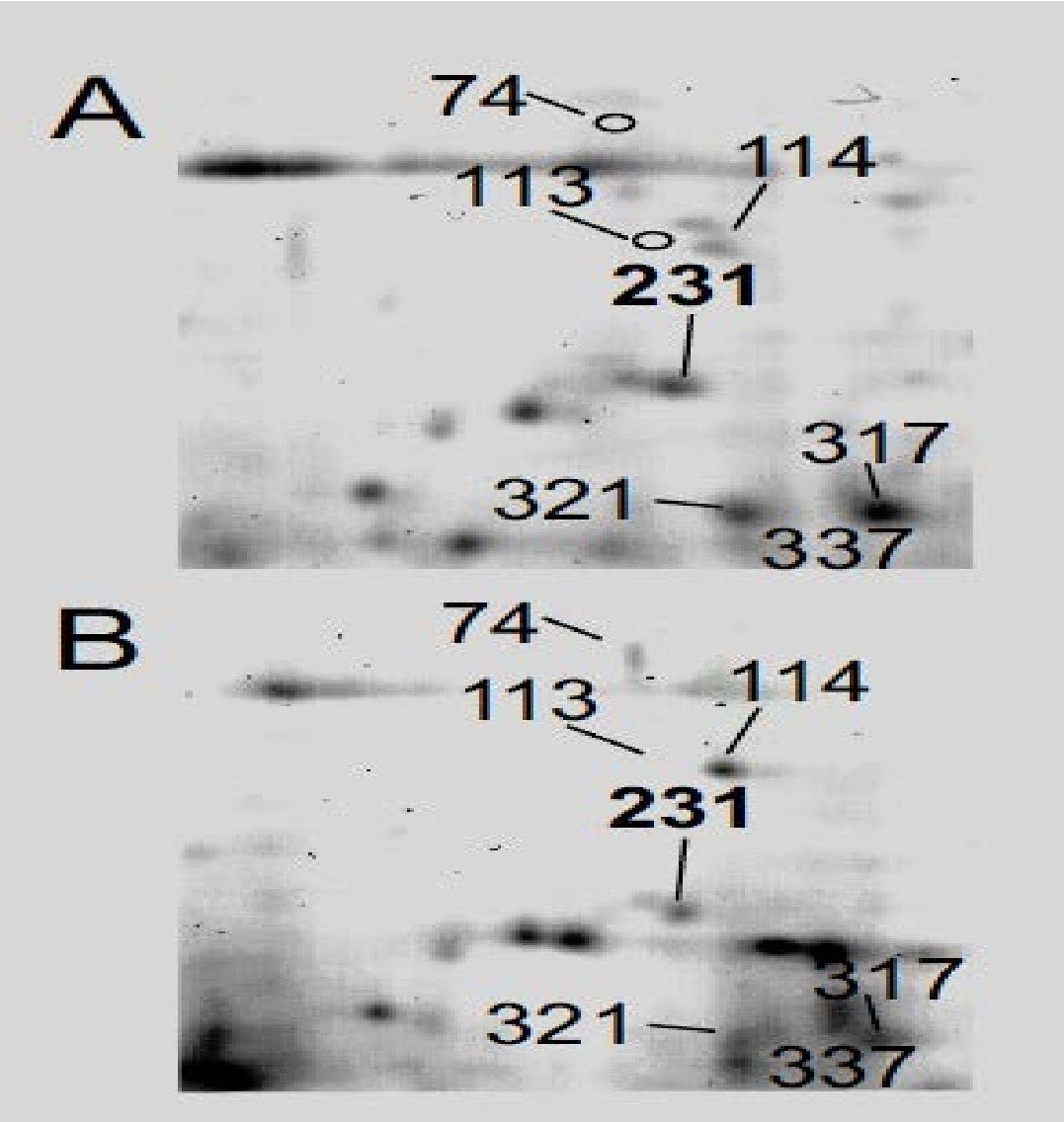


Figure 3.13. Differential protein expression analysis with zoomed view of a gel region showing an up-regulated spot of NADPH Cytochrome P450 reductase; **A:** Mardin; **B:** Control. Up-regulated NADPH Cytochrome P450 reductase protein is found in spot 231 (see **Table 3.10**).

3.3. Real-Time PCR Results

In order to analyse transcriptional level differences of certain genes from three main detoxification enzyme systems, real-time PCR (*q*RT-PCR) analysis were done with *H. armigera* samples. Therefore, it was planned to analyze individual expression levels of five suspected CYP450 genes from 3 main CYP450 gene families (CYP4S1, CYP6B6, CYP6B7, CYP9A12 and CYP9A14) together with two different GST genes (GST and GSTX01) and one esterase gene (ESTX018) for better understanding these systems in resistant *H. armigera* samples. Furthermore, elongation factor-1alpha (EF-1alpha) gene was also analyzed in *H. armigera* samples by *q*RT-PCR to normalize *q*RT-PCR data. Consequently, real-time PCR data were analyzed with Relative Expression Software Tool 2008 for comparison of susceptible and field populations.

Unfortunately, the CYP450 enzyme systems role could not be analyzed with biochemical assays. The main reason was the huge amount of individual sample requirement for each assay. Typical CYP450 assays require a lot of midguts (approximately, about 50 individual midguts for one assay duplicate measurement). Furthermore, grouping midgut samples may cause for masking the ones, showing increase in enzyme activity. Thus, it was decided not to make this type assay for analyzing CYP450s in protein level. Instead of analyzing protein level of midgut sample groups, this enzyme system was analyzed at mRNA level for individual midgut samples with *q*RT-PCR technique.

In addition, *q*RT-PCR analysis for all analyzed genes initially was done with 10 samples from each population. Nonetheless, if up regulation of any gene was detected in field population, analyzed sample number in that population increased up to 20 for ensuring up-regulation in that population.

3.3.1. Elongation Factor-1Alpha Gene Real-Time PCR Results of Susceptible and Field Populations of *Helicoverpa armigera*

In order to determine C_T values for elongation factor-1alpha gene (EF-1alpha), one of the samples was chosen from susceptible population as a reference sample. From this reference sample cDNA serial dilutions were prepared as; no dilution, 1/10, 1/50, 1/100, 1/250 and 1/1000. Then, concentrations of these dilutions were assigned arbitrarily as; 100000, 10000, 2000, 1000, 400 and 100, reciprocally. After that, it was tried to obtained C_T versus Log concentration curves for EF-1alpha gene with its slopes in a range of – 3.6 > slope > – 3.1 (**Figure 3.14**). C_T values of EF-1alpha gene in all populations were determined with this standard curve (**Table 3.11**). Average C_T values were mean of the triplicate measurements of that sample. These C_T values used for normalization of detoxification systems genes data.

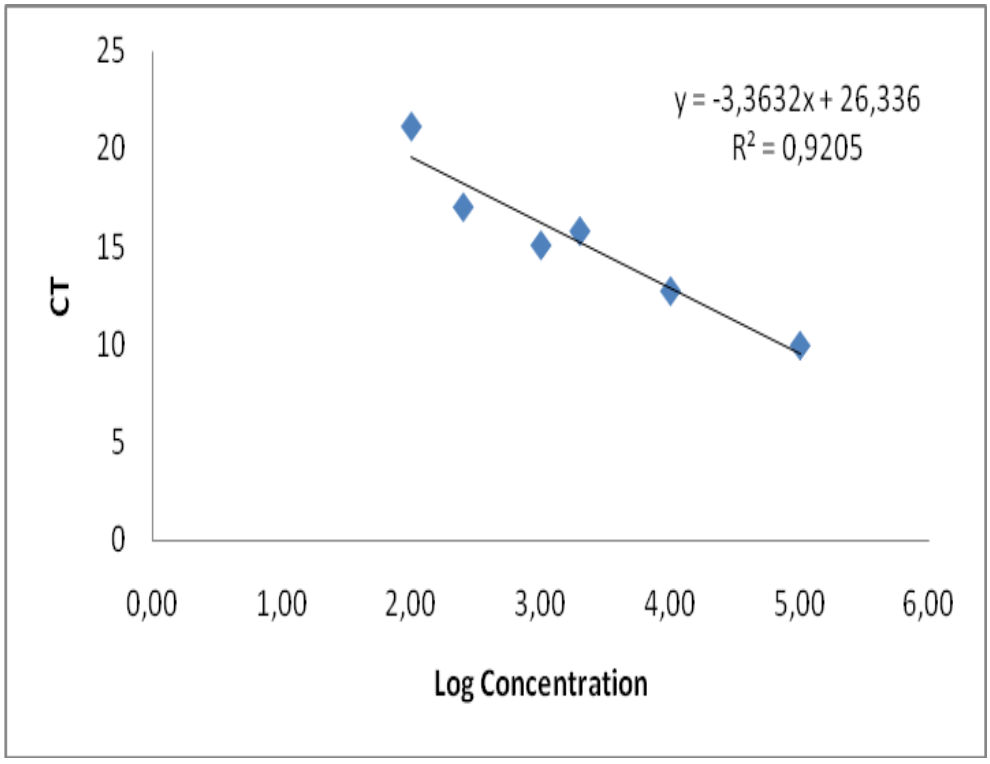


Figure 3.14. EF-1Alpha Gene Standard Curve.

Table 3.11. EF-1Alpha Gene Real-Time PCR Results of *H. armigera* Populations

Sample No	Susceptible Average C_T	Adana Average C_T	Çanakkale Average C_T	Mardin Average C_T
1	14.59	24.67	15.58	13.15
2	14.86	24.24	13.59	12.41
3	17.77	26.38	11.59	12.11
4	15.36	24.94	10.78	12.31
5	14.72	23.21	10.35	13.14
6	13.85	22.79	11.62	11.96
7	15.47	19.46	11.43	11.57
8	13.70	21.30	12.19	11.12
9	15.04	19.05	12.63	11.12
10	13.79	29.44	11.65	11.65
11		22.41	17.2	17.91
12		28.81	16.51	15.98
13			17.25	14.83
14			15.95	16.32
15			15.89	14.88
16			16.42	15.26
17			17.38	15.92
18			15.97	16.58
19			15.01	17.90
20			17.05	20.80

3.3.2. CYP4S1 Gene Real-Time PCR Results of Susceptible and Field Populations of *Helicoverpa armigera*

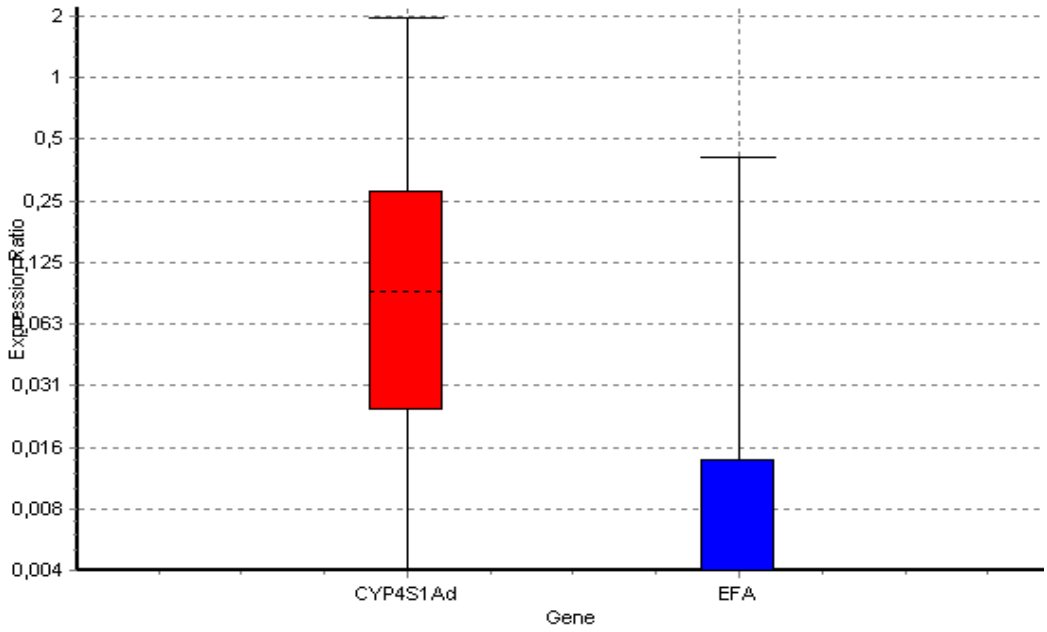
C_T values of CYP4S1 gene were determined according to the its standard curve, prepared similarly as already described in chapter III, part 3.3.1, shown at **table 3.12**. After that, these results were analyzed with REST 2008 for analyzing CYP4S1 gene expression in field populations of *H. armigera*. REST 2008 results for Adana, Çanakkale and Mardin populations were shown in the following figures (**Figure 3.15, Figure 3.16** and **Figure 3.17)** and tables (**Table 3.13**, **Table 3.14** and **Table 3.15**).

3.3.3. CYP6B6 Gene Real-Time PCR Results of Susceptible and Field Populations of *Helicoverpa armigera*

Real-time PCR analysis of CYP6B6 gene was planned to search with primer couple of Grubor *et al.*, (2007). Nonetheless, CYP6B6 gene primer couple was not worked in all analyzed populations so its expression changes in field populations could not be analyzed in this work.

Table 3.12. CYP4S1 Gene Real-Time PCR Results of *H. armigera* Populations

Sample No	Susceptible Average C_T	Adana Average C_T	Çanakkale Average C_T	Mardin Average C_T
1	26.07	27.02	21.19	21.15
2	25.71	30.12	24.61	24.85
3	26.86	28.75	29.03	26.27
4	22.63	29.71	22.07	22.31
5	22.42	27.93	29.44	19.80
6	24.21	27.41	24.79	17.37
7	24.33	25.90	29.76	20.04
8	25.44	26.73	26.61	25.78
9	23.15	25.91	25.24	19.95
10	24.09	33.16	23.57	25.73
11				21.97
12				25.01
13				20.73
14				25.48
15				16.54
16				20.69
17				24.54
18				27.45
19				18.75
20				22.37



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

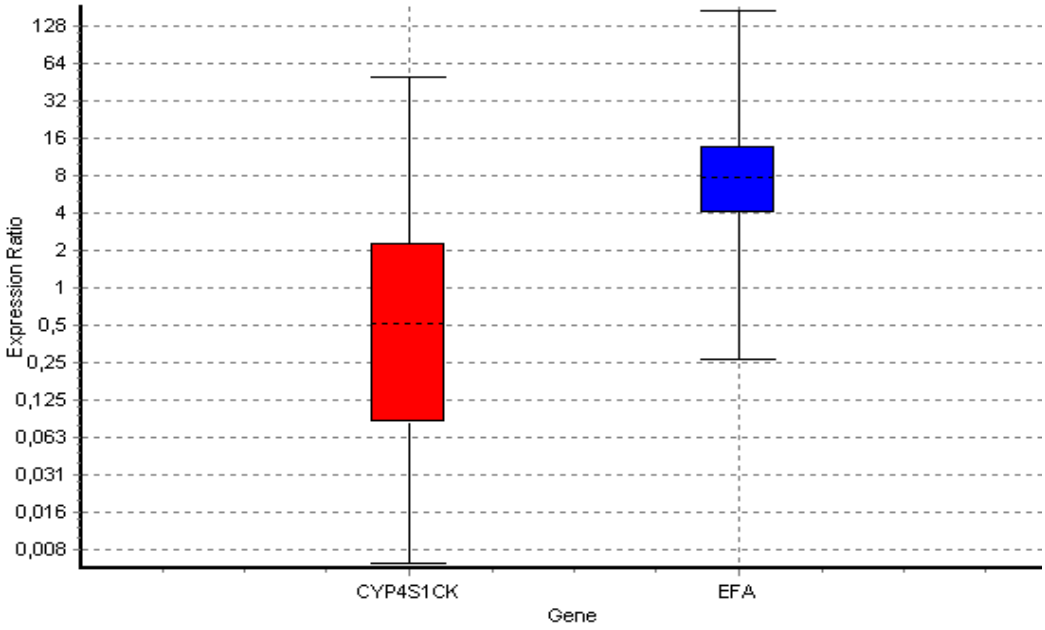
Figure 3.15. CYP4S1 Gene Whisker Box Plot of Adana Population

Table 3.13. REST 2008 Results of Adana Population for CYP4S1 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
CYP4S1 Adana	Target Gene	0.073	0.001 – 1.121	0.001	DOWN

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

CYP4S1 gene expression was 0.073 fold down-regulated in Adana population in comparison to susceptible population (P(H1)<0.05).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

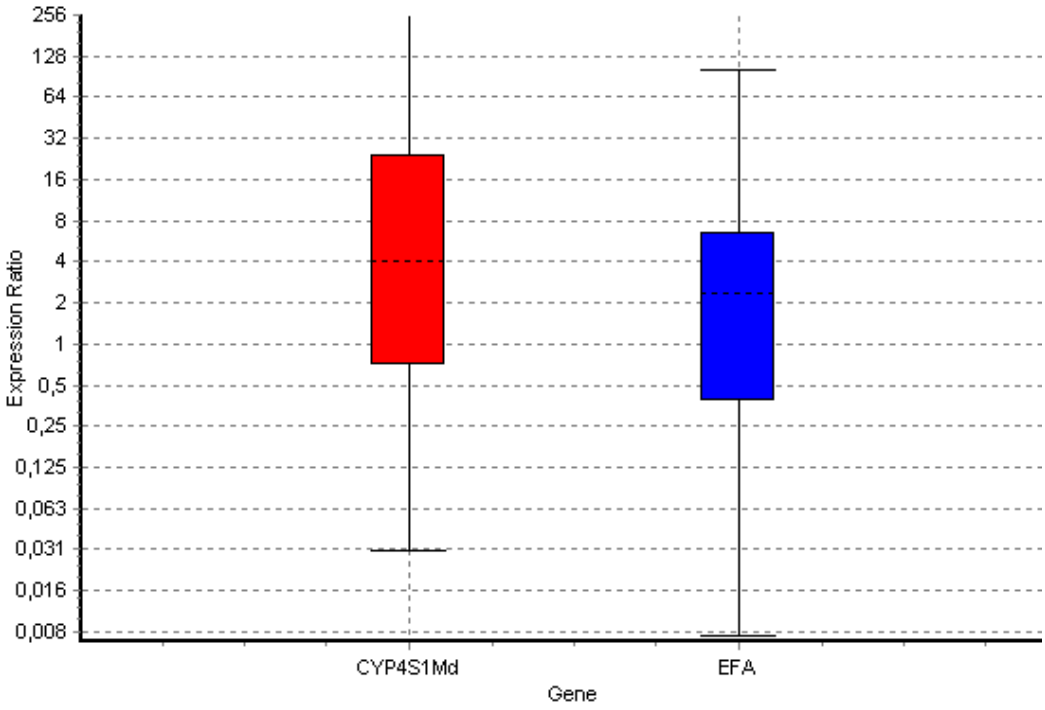
Figure 3.16. CYP4S1 Gene Whisker Box Plot of Çanakkale Population

Table 3.14. REST 2008 Results of Çanakkale Population for CYP4S1 Gene

Gene	**Type**	**Expression Ratio**	**95% Confidence Interval**	**P(H1)**	**Regulation**
CYP4S1 Çanakkale	Target Gene	0.454	0.008 – 25.422	0.284	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

CYP4S1 gene expression was not different in Çanakkale population in comparison to susceptible population (P(H1)= 0.284).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Figure 3.17. CYP4S1 Gene Whisker Box Plot of Mardin Population

Table 3.15. REST 2008 Results of Mardin Population for CYP4S1 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
CYP4S1 Mardin	Target Gene	4.491	0.097 – 426.975	0.047	UP

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

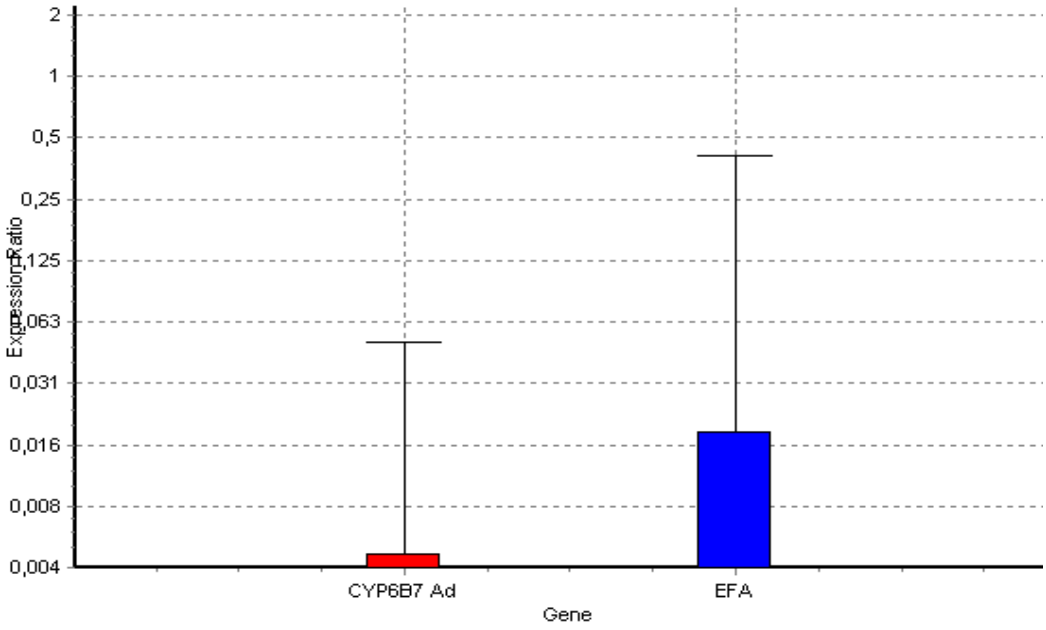
CYP4S1 gene expression was 4.491 fold up-regulated in Mardin population in comparison to susceptible population (P(H1)<0.05).

3.3.4. CYP6B7 Gene Real-Time PCR Results of Susceptible and Field Populations of *Helicoverpa armigera*

C_T values of CYP6B7 gene were determined according to its standard curve, prepared similarly as already described in chapter III, part 3.3.1, shown at **table 3.16**. After that, these results were analyzed with REST 2008 for analyzing CYP6B7 gene expression in field populations of *H. armigera*. REST 2008 results for Adana, Çanakkale and Mardin populations were shown in the following figures (**Figure 3.18, Figure 3.19** and **Figure 3.20)** and tables (**Table 3.17, Table 3.18** and **Table 3.19**).

Table 3.16. CYP6B7 Gene Real-Time PCR Results of *H. armigera* Populations

Sample No	Susceptible Average C_T	Adana Average C_T	Çanakkale Average C_T	Mardin Average C_T
1	24.32	31.51	22.28	22.68
2	22.76	29.38	22.18	27.97
3	24.48	31.99	23.12	23.40
4	20.17	30.77	20.76	24.11
5	20.34	31.21	22.77	23.83
6	17.13	30.44	25.86	21.69
7	18.46	29.91	21.07	21.63
8	19.71	30.35	21.74	19.42
9	20.17	28.80	20.64	18.89
10	20.39	----	17.91	21.09



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

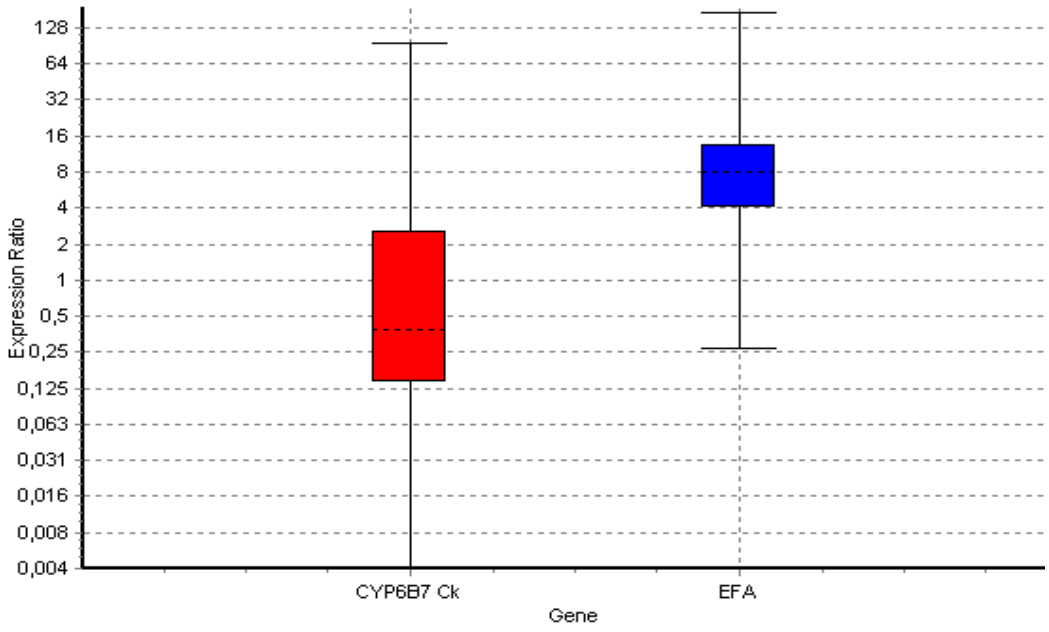
Figure 3.18. CYP6B7 Gene Whisker Box Plot of Adana Population

Table 3.17. REST 2008 Results of Adana Population for CYP6B7 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
CYP6B7 Adana	Target Gene	0.001	0.000 - 0.033	0.000	DOWN

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

CYP6B7 gene expression was 0.001 fold down-regulated in Adana population in comparison to susceptible population (P(H1)<0.05).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

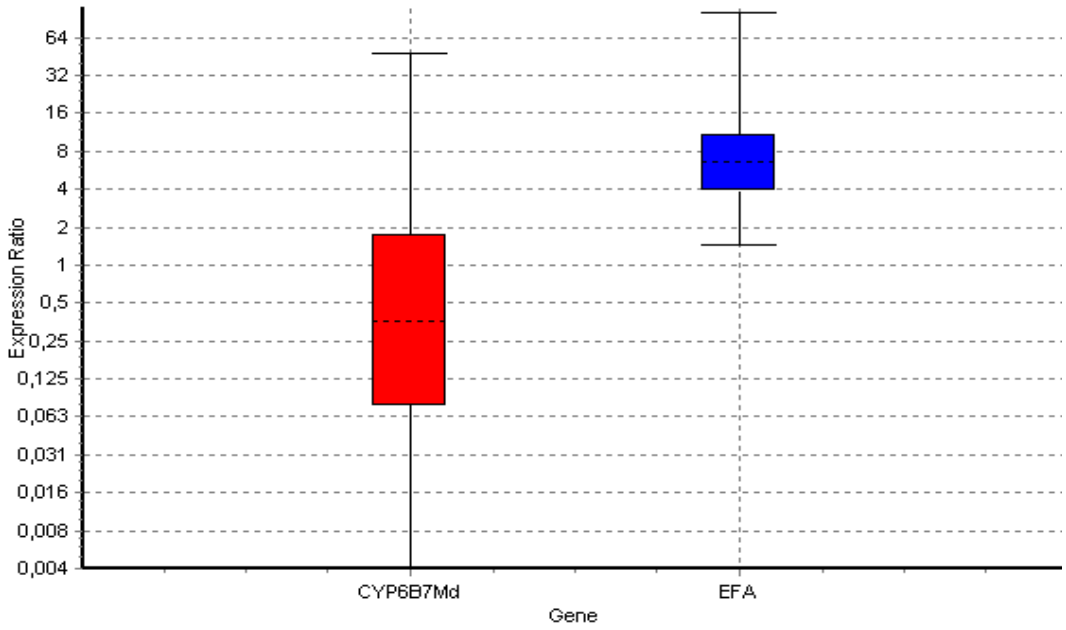
Figure 3.19. CYP6B7 Gene Whisker Box Plot of Çanakkale Population

Table 3.18. REST 2008 Results of Çanakkale Population for CYP6B7 Gene

Gene	**Type**	**Expression Ratio**	**95% Confidence Interval**	**P(H1)**	**Regulation**
CYP6B7 Çanakkale	Target Gene	0.486	0.015 - 21.943	0.303	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

CYP6B7 gene expression was not different in Çanakkale population in comparison to susceptible population (P(H1)= 0.303).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Figure 3.20. CYP6B7 Gene Whisker Box Plot of Mardin Population

Table 3.19. REST 2008 Results of Mardin Population for CYP6B7 Gene

Gene	**Type**	**Expression Ratio**	**95% Confidence Interval**	**P(H1)**	**Regulation**
CYP6B7 Mardin	Target Gene	0.313	0.004 – 31.696	0.153	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

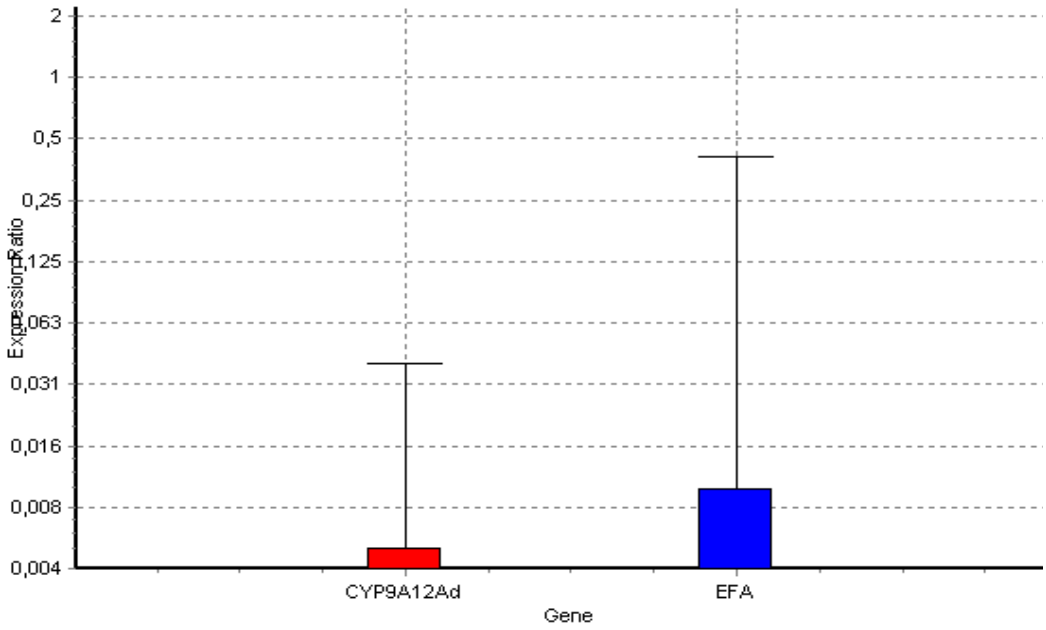
CYP6B7 gene expression was not different in Mardin population in comparison to susceptible population (P(H1)= 0.153).

3.3.5. CYP9A12 Gene Real-Time PCR Results of Susceptible and Field Populations of *Helicoverpa armigera*

C_T values of CYP9A12 gene were determined according to its standard curve, prepared similarly as already described in chapter III, part 3.3.1, shown at **table 3.20**. After that, these results were analyzed with REST 2008 for analyzing CYP9A12 gene expression in field populations of *H. armigera*. REST 2008 results for Adana, Çanakkale and Mardin populations were shown at following figures (**Figure 3.21, Figure 3.22** and **Figure 3.23)** and tables (**Table 3.21**, **Table 3.22** and **Table 3.23**).

Table 3.20. CYP9A12 Gene Real-Time PCR Results of *H. armigera* Populations

Sample No	Susceptible Average C_T	Adana Average C_T	Çanakkale Average C_T	Mardin Average C_T
1	20.71	27.24	17.07	14.95
2	18.84	29.25	13.86	20.02
3	20.76	26.51	13.90	16.34
4	19.49	30.38	13.79	15.61
5	15.47	28.77	17.88	15.62
6	13.17	27.74	19.78	19.33
7	15.55	26.35	13.57	17.39
8	17.09	27.61	15.16	12.09
9	20.17	25.42	17.79	12.85
10	19.77	30.37	15.38	17.07
11		27.57	15.82	17.32
12		28.85	17.36	16.16
13			17.62	14.62
14			17.98	16.63
15			12.52	15.63
16			15.17	15.26
17			16.92	16.43
18			12.51	17.36
19			14.68	16.22
20			18.45	20.05



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

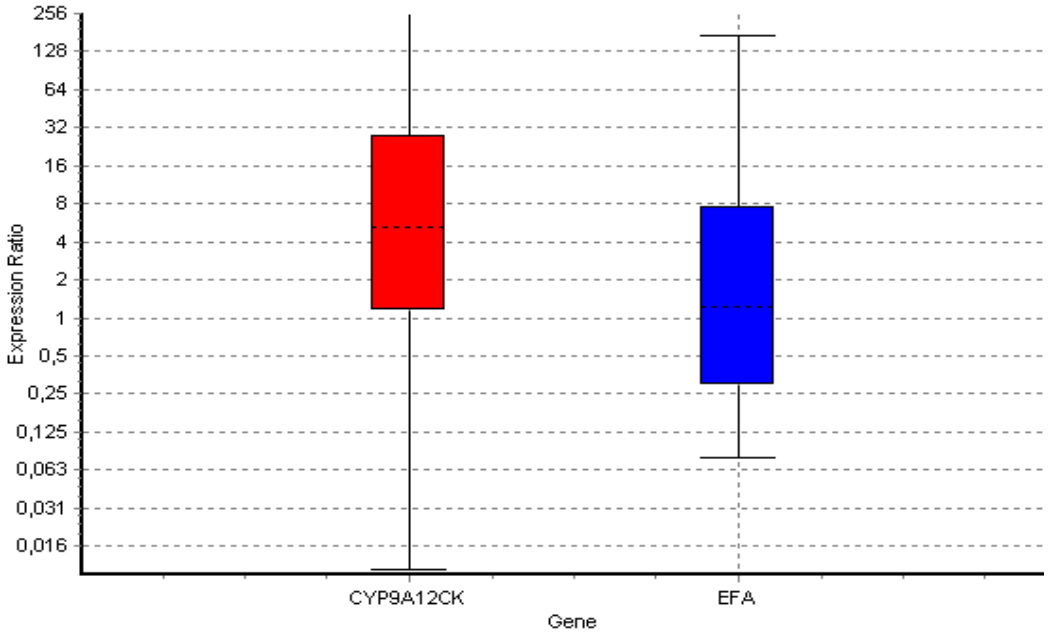
Figure 3.21. CYP9A12 Gene Whisker Box Plot of Adana Population

Table 3.21. REST 2008 Results of Adana Population for CYP9A12 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
CYP9A12 Adana	Target Gene	0.001	0.000 – 0.021	0.000	DOWN

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

CYP9A12 gene expression was 0.001 fold down-regulated in Adana population in comparison to susceptible population (P(H1)<0.05).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

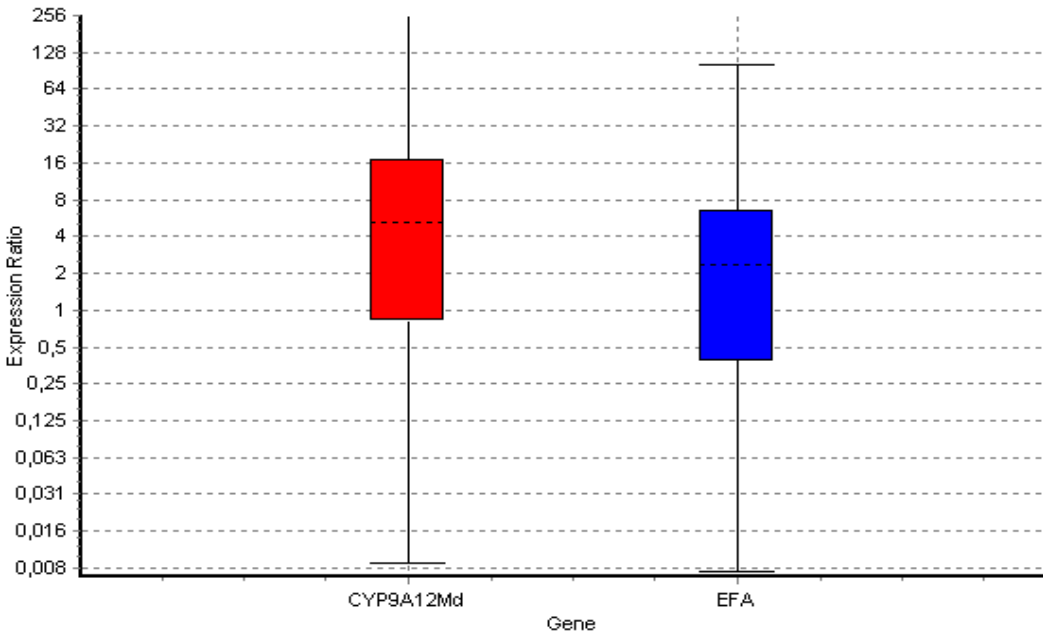
Figure 3.22. CYP9A12 Gene Whisker Box Plot of Çanakkale Population

Table 3.22. REST 2008 Results of Çanakkale Population for CYP9A12 Gene

Gene	**Type**	**Expression Ratio**	**95% Confidence Interval**	**P(H1)**	**Regulation**
CYP9A12 Çanakkale	Target Gene	4.729	0.046 – 200.888	0.019	UP

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

CYP9A12 gene expression was 4.729 fold up-regulated in Çanakkale population in comparison to susceptible population (P(H1)<0.05) .



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Figure 3.23. CYP9A12 Gene Whisker Box Plot of Mardin Population

Table 3.23. REST 2008 Results of Mardin Population for CYP9A12Gene

Gene	**Type**	**Expression Ratio**	**95% Confidence Interval**	**P(H1)**	**Regulation**
CYP9A12 Mardin	Target Gene	3.374	0.044 – 205.755	0.046	UP

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

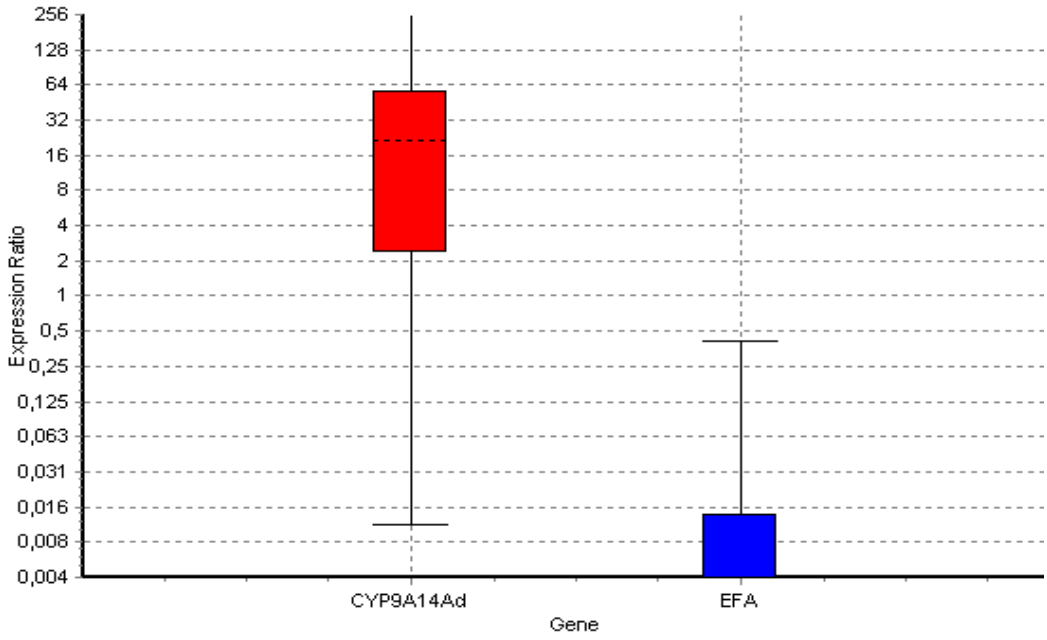
CYP9A12 gene expression was 3.374 fold up-regulated in Mardin population in comparison to susceptible population (P(H1)<0.05) .

3.3.6. CYP9A14 Gene Real-Time PCR Results of Susceptible and Field Populations of *Helicoverpa armigera*

C_T values of CYP9A14 gene were determined according to its standard curve, prepared similarly as already described in chapter III, part 3.3.1, shown at **table 3.24**. After that, these results were analyzed with REST 2008 for analyzing CYP9A12 gene expression in field populations of *H. armigera*. REST 2008 results for Adana, Çanakkale and Mardin populations were shown at following figures (**Figure 3.24, Figure 3.25** and **Figure 3.26)** and tables (**Table 3.25**, **Table 3.26** and **Table 3.27**).

Table 3.24. CYP9A14 Gene Real-Time PCR Results of *H. armigera* Populations

Sample No	Susceptible Average C_T	Adana Average C_T	Çanakkale Average C_T	Mardin Average C_T
1	26.76	20.65	18.23	17.30
2	28.18	24.37	20.45	20.72
3	30.57	23.67	20.14	19.24
4	29.52	27.96	16.59	16.89
5	28.67	21.30	21.97	16.51
6	22.52	24.17	21.29	20.05
7	21.48	24.92	19.73	15.95
8	29.20	21.96	17.84	17.44
9	25.75	23.53	20.01	18.11
10	29.28	24.67	19.70	18.31
11		22.75	20.37	17.61
12		26.38	24.06	18.74
13			18.58	15.13
14			21.04	16.56
15			19.14	15.82
16			19.29	14.20
17			19.58	17.59
18			19.38	19.98
19			20.43	16.42
20			20.08	15.85



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

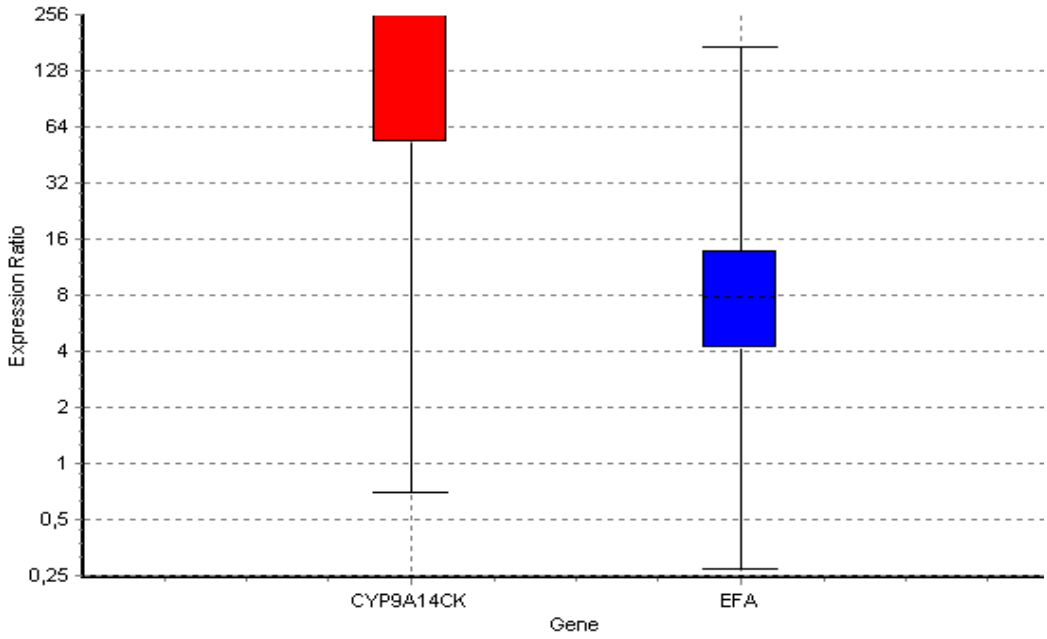
Figure 3.24. CYP9A14 Gene Whisker Box Plot of Adana Population

Table 3.25. REST 2008 Results of Adana Population for CYP9A14 Gene

Gene	**Type**	**Expression Ratio**	**95% Confidence Interval**	**P(H1)**	**Regulation**
CYP9A14 Adana	Target Gene	11.104	0.100 – 433.822	0.011	UP

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

CYP9A14 gene expression was 11.104 fold up-regulated in Adana population in comparison to susceptible population (P(H1)<0.05).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

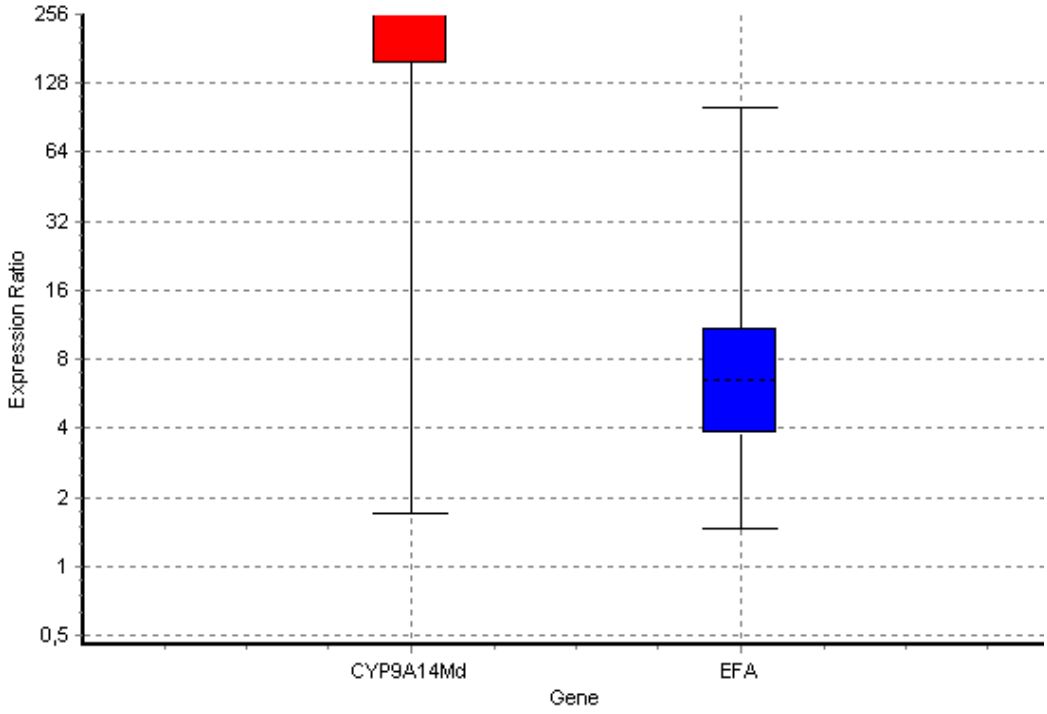
Figure 3.25. CYP9A14 Gene Whisker Box Plot of Çanakkale Population

Table 3.26. REST 2008 Results of Çanakkale Population for CYP9A14 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
CYP9A14 Çanakkale	Target Gene	193.743	1.739 - 6.705.542	0.000	UP

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

CYP9A14 gene expression was 193.743 fold up-regulated in Çanakkale population in comparison to susceptible population (P(H1)<0.05).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Figure 3.26. CYP9A14 Gene Whisker Box Plot of Mardin Population

Table 3.27. REST 2008 Results of Mardin Population for CYP9A14 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
CYP9A14 Mardin	Target Gene	564.567	4.072 - 12.667.061	0.000	UP

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

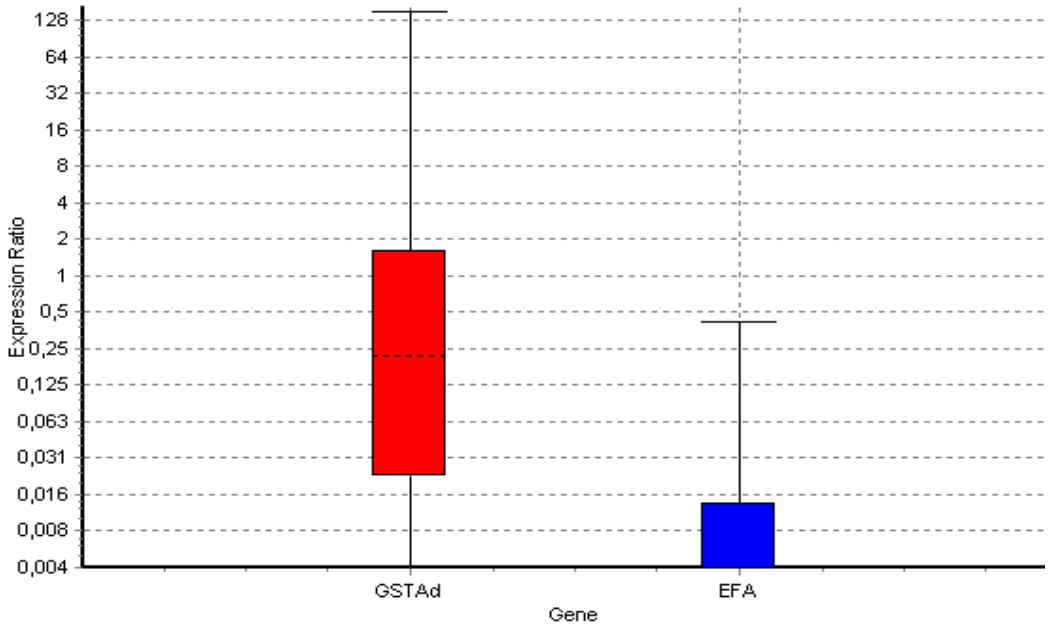
CYP9A14 gene expression was 564.567 fold up-regulated in Mardin population in comparison to susceptible population (P(H1)<0.05).

3.3.7. GST (isozyme type not defined) Gene Real-Time PCR Results of Susceptible and Field Populations of *Helicoverpa armigera*

C_T values of GST (isozyme type not defined) gene were determined according to its standard curve, prepared similarly as already described in chapter III, part 3.3.1, shown at **table 3.28**. After that, these results were analyzed with REST 2008 for analyzing GST (isozyme type not defined) gene expression in field populations of *H. armigera*. REST 2008 results for Adana, Çanakkale and Mardin populations were shown at following figures (**Figure 3.27, Figure 3.28** and **Figure 3.29)** and tables (**Table 3.29**, **Table 3.30** and **Table 3.31**).

Table 3.28. GST (isozyme type not defined) Gene Real-Time PCR Results of *H. armigera* Populations

Sample No	Susceptible Average C_T	Adana Average C_T	Çanakkale Average C_T	Mardin Average C_T
1	29.47	22.93	23.77	27.74
2	27.73	25.54	29.95	26.86
3	30.18	28.96	26.27	27.95
4	24.04	28.60	21.56	20.44
5	20.00	27.74	28.61	21.73
6	23.15	29.75	30.19	22.32
7	22.44	23.29	28.02	22.21
8	22.09	26.44	28.63	27.68
9	23.85	23.23	20.05	24.56
10	22.13	28.74	27.72	22.13



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

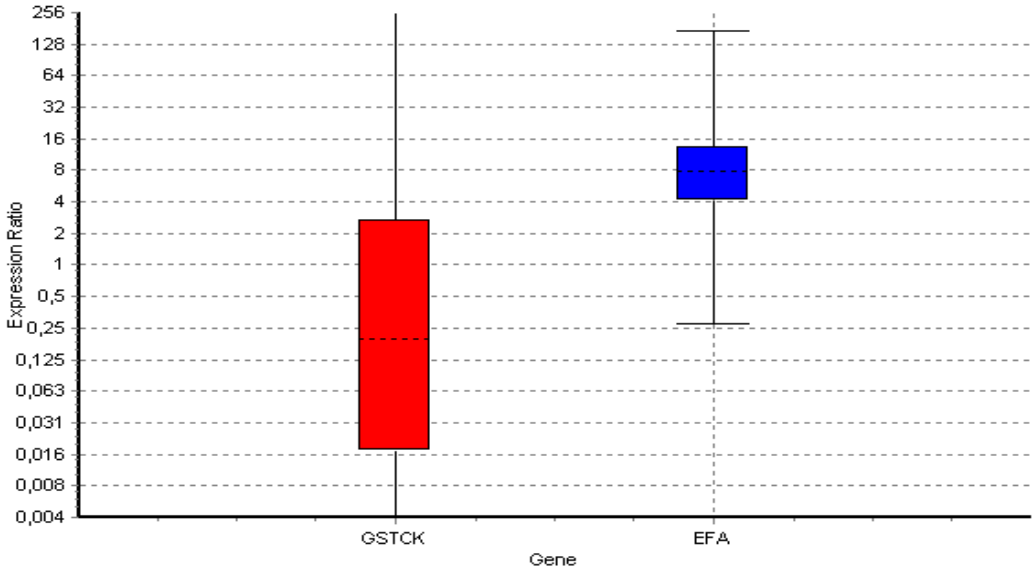
Figure 3.27. GST (isozyme type not defined) Gene Whisker Box Plot of Adana Population

Table 3.29. REST 2008 Results of Adana Population for GST (isozyme type not defined) Gene

Gene	**Type**	**Expression Ratio**	**95% Confidence Interval**	**P(H1)**	**Regulation**
GST Adana	Target Gene	0.248	0.002 – 106.467	0.160	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

GST (isozyme type not defined) gene expression was not different in Adana population in comparison to susceptible population (P(H1)= 0.160).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

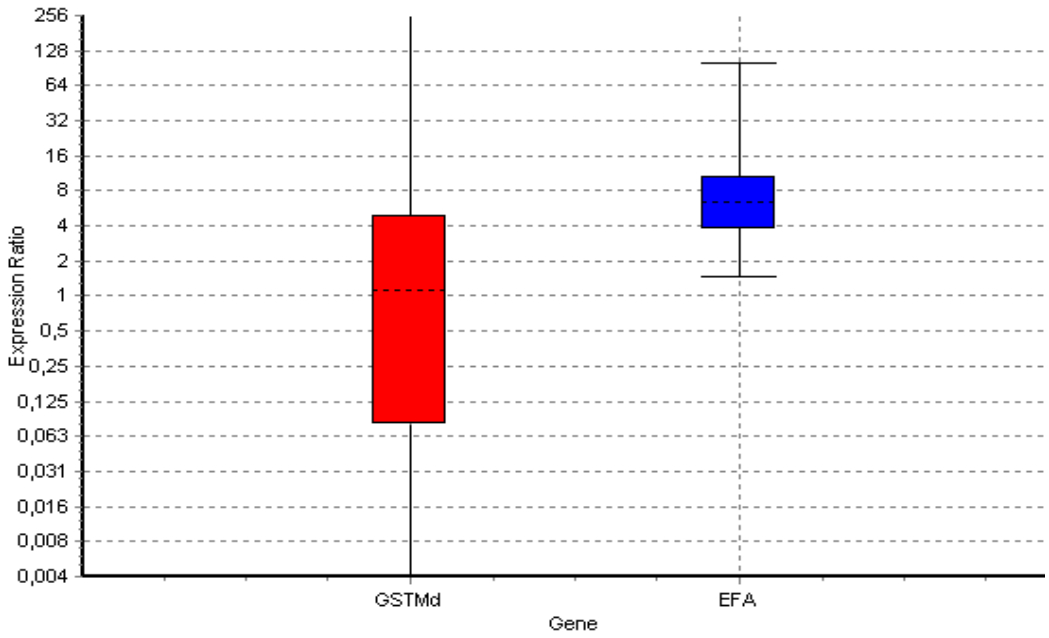
Figure 3.28. *GST (isozyme type not defined) Gene Whisker Box Plot of Çanakkale Population*

Table 3.30. REST 2008 Results of Çanakkale Population for GST (isozyme type not defined) Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
GST Çanakkale	Target Gene	0.255	0.003 – 320.802	0.215	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

GST (isozyme type not defined) gene expression was not different in Çanakkale population in comparison to susceptible population (P(H1)= 0.215).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Figure 3.29. GST (isozyme type not defined) Gene Whisker Box Plot of Mardin Population

Table 3.31. REST 2008 Results of Mardin Population for GST (isozyme type not defined) Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
GST Mardin	Target Gene	1.106	0.007 – 309.484	0.925	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

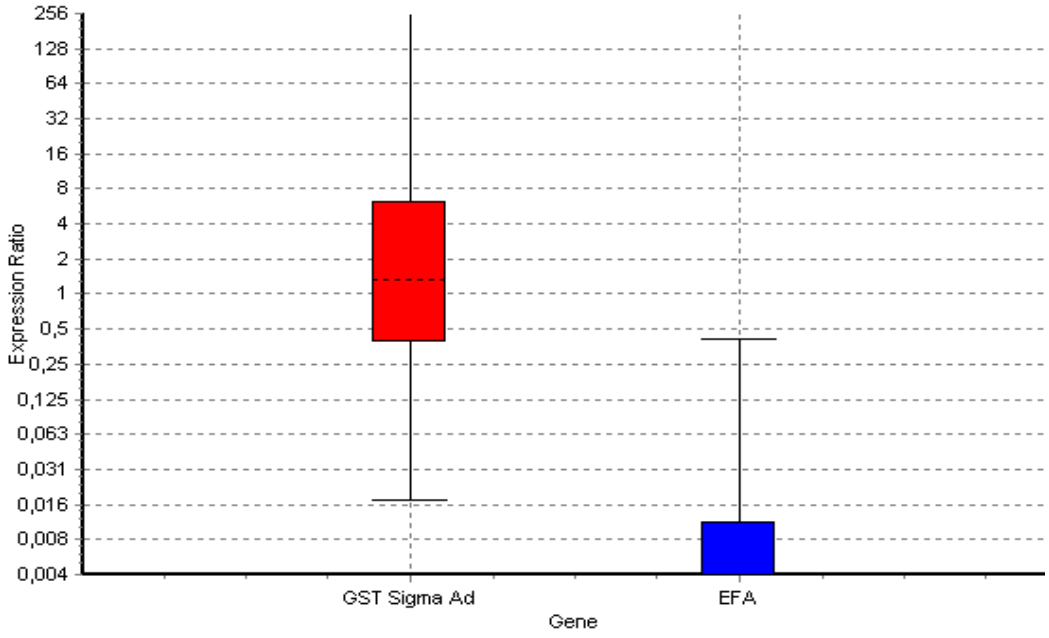
GST (isozyme type not defined) gene expression was not different in Mardin population in comparison to susceptible population (P(H1)= 0.925).

3.3.8. GSTX01 Gene Real-Time PCR Results of Susceptible and Field Populations of *Helicoverpa armigera*

C_T values of GSTX01 gene, indicated as a GST sigma like (Wee *et al.,* 2008), were determined according to its standard curve, prepared similarly as already described in chapter III, part 3.3.1, shown at **table 3.32**. After that, these results were analyzed with REST 2008 for analyzing GSTX01 gene expression in field populations of *H. armigera*. REST 2008 results for Adana, Çanakkale and Mardin populations were shown at following figures (**Figure 3.30, Figure 3.31** and **Figure 3.32)** and tables (**Table 3.33**, **Table 3.34** and **Table 3.35**).

Table 3.32. GSTX01 Gene Real-Time PCR Results of *H. armigera* Populations

Sample No	Susceptible Average C_T	Adana Average C_T	Çanakkale Average C_T	Mardin Average C_T
1	29.20	23.67	24.83	28.33
2	28.12	25.01	25.33	28.36
3	30.58	30.16	27.93	27.90
4	24.83	28.95	22.21	23.99
5	---	28.04	27.75	27.60
6	25.54	25.79	28.40	22.96
7	26.10	22.30	27.72	25.62
8	24.31	24.73	26.03	26.58
9	25.80	25.80	24.36	23.22
10	27.81	26.07	28.87	26.84
11		----	27.73	20.75
12		28.34	31.79	30.08
13			28.03	26.52
14			30.68	29.33
15			26.16	26.42
16			22.75	26.97
17			28.85	29.32
18			26.35	30.56
19			26.07	23.49
20			29.86	24.78



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

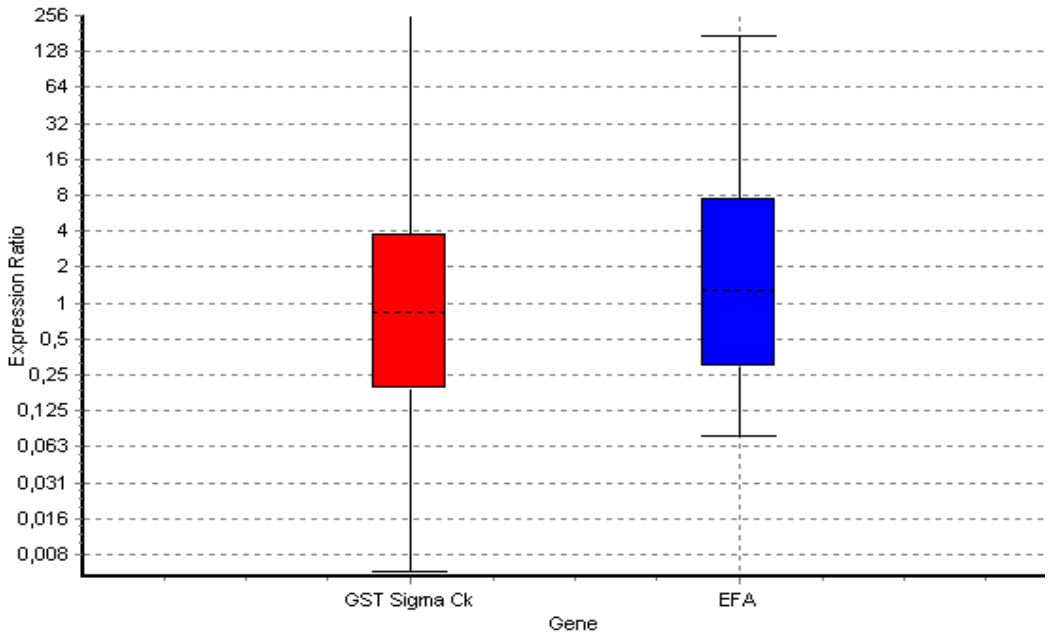
Figure 3.30. GSTX01 Gene Whisker Box Plot of Adana Population

Table 3.33. REST 2008 Results of Adana Population for GSTX01 Gene

Gene	**Type**	**Expression Ratio**	**95% Confidence Interval**	**P(H1)**	**Regulation**
GSTX01 Adana	Target Gene	1.581	0.040 – 91.642	0.522	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

GST (GST Sigma Like) gene expression was not different in Adana population in comparison to susceptible population (P(H1)= 0.522).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

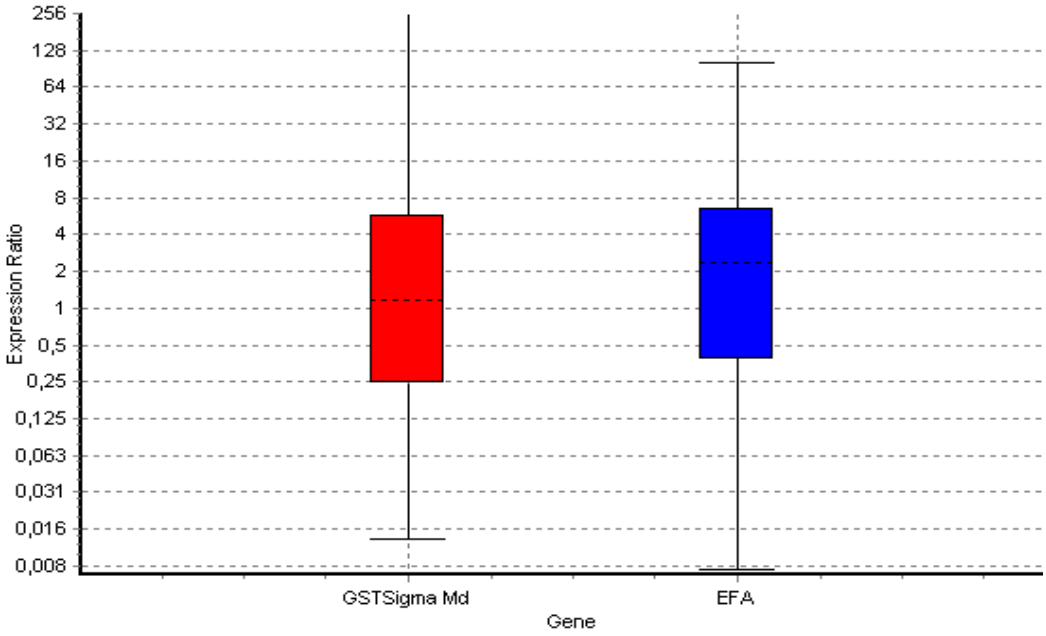
Figure 3.31. GSTX01 Gene Whisker Box Plot of Çanakkale Population

Table 3.34. REST 2008 Results of Çanakkale Population for GSTX01 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
GSTX01 Çanakkale	Target Gene	0.893	0.016 – 67.697	0.863	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

GST (isozyme type not defined) gene expression was not different in Çanakkale population in comparison to susceptible population (P(H1)= 0.863).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Figure 3.32. GSTX01 Gene Whisker Box Plot of Mardin Population

Table 3.35. REST 2008 Results of Mardin Population for GSTX01 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
GSTX01 Mardin	Target Gene	1.357	0.031 – 150.960	0.676	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

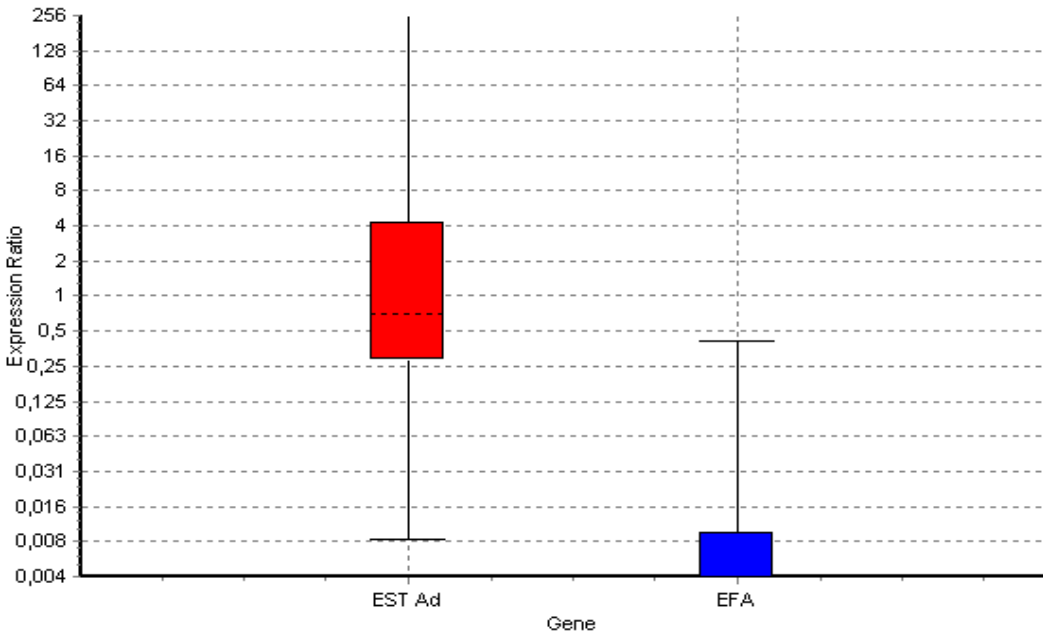
GST (isozyme type not defined) gene expression was not different in Mardin population in comparison to susceptible population (P(H1)= 0.676).

3.3.9. ESTX018 Gene Real-Time PCR Results of Susceptible and Field Populations of *Helicoverpa armigera*

C_T values of ESTX018 gene, indicated as a carboxylesterase like (Wee *et al.*, 2008), were determined according to its standard curve, prepared similarly as already described in chapter III, part 3.3.1, shown at **table 3.36**. After that, these results were analyzed with REST 2008 for analyzing ESTX018 gene expression in field populations of *H. armigera*. REST 2008 results for Adana, Çanakkale and Mardin populations were shown at following figures (**Figure 3.27, Figure 3.28** and **Figure 3.29)** and tables (**Table 3.37**, **Table 3.38** and **Table 3.39**).

Table 3.36. ESTX018 Gene Real-Time PCR Results of *H. armigera* Populations

Sample No	**Susceptible Average C_T**	**Adana Average C_T**	**Çanakkale Average C_T**	**Mardin Average C_T**
1	36.23	32.38	35.86	42.31
2	34.71	33.13	37.28	40.95
3	37.72	37.84	39.69	36.99
4	30.98	33.12	27.55	34.69
5	32.25	30.47	36.43	37.64
6	31.55	29.33	39.49	33.26
7	31.30	36.64	39.73	36.88
8	31.54	30.46	34.28	35.41
9	31.62	29.41	34.65	32.57
10	31.26	32.90	35.92	28.19



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

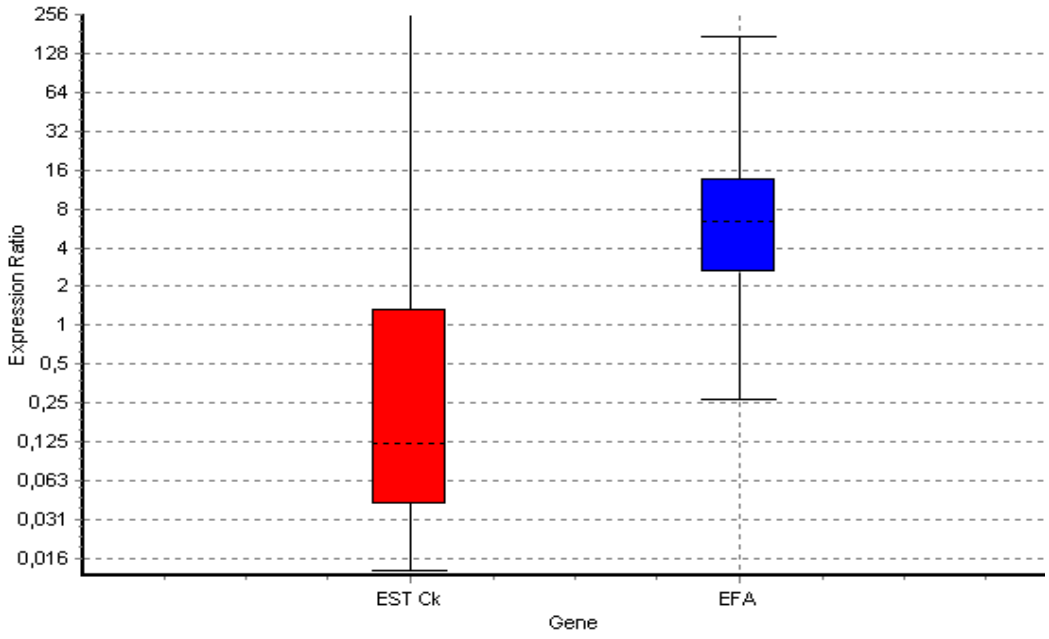
Figure 3.33. ESTX018 Gene Whisker Box Plot of Adana Population

Table 3.37. REST 2008 Results of Adana Population for ESTX018 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
ESTX018 Adana	Target Gene	0.916	0.010 – 152.245	0.920	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

ESTX018 gene expression was not different in Adana population in comparison to susceptible population (P(H1)= 0.920).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

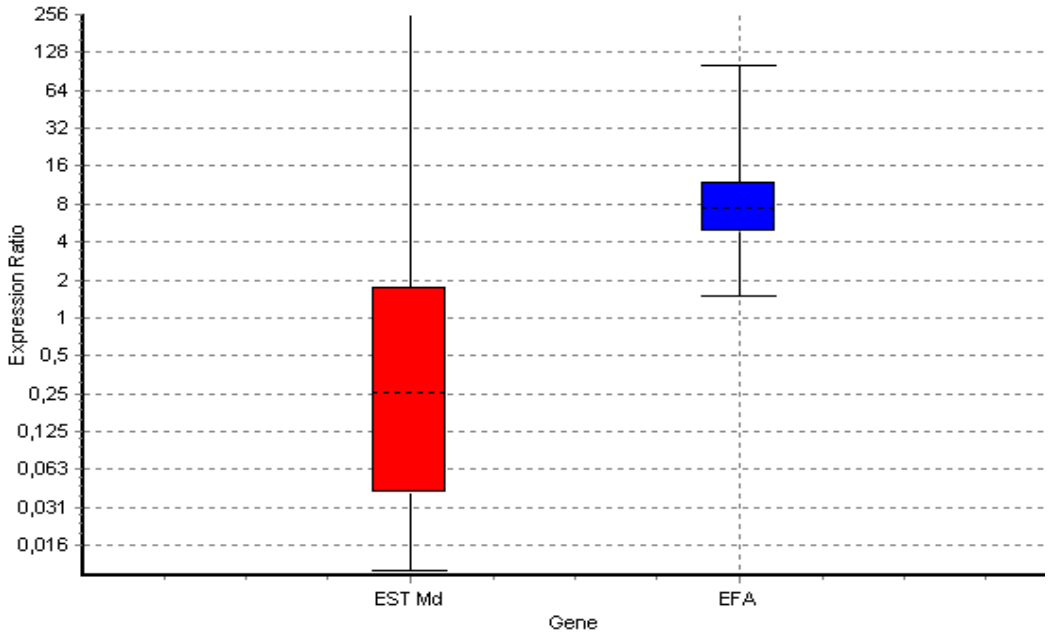
Figure 3.34. ESTX018 Gene Whisker Box Plot of Çanakkale Population

Table 3.38. REST 2008 Results of Çanakkale Population for ESTX018 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
ESTX018 Çanakkale	Target Gene	0.318	0.016 – 216.475	0.243	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

ESTX018 gene expression was not different in Çanakkale population in comparison to susceptible population (P(H1)= 0.243).



A box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Figure 3.35. ESTX018 Gene Whisker Box Plot of Mardin Population

Table 3.39. REST 2008 Results of Mardin Population for ESTX018 Gene

Gene	Type	Expression Ratio	95% Confidence Interval	P(H1)	Regulation
ESTX018 Mardin	Target Gene	0.344	0.012 – 96.059	0.245	NOT DIFFERENT

P(H1) - Probability of alternate hypothesis that difference between sample and control populations is due only to chance.

ESTX018 gene expression was not different in Mardin population in comparison to susceptible population (P(H1)= 0.245).

Table 3.40. Real-time PCR Results of *Helicoverpa armigera* Strains

STRAIN	CYP 4S1	CYP 6B7	CYP 9A12	CYP 9A14	GST	GSTX01	ESTX018
Adana	0.073*↓	0.001*↓	0.001*↓	10.1*↑	0.25	1.58	0.92
Çanakkale	0.454	0.49	4.73*↑	157.4*↑	0.26	0.89	0.32
Mardin	4.49 *↑	0.31	3.37 *↑	874.3*↑	1.11	1.36	0.34

Numerical values indicate that expression ratio of that gene in field population compare to the susceptible population.

Arrows indicate that up (↑) or down (↓) regulation of searched gene expression.

*Value significantly different from the susceptible strain ($p<0.05$) with REST 2008

CHAPTER IV

DISCUSSION

In this study, the scope of proteome analysis was detecting differences at protein level in midgut tissues of *H. armigera* strains. It was tried to do comparative proteomics by analysing expression differences of identified proteins between susceptible and field populations of *H. armigera* from Turkey. As proteins are functional biomolecules in metabolic processes, differences in their expressions are likely to improve our understandings in pyrethroid resistance mechanism. However, proteins are produced from genes, before functioning in biological process, most commonly; they are posttranslationally modified according to the functional requirements. Thus, there are more proteins than genes in the organisms. This diversity made it difficult to do proteome analysis.

In addition, the necessity of using two different sample preparation protocols in this work resulted in additional difficulties in proteome analysis. The main difference between these protocols was lyophilisation process of samples from Turkey. In order to make sample material more convenient to travel from Turkey to Rostock Proteome Centrum, they were lyophilized. As lyophilisation process may cause changes in ionic strength and pH, this generally leads to loss of that protein in lyophilized samples. Consequently, there were faint 2D-gels observed with lyophilized samples compared to fresh-frozen tissue samples. Furthermore, there was less protein spots detected in these gels with Coomassie Brilliant Blue G250 (CBB G250) staining method. This might be caused from completely loss of some proteins, so prone to damage during lyophilisation, and/or partially losses in some proteins resulted in its amount lesser than <15 ng protein, detection limit of CBB G250 stain.

Insects generally develop resistance against applied insecticides by following three main mechanisms; (i) by reducing the amount of insecticide entering into the insect body, (ii) developing insensitivity of the insecticide effective site and (iii) increasing detoxification metabolism of insecticides using major enzyme systems such as esterases (EST), glutathione S-transferases (GST) and cytochrome P-450 monooxygenases (CYP450).

Biochemical analysis of esterases and glutathione S-transferases together with comparative proteomics analysis were done to characterize pyrethroid resistance mechanisms in two field populations of *H. armigera* from Turkey. According to the enzyme activities results except GST-EPNP activity, EST-PNPA, EST-α-NA, GST-CDNB, GST-DCNB and GST-PNBC enzyme activities were increased statistically significant ($p<0.05$, student-t test) in the Adana field population. Moreover, Mardin field population showed similar biochemical activities pattern except EST-PNPA, not statistically significant from susceptible population. While esterase enzyme activities were increasing 1.54-2.11 fold ranges in field populations of *H. armigera* GST activities were increasing between 1.51-4.80 fold ranges in these populations. GST-DCNB and GST-PNBC activities in the Çanakkale field population, not used in comparative proteomic analysis, were also significantly increased 1.97 and 2.16 folds, reciprocally. However, increases in EST and GST activities would not be supported by comparative proteomic analysis. Nevertheless, there was no esterase protein identified during reference gel preparation list, as well. But, there was only two different GSTs protein identified (spot no: 623 and 401 in **figure 2.1**) in reference gel formation. This might be resulted from loss of those proteins in lyophilisation process and limitations of proteome analysis, including unknown genome sequence of *H. armigera*, as well. Although, it seemed that there was no up regulation in protein amount of identified EST and GSTs, significant increases in these enzymatic activities were determined. This could be explained by posttranslational modifications of esterase and glutathione S-transferases enzymes, isozyme diversity of EST and GSTs in *H. armigera* and methodological differences between biochemical assays and comparative proteome analysis.

According to these results, both EST and GSTs might have a role in pyrethroid resistance development in the Adana and Mardin field populations. In addition, GSTs also seemed to participate in pyrethroid resistance development in the Çanakkale field population.

Pyrethroids are synthetic esters composed of an acid and alcohol moiety. They are roughly categorized as type I and type II pyrethroids (Soderlund *et al.*, 2002). Type I pyrethroids are esters of primary or secondary alcohols. Type II pyrethroids are esters of secondary alcohols, containing a cyano group at the α-carbon (Scollon et al., 2009). As type II pyrethroids are so effective for killing insects compared to type I pyrethroids (Bloomquist, J. R. 1996), they have been commonly used in agriculture areas. Moreover, Ugurlu S. (sugurlu@hotmail.com) found that type II pyrethroids (beta-cyfluthrin, bifenthrin, esfenvalerate and lambda cyhalothrin) showed resistance ratio 6-67 fold range in Adana and Mardin field populations. Ugurlu S. was also found that Çanakkale field population of *H. armigera* showed 14.5 folds resistance ratio to lambda-cyhalothrin. Thus, one of the type II pyrethroid insecticides "deltamethrin" was selected as a model for generating possible metabolizing pathways in field populations of *H. armigera* from Turkey according to the differential proteome analysis and enzyme activity assays results (**Figure 4.1** and **Figure 4.2**).

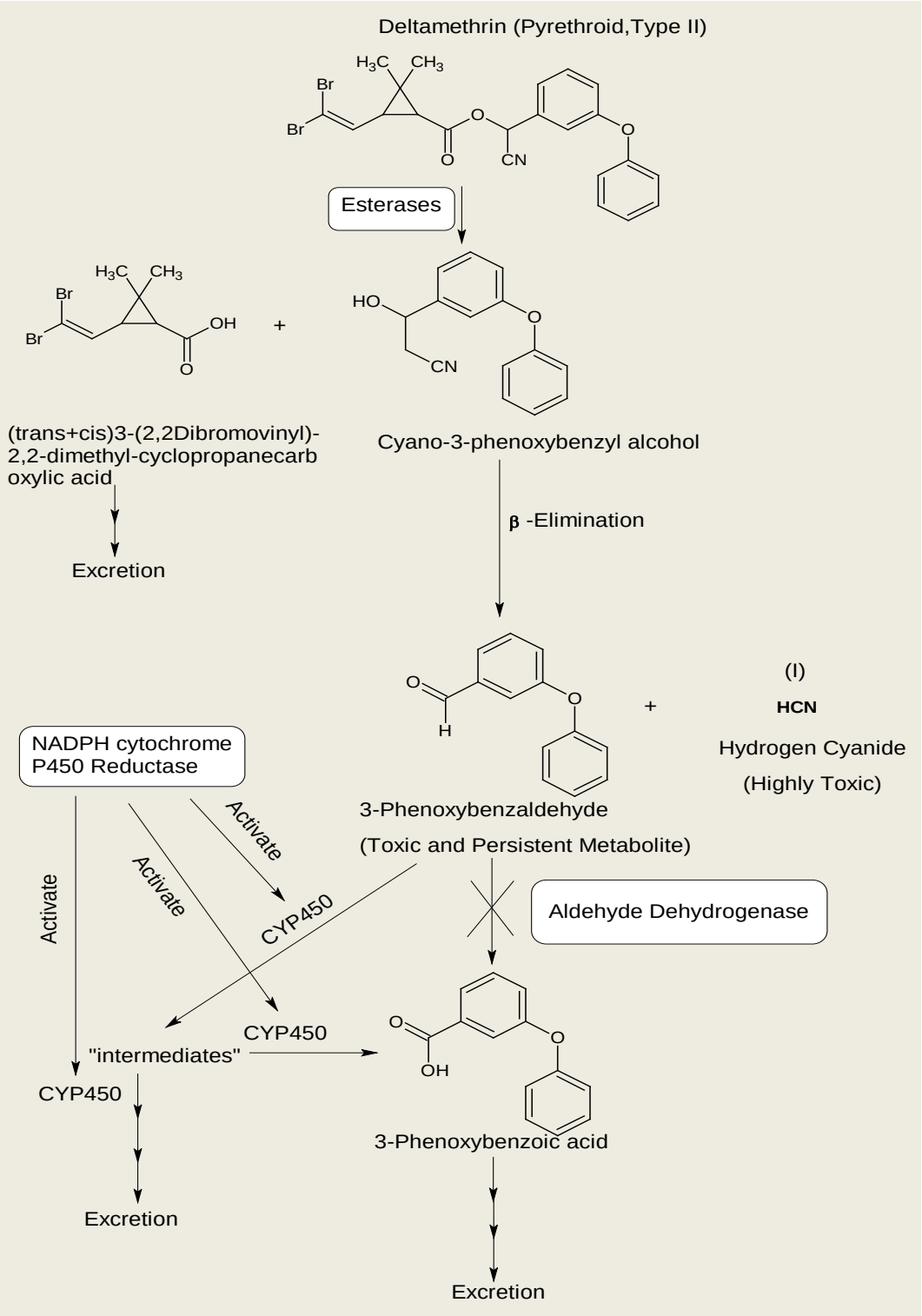


Figure 4.1. Scheme for Metabolism of Deltamethrin (pyrethroid type II) in *H. armigera* Midgut.

Esterases are enzymes that hydrolyse esters into acid and alcohol. They initiate metabolism of pyrethroid esters such as deltamethrin (pyrethroid type II) into cyano-3-phenoxybenzyl alcohol and (trans+cis)3-(2,2Dibromovinyl)-2,2-dimethyl-cyclopropane carboxylic acid (**Figure 4.1**). Trans and cis isomers of acid compounds would be eliminated via excretion of these acidic metabolites. On the other hand, cyano-3-phenoxybenzyl alcohol would be converted to hydrogen cyanide (toxic) and 3-phenoxybenzaldeyhde (toxic) via β-elimination. 3-phenoxybenzaldeyhde could be eliminated by aldehyde dehydrogenase (spot no: 393 in **figure 3.12**), catalyzing conversion of 3-phenoxybenzaldehyde to 3-phenoxybenzoicacid (Ai *et al.*, 2010) which could be excretes as other corresponding acid compounds. As aldehyde dehydrogenase was found down-regulated in both field populations, it was concluded that this route was not used or preferred for elimination of these toxic compounds. Instead it is suggested that other enzyme(s) play a role in elimination of toxic 3-phenoxybenzaldehyde product. In particular, cytochrome P450 monooxygenases (Nakamura *et al.,* 2007; Scollon *et al.,* 2009) could play an important role in elimination of toxic aldehyde compounds by converting them into another intermediate that then would be converted into the corresponding acid that would be excreted. It might be also possible to excrete an intermediate without converting into acid compound. According to the comparative proteomic analysis, NADPH cytochrome P450 reductase (spot no: 231 in **figure 3.13**), responsible for activation of CYP450 enzymes, showed 2.52-fold up-regulation in the Mardin population as compared to susceptible population. It should be noted that in the Adana population NADPH cytochrome P450 reductase was expressed 1.83-folds more. As the cut off for differential protein expression was set to minimally 2-fold, NADPH cytochrome P450 reductase was not regarded as differentially regulated in this population. Two additional spots of NADPH cytochrome P450 reductase were identified in the reference gel, but these spots were missing in all gels of lyophilized samples from control and field populations. Nevertheless, it can be concluded that NADPH cytochrome P450 reductase may be importantly involved in pyrethroid resistance and in the detoxification toxic aldehyde metabolites.

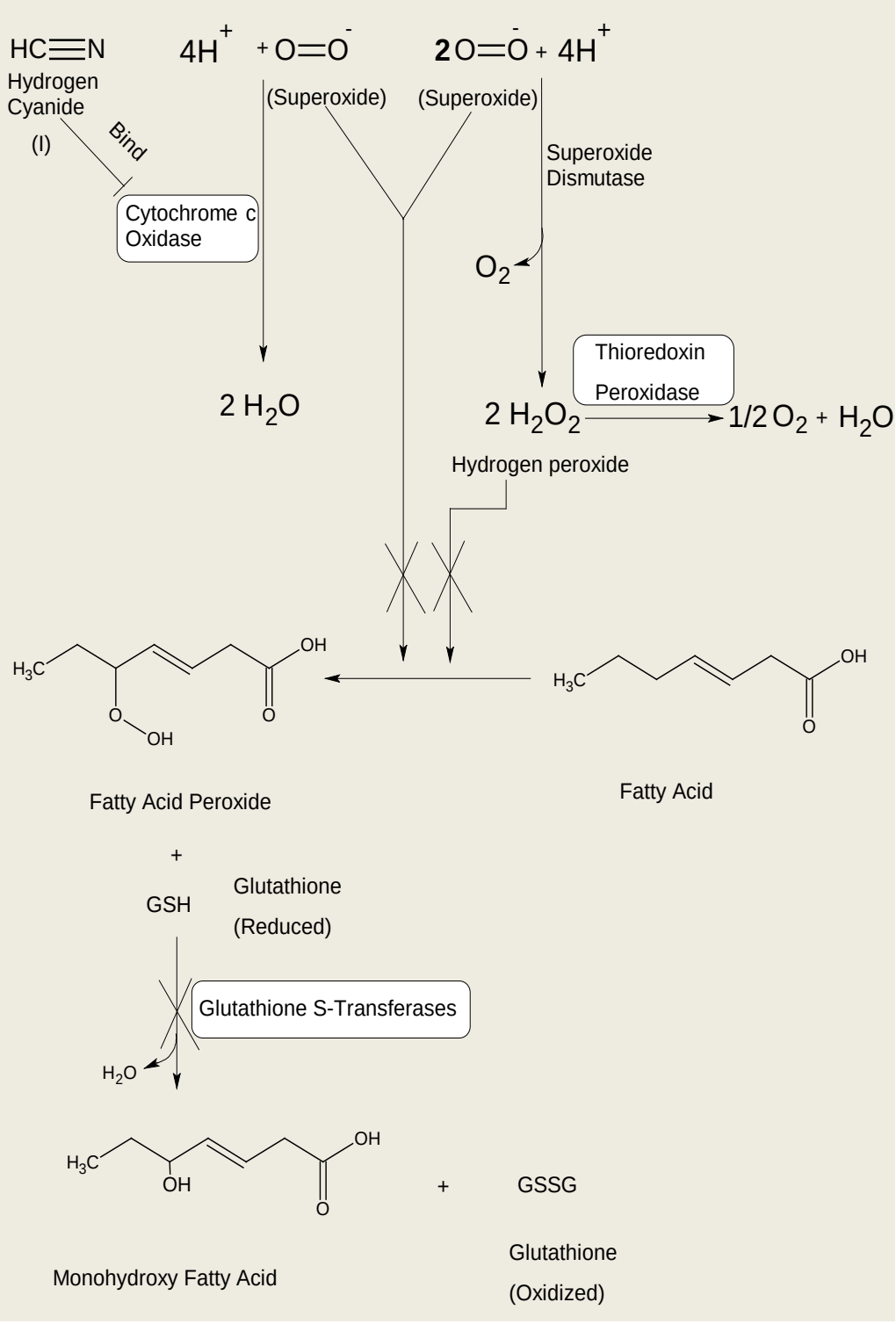


Figure 4.2. Scheme for Formation and Elimination Pathways of Reactive Oxygen Species Formed During Metabolism of Deltamethrin (pyrethroid type II) in *H. armigera* Midgut.

As mentioned above, toxic hydrogen cyanide is also formed during metabolism of cyano group containing pyrethroid insecticides such as deltamethrin. Hydrogen cyanide blocks oxidative phosphorylation by inhibiting mitochondrial cytochrome c oxidase enzyme, and thus, ATP can not be produced. According to the findings from our differential proteome analysis, energy production related protein expression was up-regulated; presumably to overcome the ATP supply shortage in field populations of *H. armigera*. These up-regulated proteins were mostly ATP synthase, vacuolar-type ATPase A/B (fragment), arginine kinase (**Table 3.9** and **Table 3.10**). For example, while there were five up-regulated arginine kinase spots identified in Mardin population, there were three non-regulated arginine kinase spots identified in Mardin population, as well. In addition, expression putative mitochondrial cytochrome c oxidase polypeptide Va (spot no: 566 in **Figure 3.1**), a target protein of hydrogen cyanide, were increased 3.05-fold in Adana field population.

Interestingly, certain spots of enzymes of glycolysis pathway, such as enolase and triosephosphate isomerase, were also found up-regulated. In other words, the pyrethroid metabolism related energy problem in field populations of *H. armigera* might be overcome with increase in production of ATP synthase, vacuolar-type ATPase A/B and arginine kinase together with glycolytic pathway enzymes such as triosephosphate isomerase and enolase.

It was emphasized that cyanide inhibition of mitochondrial electron transport bring about formation of excess reactive oxygen species (ROS) at complexes I and III in the mitochondria inner membrane, inducing intense oxidative stress that alloy to cellular dysfunction (Chen *et al.*, 2003; Gunasekar *et al.*, 1998; Jones *et al.*, 2000). Accumulations of ROS in cells such as superoxide, one of the main harmful ROS, commonly cause DNA damage, protein oxidation and lipid peroxidation. As superoxide is quite toxic for organisms, most of the living organisms have superoxide dismutase enzyme that catalyze dismutation reaction of toxic superoxide into lesser toxic compounds oxygen and hydrogen peroxides (H_2O_2). However, H_2O_2 is another compound that contributes into oxidative damage such as the initiation of lipid peroxidation (Kellog and Fridovich, 1977). Therefore, it needs to

be converted non toxic molecules, commonly by catalases and peroxidases such as thioredoxin peroxidases such as thioredoxin peroxidase. Lee *et al.,* 2005 found that thioredoxin peroxidase protein were present in fat body and midgut in lepidopterian insect *Bombxy mori* and when H_2O_2 injected into *B. mori*, thioredoxin peroxidase mRNA expression was highly up-regulated especially in fat tissues. According to the comparative proteomic analysis, thioredoxin peroxidase enzyme (spot: 413 in **figure 3.1**) amount was up-regulated infinitely in the Mardin population as it was not detected in the susceptible population. However, it was not changed in Adana field population. This result suggests that cyanide blocking of electron transport might produce more oxidative stress in the Mardin field population as compared to Adana field population. While up-regulation of ATP synthase, vacuolar-type ATPase A/B proteins and arginine kinases were major proteins contributing to solve energy problems in the Mardin population, up-regulation of similar proteins were accompanied with putative mitochondrial cytochrome c oxidase polypeptide Va in the Adana field population. Up-regulation of the cytochrome c oxidase protein seems to exert an inhibitory effect on production of reactive oxygen species in the Adana field population compared to the Mardin field population.

Vontas *et al.,* 2001 demonstrated that pyrethroids induce oxidative stress and lipid peroxidation in insects as it is shown in **figure 4.2**. It was also indicated that GSTs were involved in pyrethroid resistance through antioxidant defence in insects by reducing pyrethroid induced lipid peroxides (Vontas *et al.,* 2001). Nonetheless, increases in biochemical glutathione S-transferase activities with three different substrates (CDNB, DCNB and PNBC) indicate that some GST isozymes may catalyze detoxification reactions of these toxic lipid peroxide compounds in *H. armigera* during metabolism of pyrethroid insecticides.

In addition, although the Adana population showed higher GST activity than the Mardin population with the general substrate CDNB, it showed less GST activities than the Mardin population with specified DCNB and PNBC substrates. Therefore, it was concluded that GST-DCNB and GST-PNBC activities support the above demonstrated comparative proteomic results as they seem to function as better

indicators of oxidative stress in *H. armigera* (**Table 3.3** and **Table 3.4**). However, these findings need further confirmation at the transcriptome and protein level to help to understand the molecular mechanism of pyrethroid resistance in *H. armigera* in more detail.

As it was looked at *q*-RT-PCR results of Adana population, analysed GST and EST genes expressions (GST, GSTX01 and ESTX018) were not changed significantly ($p<0.05$, REST 2008). However, only CYP9A14 gene expression was up-regulated (10.1 fold up regulation, $p<0.05$, REST 2008) compared to susceptible population. Furthermore, other measured CYP4S1, CYP6B7 and CYP9A12 genes expressions showed 0.073, 0.001 and 0.001 fold ($p<0.05$, REST 2008) down regulation, reciprocally. According to these results, it would be understood that CYP9A14 gene could play an active role in developing pyrethroid resistance in Adana population.

Considering the *q*-RT-PCR results of Çanakkale population, it was seen that *H. armigera* insects from Çanakkale field showed no significant changes in mRNA levels of searched GST and EST genes. Furthermore, CYP450 enzyme system members from CYP4 (CYP4S1) and CYP6 (CYP6B6 and CYP6B7) families did not detected any significant changes in *q*-RT-PCR analysis, as well. However, only CYP9 family member genes, CYP9A12 (4.73 fold up regulation) and CYP9A14 (157.4 fold up regulation) mRNA copy numbers were significantly ($p<0.05$, REST 2008) up-regulated compared to susceptible population. These results indicate that only CYP9 family of cytochrome P450 enzyme system seemed to participate actively in resistance development of Çanakkale field population with CYP9A12 and CYP9A14 genes.

Analysed GST and EST genes expressions were not changed significantly ($p<0.05$, REST 2008) in Mardin population similar to Adana and Çanakkale populations, as well. Nonetheless, *q*-RT-PCR analysis results of CYP4S1, CYP9A12 and CYP9A14 genes, except for the CYP6 family, showed 4.49, 3.37 and 874.3 fold ($p<0.05$, REST 2008) up regulation, reciprocally. These results demonstrate that CYP4 and CYP9

family member these genes participate actively in pyrethroid metabolism to overcome hazardous effect of these pesticides in Mardin field population.

According to the overall *q*-RT-PCR findings related with EST, GST and CYP450 enzyme systems, there was no up regulation in expressions of GST, GSTX01, ESTX018 and CYP6B7 genes in all analysed field populations. However, while CYP4S1 gene increased expression only in Mardin population, CYP9A12 gene expression increased in both Çanakkale and Mardin populations. Furthermore, CYP9A14 gene expression showed increase in all field samples of *H. armigera*. Increases in gene expression of CYP9A14 were dramatically high especially in Çanakkale and Mardin population as a 157.4 and 874.3 fold ($p<0.05$, REST 2008) compared to susceptible population, respectively. According to *q*-RT-PCR results, it seems that CYP9A14 gene might have an important role in developing pyrethroid resistance in field population *H. armigera* from Turkey. Furthermore, CYP9A12 and CYP4S1 genes may have a role in developing pyrethroid resistance in *H. armigera* from Turkey, too. Although, there were no supportive comparative proteomic analysis data and biochemical assay results for these up-regulated CYP450 genes, these genes possibly take role in detoxifying toxic 3-phenoxybenzaldehyde compound that produced from metabolism of pyrethroids, as described at **figure 4.2**. Consequently, it was concluded that CYP9A14, CYP9A12 and CYP4S1 were possible candidate enzymes, may be activated by NADPH cytochrome P450 reductase, take a role in elimination of toxic pyrethroid metabolites in *H. armigera* from Turkey (**Figure 4.3**).

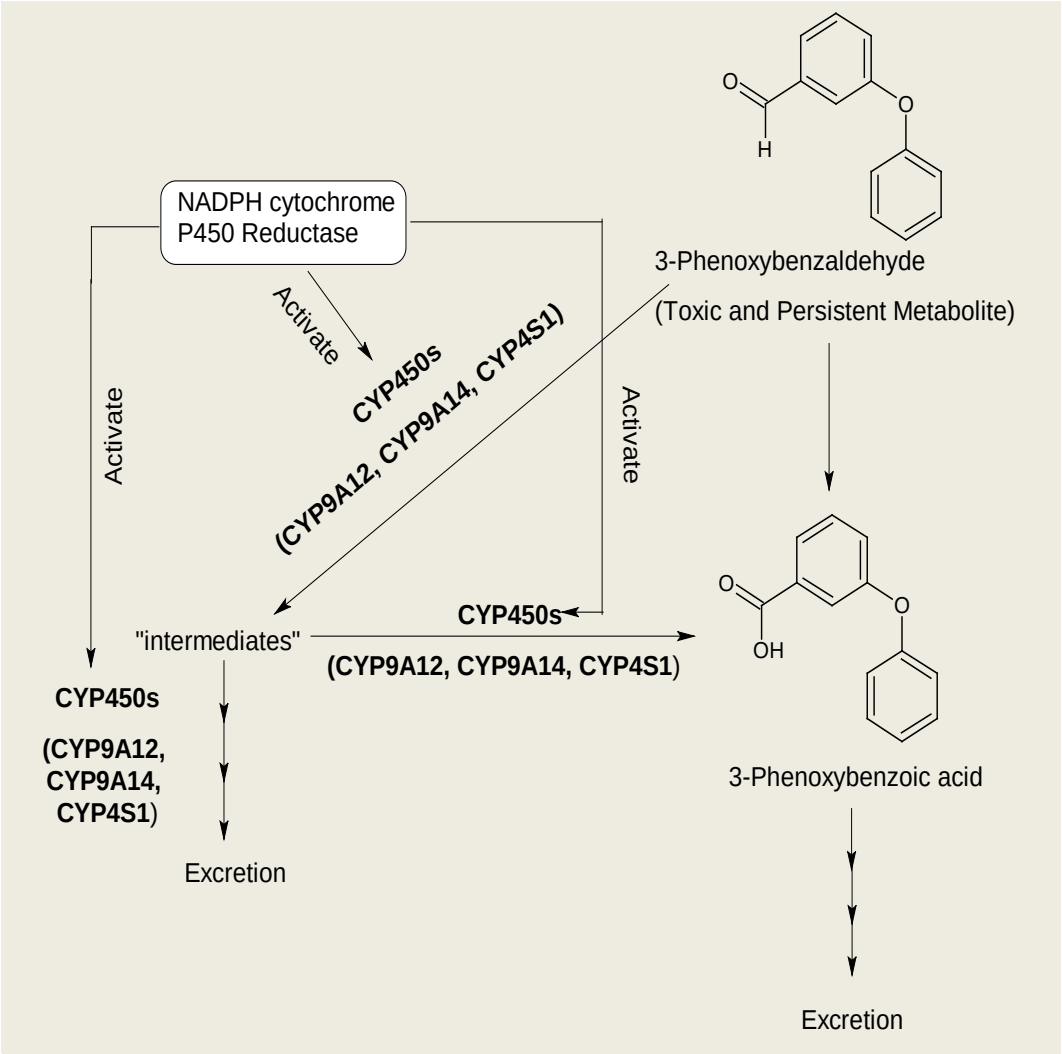


Figure 4.3. Scheme for Elimination of Toxic Metabolic Compounds That Produced During Pyrethroid Metabolism in *H. armigera* Midgut.

CHAPTER V

CONCLUSION

Esterase enzyme activities (EST-PNPA and EST-α-NA) of *H. armigera* midgut were determined with biochemical assays in the Adana, Çanakkale and Mardin field populations. Both Adana and Mardin field samples showed statistically significant ($p<0.05$) increase in esterase activities (in the range of 1.54 to 2.11 folds) compared to the susceptible samples. However, Çanakkale field samples did not show statistically significant ($p<0.05$) increase in esterase activities than susceptible samples.

Glutathione S-transferases enzyme activities, GST-CDNB, GST-DCNB, GST-EPNP and GST-PNBC activities of *H. armigera* midgut were determined with biochemical assays in the Adana, Çanakkale and Mardin field populations. Both Adana and Mardin field samples showed statistically significant ($p<0.05$) increase in GST-CDNB, GST-DCNB and GST-PNBC activities (in the range of 1.51 to 4.8 folds) compared to the susceptible samples. However, Çanakkale samples showed statistically significant ($p<0.05$) increase only in GST-DCNB and GST-PNBC activities (in the range of 1.97 to 2.16 folds) than susceptible samples.

According to the overall biochemical assay data, it can be said that increased EST and GST activity in the Adana and Mardin field populations associated with the resistance through synthetic pyrethroid insecticides. Furthermore, GSTs also seemed to play a role in developed lambda-cyhalothrin resistance in the Çanakkale field population.

According to the comparative mass spectrophotometric analysis in the Adana field population, most of the up-regulated proteins belong to energy system related proteins (**Table 3.9**). These up-regulated proteins consist of mitochondrial proteins (e.g. spots 293, 311, 470 see in **table 3.9**), cytosolic proteins (spots 1005, 1009 and 712) and kinase/phosphatase proteins (spots 598, 618, 1016 and 1017). Furthermore, protein metabolism proteins (spots 349, 475, 1010 and 1020), cytoskeleton proteins (spots 383, 441 and 492) and translational controlled tumor protein (spot 498) were up regulated in the Adana field population. In addition, most of the identified down regulated proteins belong to protein metabolism (spots 1011, 334, 79, 1022 and 1002), cytosolic energy proteins (spots 137, 426, 673 and 723). Aldehyde dehydrogenase (spot 393) and putative tubulin beta-1 chain (spot 379) were also down regulated in the Adana population.

Comparative mass spectrophotometric results of the Mardin field population showed similar pattern to Adana field population especially for energy system related and protein metabolism and cytoskeleton proteins. Likewise Adana field population, up-regulated energy system related proteins in the Mardin field population consist of mitochondrial proteins (e.g. spots 61, 293, 311, 470 see in **table 3.10**), cytosolic proteins (spots 712, 1000, 1005, 1006 and 1019) and kinase/phosphatase proteins (spots 246, 278, 394, 598 and 1016). There were also up-regulated spots identified related with protein metabolism (spots 92, 247, 321, 349, 475, 606, 1001 and 1014) and cytoskeletal system (spots 118, 379, 383, 441 and 492) in the Mardin population. In addition, translational controlled tumor protein (spot 498) and detoxification proteins such as NADPH cytochrome P450 reductase (spot 231) and thioredoxin peroxidase (spot 413) were also up-regulated. In the Mardin population most of the identified down regulated proteins, similar to Adana population, were related to protein metabolism (spots 309, 334, 1010, 1011 and 1022), cytosolic energy proteins (spots 426, 636, 673 and 1018). Furthermore, cytidine deaminase (spot 592), ATP synthase (spot 616) and myosin heavy chain (spot 337) proteins were down regulated, as well. In addition, detoxification proteins such as aldehyde dehydrogenase (spot 393) and glutathione S-transferases (spot 623) and thioredoxin

peroxidase, incomplete form, (spot 1012) were also down regulated in the Mardin population.

Only CYP9A14 gene expression was up-regulated (10.1 fold up regulation, $p<0.05$) in the Adana population compared to susceptible population. However, CYP4S1, CYP6B7 and CYP9A12 genes expressions showed 0.073, 0.001 and 0.001 fold ($p<0.05$) down regulation, reciprocally. Moreover, GST, GSTX01 and ESTX018 genes expressions were not changed significantly ($p<0.05$, REST 2008) in the Adana population.

H. armigera insects from Çanakkale field population showed no significant changes in both activity and mRNA levels of GST, GSTX01 and ESTX018 genes. Furthermore, CYP4S1 and CYP6B7 genes expressions did not change significantly, as well. Nonetheless, CYP9A12 (4.73 fold up regulation) and CYP9A14 (157.4 fold up regulation) mRNA expression levels were significantly ($p<0.05$, REST 2008) up-regulated compared to susceptible population.

Mardin field population real time-PCR results demonstrated that GST, GSTX01 and ESTX018 genes expressions were not changed significantly similar to Adana and Çanakkale field populations. In contrast, CYP4S1, CYP9A12 and CYP9A14 genes showed 4.49, 3.37 and 874.3 fold ($p<0.05$) up regulation, reciprocally.

As a consequence of overall *q*RT-PCR results for GST and EST system related genes, there were not determined any up regulation in expressions of GST, GSTX01 and ESTX018 and CYP6B7 genes. However, while CYP4S1 gene increased expression only in the Mardin population, CYP9A12 gene expression increased in both Çanakkale and Mardin populations. Furthermore, CYP9A14 gene expression showed increase in all field samples of *H. armigera*. The most dramatic increases in analyzed gene expression was detected at CYP9A14 gene expression, dramatically high especially in the Çanakkale and Mardin population as a 157.4 and 874.3 fold ($p<0.05$) compared to susceptible population, respectively. Therefore, CYP9A14 gene possibly had an important role in developing pyrethroid resistance in the field

populations of *H. armigera* from Turkey. In addition, CYP9A12 and CYP4S1 genes might have a contributory role in pyrethroid resistance in *H. armigera* from Turkey.

CHAPTER VI

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APPENDIX

A. MASCOT PEPTIDE MASS FINGERPRINT SEARCH

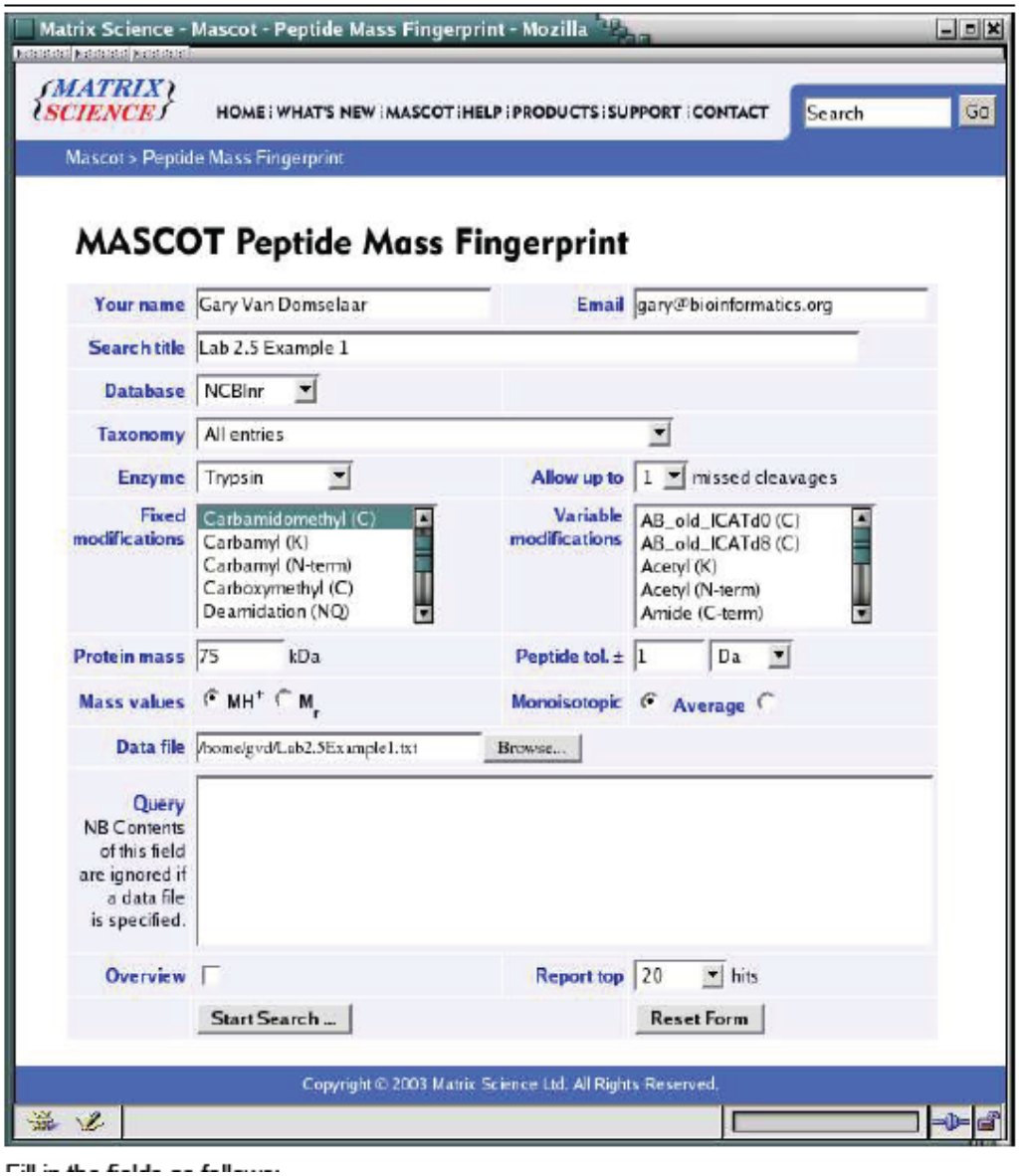

Figure A.1. MASCOT Peptide Mass Fingerprint Search in MATRIX SCIENCE Website (Image from MATRIX SCIENCE, http://www.matrixscience.com)

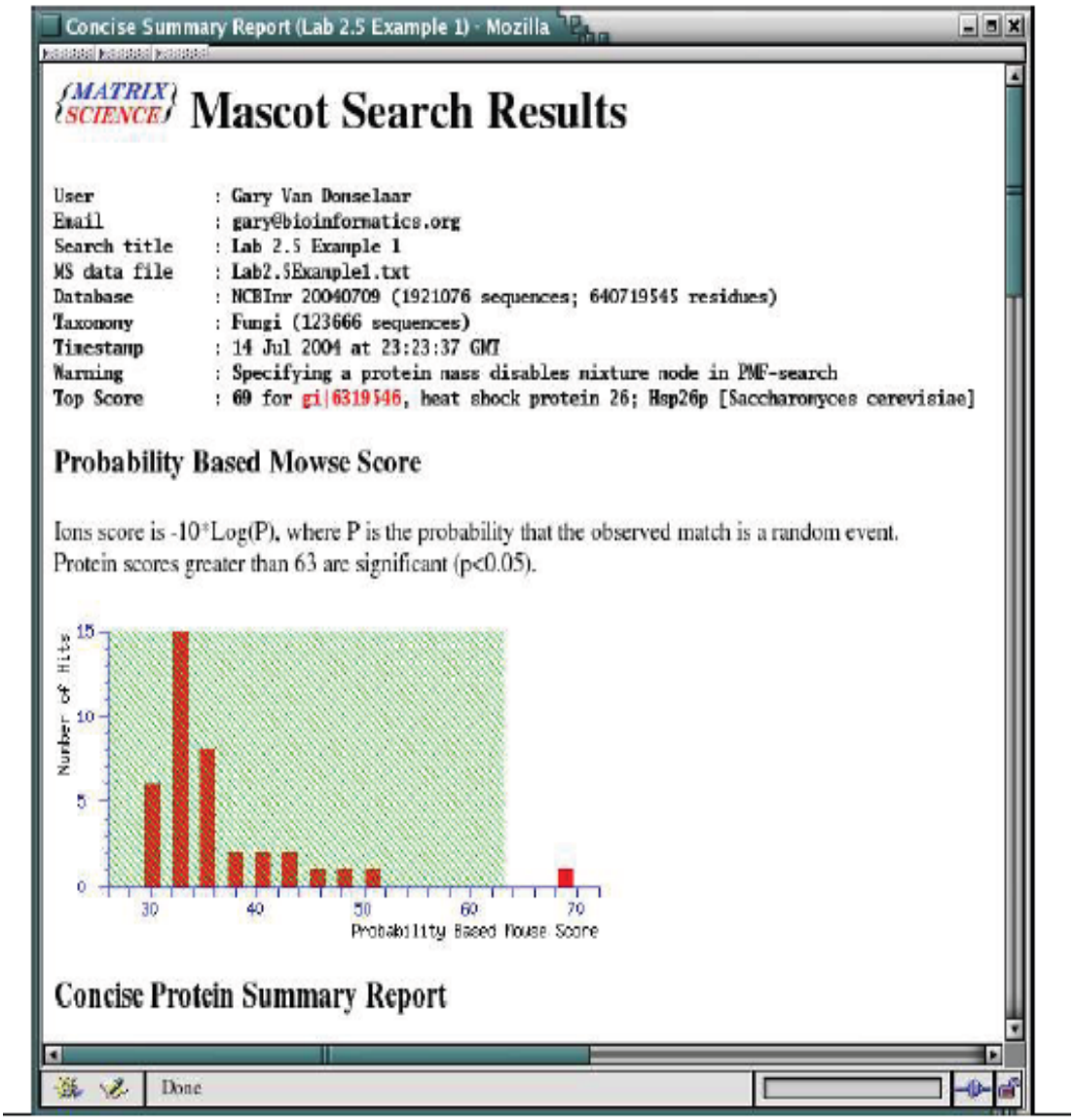

Figure A.2. Peptide Mass Fingerprint Search Result in MATRIX SCIENCE Website (Image from MATRIX SCIENCE, http://www.matrixscience.com)

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ACADEMIC PUBLICATIONS

Journal Papers

1. Uğurlu S., **Konuş M**., Isgör B., İşcan M. (2007) Pyrethroid Resistance and Possible Involvement of Glutathione S-transferases in *Helicoverpa armigera* from Turkey, *Phytoparasitica*, 35, (1):23-26.

2. **Konus M.**, Koy C., Mikkat S., Zimmermann R., İşcan M and M. O. Glocker, (2012), Molecular Resistance Mechanisms of *Helicoverpa armigera* against Pyrethroid Insecticides Analyzed by Differential Proteome Analysis and Enzyme Activity Assays (Manuscript in Preparation)

Conference Papers

1. **Konuş M**., Uğurlu S., İşcan M., (2003) Biochemical Characterization of *Helicoverpa armigera*, 13th Balkan Biochemical and Biophysical Days and meeting on metabolic disorders.

2. **Konuş M**., Uğurlu S., İşcan M., (2004) Analysis of Glutathione S-Transferases in susceptible and field populations of *Helicoverpa armigera*, XVIII. National Biochemistry Congress, Nevşehir, Turkey.

3. **Konuş M**., Uğurlu S., İşcan M., (2005) Effects of synthetic pyrethroid insecticides on *Helicoverpa armigera*, Glutathione S-Transferases, XIX. National Biochemistry Congress, Antalya, Turkey.

4. **Konuş M**., Uğurlu S., İşcan M., (2006) Effects of Lambda Cyhalothrin insecticide on *Helicoverpa armigera*, Glutathione S-Transferases isozymes, 31. FEBS Congress, İstanbul, Turkey.

5. **Konuş M**., Uğurlu S., İşcan M., (2008) Roles of Detoxification Enzymes in Pyrethroid Resistance of *Helicoverpa armigera* from Turkey, 45th Eurotox2008 Congress, Rhodos, Greece.

6. **Konuş M**., Uğurlu S., İşcan M., (2008) Real Time PCR Analysis of the Roles of CYP6B7 and CYP9A12 Genes in Pyrethroid Resistance of *Helicoverpa armigera,* XX. National Biochemistry Congress, Nevşehir, Turkey.

7. **Konuş M**., Uğurlu S., İşcan M., (2009) Distinct Effects of Pyrethroids on Cytochrome P450s, Glutathione S-Transferases and Carboxylesterase Gene Expressions in *Helicoverpa armigera* from Turkey, ISSX 11th European Regional Meeting, Lisbon, Portugal

8. **Konuş M**., Uğurlu S., İşcan M., (2009) Roles of GST-Theta in pyrethroid resistance in Helicoverpa armigera, 7th Turkish Toxicology Congress, Ankara, Turkey

9. **Konuş M**., Uğurlu S., İşcan M., (2009) Glutathione S-Transferases in Pyrethroid Resistance of *Helicoverpa armigera* from Turkey34th FEBS Congress, Prague, Czech Republic

10. C. Koy, M. Linnebacher, **M. Konus**, P. Lorenz, E. Klar, H.-J. Thiesen and M. O. Glocker, (2010) Towards the Determination of Neoepitope-Specific Antibody Signatures in Patients with Colorectal Carcinoma, The 43th Annual Meeting of the German Society for Mass Spectrometry (DGMS), Saale, Germany.

11. C. Koy, **M. Konus**, P. Lorenz, S. Drynda, J. Kekow, H.-J. Thiesen and M. O. Glocker, (2011) A Mass Spectrometric Approach for the Quantification of Therapeutic Antibodies in Plasma samples of Rheumatoid Arthritis Patients, The 44th Annual Meeting of the German Society for Mass Spectrometry (DGMS), Dortmund, Germany.

12. C. Koy, **M. Konus**, P. Lorenz, S. Drynda, J. Kekow, H.-J. Thiesen and M. O. Glocker, (2011) A Quantitation Method for Therapeutic Antibodies in Patient Plasma Samples Using Mass Spectrometry, 10th German Peptide Symposium, Berlin, Germany.

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PROJECTS (worked on as Researcher)

1. Tubitak projects between 2002-2007 years
Project name: **Helicoverpa (=Heliothis) armigera (Hubn.) (Lepidoptera: Noctuidae)' da sentetik piretroidli insektisitlere karşı dayanıklılığın biyokimyasal mekanizmalarının belirlenmesi üzerinde araştırmalar**

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Project name: **Effects of Synthetic Pyrethroid Insecticide Lambda-Cyhalothrin on *Helicoverpa armigera* Drug Metabolizing Enzyme Systems**

TEACHING EXPERIENCE

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