BEHAVIORAL AND MOLECULAR IMPACT OF A STRESS FACTOR, ACARICIDE PERIZIN ON HONEY BEES (*APIS MELLIFERA*) OF TURKEY

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BEHAVIORAL AND MOLECULAR IMPACT OF A STRESS FACTOR, ACARICIDE PERIZIN ON HONEY BEES (*APIS MELLIFERA*) OF TURKEY

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ABSTRACT

BEHAVIORAL AND MOLECULAR IMPACT OF A STRESS FACTOR, ACARICIDE PERIZIN ON HONEY BEES (*APIS MELLIFERA*) OF TURKEY

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Doctor of Philosophy, Biology
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The effects of acaricide Perizin (coumaphos as the active ingredient) which is used against parasitic mite, *Varroa destructor*, on three native honey bee subspecies; *Apis mellifera caucasica, A. m. carnica* and *A. m. syriaca*. in terms of behavioral, molecular, and genetic aspects were investigated. After acute sub-lethal exposure to various doses of coumaphos, the worker bees of control and treatment groups were subjected to activity monitoring tests for evaluation of locomotor activity, electric shock avoidance tests for aversive behavior, proboscis extension reflex tests for olfactory learning behavior and acetylcholinesterase (AChE) activity assay in brain and midgut tissues. The expressions of six genes related to action mechanism of coumaphos in the nervous system were measured in brain samples while three cytochrome p-450 genes specifically related to coumaphos metabolism were measured in midgut samples. Coumaphos treatment significantly increased locomotor activity and decreased aversive and olfactory learning behavior in *syriaca* whereas no significant changes were observed in terms of these assays in *caucasica*. Increased locomotor activity and decreased aversive behavior were

observed in *carnica* while. Decreased midgut AChE activities were observed in all three subspecies while this decrease was more intense in *syriaca* compared to that of the *carnica* and *caucasica*. Gene expression studies showed no significant difference in brain expressions between control and treatment groups while in midguts, expressions of CYP9Q1 was observed to be significantly decreased in *syriaca* compared to that of the *caucasica*. In terms of behavioral alterations, *caucasica* bees appeared to be the most resistant to the sublethal coumaphos exposure while *syriaca* bees were observed to be the most susceptible. Whereas *carnica* had a moderate susceptibility compared to the other two. All the results indicate that coumaphos detoxification efficiency may be a factor underlying the resistance or susceptibility to behavior altering effects of sublethal coumaphos exposure in honeybee subspecies.

Keywords: Honey Bee (*Apis mellifera*), *Varroa destructor*, Coumaphos, Learning, Acaricides, Acetylcholinesterase (AChE).

ÖZ

STRES FAKTÖRÜ AKARİSİT PERİZİN'İN TÜRKİYE BAL ARILARINA (*APIS MELLIFERA*) OLAN DAVRANIŞSAL VE MOLEKÜLER ETKİLERİ

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Varroa destructor parazitine karşı kullanılan bir akarisit olan Perizinin (coumaphos etken maddesi) üç yerli bal arısı alttürü *Apis mellifera caucasica, A. m. carnica* ve *A. m. syriaca* üzerindeki etkileri davranışsal ve biyokimyasal açıdan incelenmiştir. Farklı akut ölümcül olmayan dozlarda coumaphos uygulamasından sonra, işçi arıların kontrol ve deney grupları üzerinde lokomotor aktivitesi ölçümü için monitor deneyleri, kaçınma davranışı için elektrik şok deneyleri, öğrenme davranışı ölçümü için Proboskis uzatma deneyleri ve biyokimyasal değerlendirme içinse Asetilkolinesteraz (AChE) aktivitesi ölçümü deneyleri uygulanmıştır. Coumaphos'un sinir sistemindeki etki mekanizması ile ilişkili 6 genin ifadesi beyin örneklerinde; coumaphosu spesifik olarak metabolize eden üç sitokrom p450 geninin ifadesi de orta bağırsak örneklerinde ölçülmüştür. Coumaphos uygulanması *syriaca* için lokomotor aktivitede belirgin bir artış, kaçınma ve öğrenme davranışlarında ise belirgin bir azalma ile kendini göstermiştir. *caucasica*'da ise bu deneyler açısından belirgin herhangi bir değişiklik gözlemlenmemiştir. *carnica*'da locomotor aktivite artışı ve elektrik şoku kaçınma davranışında azalma

gözlenmiştir. Her üç alttürde de coumaphos uygulanmasının orta bağırsak AChE enzim aktivitesinde düşüşe yol açtığı gözlemlenmekle birlikte bu düşüş *syriaca*'da diğerlerine göre daha fazladır. Gen ekspresyounu çalışmalarında beyin gen ifadeleri açısında gruplar arasında herhangi bir fark gözlemlenmemiş ancak orta bağırsak analizlerinde, CYP9Q1 geninin ifade oranının *syriaca*'da diğer alttürlere göre daha düşük olduğu görülmüştür. Sonuç olarak, ölümcül olmayan coumaphos kaynaklı davranış değişimlerine en dirençli alttür *caucasica* olarak belirlenmişken *syriaca'nın* perizin kaynaklı davranışsal etkilere en hassas alttür olduğu saptanmıştır. *carnica* ise bu iki alttürün arasında bir seviyede yer almaktadır. Bütün bu sonuçlar, balarısı alttürlerinin subletal coumaphos kaynaklı davranışsal etkilere karşı dirençli ya da hassas olma durumlarının coumaphosun detoksifikasyon etkinliğine bağlı olabileceğine işaret etmektedir.

Anahtar Kelimeler: Bal Arısı (*Apis mellifera*), *Varroa destructor,* Coumaphos Pestisitler, Öğrenme, Asetilkolinesteraz (AChE).

To My Biological Father Hidayet Arslan and

Doctoral Father Aykut Kence.

May You Both Rest in Peace…

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LIST OF ABBREVIATIONS

DAM	*Drosophila* Activity Monitors
LMA	Locomotor activity
ESA	Electric shock avoidance
PER	Proboscis Extension Reflex
qRT-PCR	Quantitative real time polymerase chain reaction
$GABA_A$ beta	Gamma-aminobutyric acid receptor subunit beta
($GABA_B$ sub1	GABA type B receptor subunit 1
$GABA_B$ sub2	GABA type B receptor subunit 2
mAChR	Muscarinic acetylcholine receptor
nAChR	Nicotinicacetylcholine receptor
nAChRa5	nicotinic acetylcholine receptor alpha 10 subunit
VAChT	Vesicular acetylcholine transporter

CHAPTER 1

INTRODUCTION

1.1.1 Ecologic and Economic Importance of Honey bees

Honey bees have very important ecosystem services as pollination of wild flowering plants thus contributing to the well-being of nature. They perpetuate the plants directly and support the existence of animal species which feed on those plants indirectly. Bee species are the most reliable and effective plant pollinators in wild habitats as they regularly visit flowers for nectar and pollen collection and do not give harm to the flowers in that process (Devillers and Pham-Delaque, 2002). Western honeybees (*Apis mellifera*) were estimated to carry out 13% of insect floral activities which make them the most frequent visitors of flowers in natural ecosystems. Also, 5 % of insect-pollinated plant species were estimated to be exclusively visited by honey bees (Hung *et al.,* 2018). Honey bees are managed by humans since ancient times (Crane *et al.,* 2015). Honey bees are key pollinators for many agricultural products and this makes them more important in terms of an economical perspective (Moritz *et al.*, 2010). Approximately 35 % of agricultural food production was estimated to rely on pollination by insects through direct or indirect ways (Delaplane and Mayer, 2000). In the US, the value of pollination by honeybees was estimated as 11.68 billion Dollars in 2009 (Calderone, 2012). In Europe, although the economic value of honey production is estimated as 140 million Euros, the economic contribution to crop production is nearly ten folds as 14.2 billion Euros (Moritz *et al.*, 2010). Insect pollination in the whole world formed an economical share of 153 billion Euros in 2005 (Gallai *et al.*, 2008). Nutritional, medical, and pharmaceutical applications of honeybee products form a significant market.

1.1.2 Basic Honey Bee Biology

Honey bees are eusocial insects living as colonies in their nests called hives. Each colony consists of a single mated queen, sterile female worker bees in tens of thousands and male drones in several hundred. Mating is the sole function of a drone. Matings between drones and queens occur in flight at specific sites called drone congregation areas. Queen bees mate with as many as 15 or more drones, storing sperms in their spermatheca, return to their hives and spend the rest of their life as egg layers Drones die after mating. Earlier it was known that queens lay two types of eggs; unfertilized haploid eggs hatch into drones while diploid eggs fertilized with sperm hatch into workers (Winston, 1987). Now it is known that a gene named as complementary sex determiner (csd) determines the sex of bees. At least 15 alleles of this gene are present and if an egg contains two different alleles, it develops into a female whereas an egg possessing one sex allele develop into a male. Embryos containing two same alleles develop into diploid males, but these are eaten by workers (Beye *et al*., 2003; Hasselmann and Beye, 2004). Worker bees perform all the tasks essential to the vitality of the colony. While carrying out these tasks, workers show a labor division according to their ages. Newly emerged worker bees (1 to 4 days) perform cleaning of honeycomb cells. These young workers then shift to nursing and feeding of larvae (4 to 12 days). Middle-aged workers (12 to 21 days) carry out responsibilities like construction and maintenance of the hive nests, processing flower nectar into honey, and as guards against intruders in the hive entrances. Middle-aged workers then start foraging on the field until their death after usually a couple of weeks. Foragers collect four essential components for the colony: nectar, pollen, propolis (bee gum), and water (Johnson, 2010).

1.1.3 Honey bee Diversity and Distribution

The genus *Apis* contains 10 species and among them *Apis mellifera* (Western Honeybee) and *Apis cerana* (Eastern Honeybee) are considered sister taxa (Arias and Sheppard, 2005) and these two species are domesticated by humans (Thakar, 1973) .*A. cerana* is located in the south and southeast Asia and China with 8 subspecies while *A. mellifera* is distributed throughout the rest of the world by human migration and has 28 designated subspecies in the world (Engel, 1999). Turkey has 5 subspecies namely *A. m. caucasica* at northeastern Anatolia, *A. m. anatoliaca*, at western and central Anatolia, *A. m. carnica* at Thrace region , *A. m. meda* at southeastern Anatolia, and *A. m. syriaca* at Syria border region of southeastern Anatolia, (Ruttner, 1988; Kandemir *et al*., 2000, 2005; Figure 1.1). Studies showed considerable genetic diversity and differentiation between honey bee populations from various regions of despite the homogenizing effects of migratory beekeeping (Tunca, 2009, Kükrer, 2013).

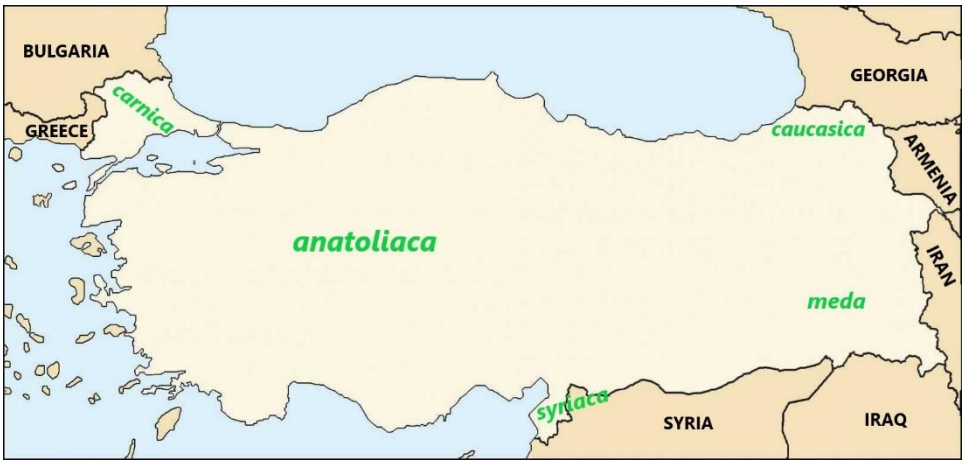

Figure 1.1. Honey bee subspecies present in Turkey

1.1.4 Colony Collapse Disorder, *Varroa* Infestation and Acaricides

Recently, an increased rate of sudden and heavy colony losses was observed firstly in Europe and later, the US. (Faucon *et al.,* 2002; van Engelsdorp *et al.,* 2007) these colony losses were defined by the sudden disappearance of adult worker bees with lack of dead worker bees in or around the hives and, also the lack of parasites such as wax moths (*Galleria melonella*) and small hive beetles (*Aethinatumida*) which usually infest dead honey bee colonies. This phenomenon was named as Colony Collapse Disorder, CCD, (Oldroyd, 2007). Several studies proposed causes for CCD such as viruses (Cox-Foster *et al.,* 2007), parasitic *Varroa* mites (Le Conte *et al.,* 2010) microsporidian pathogen *Nosema ceranae* (Higes *et al.* 2009; Paxton, 2010) and pesticides (Gross, 2008; Johnson *et al.*,2010). It is generally accepted that CCD is not developed by a single cause but complex interactions of pathogens, parasites, and other stress factors (vanEngelsdorp *et al.,* 2009). One of these, interactions between *Varroa* parasite and viruses were shown to have significant involvement in colony losses (Le Conte *et al.* 2010; Martin *et al.,* 2012).

Varroa mite is one of the most harmful parasites of honey bees. *Varroa* infests both the brood and adult feeding with their hemolymph. Loss of hemolymph loss causes reductions in organ development, weight loss, and reduced life span in bees (De Jong, 1990). *Varroa mite* causes a serious nutritional deficiency in honeybees which have a negative role in their vitality (Garedew et al., 2004). Parazitation also negatively affects the flight duration and navigational capacities of foragers (Kralj and Fuchs, 2006). *Varroa* also acts as a vector for secondary infections, especially viruses such as KBV (Kashmir bee virus, Chen et al., 2004), SBV (Sacbrood virus, Shen *et al,* 2005), ABPV (Acute bee paralysis virus, Bekesi *et al,* 1999), IAPV (Israeli acute paralysis virus, DiPrisco *et al.,* 2011), and DWV (Deformed wing virus, Bowen-Walker *et al.,* 1999). Simultaneous infection of *Varroa* and viruses is called parasitic mite syndrome which causes the ultimate death of the colony (Shimanuki *et al.,* 1994). Infected bees spread the mite to other colonies through behaviors such as drone adoption, bee drifting, and robbing. Transporting of

colonies throughout the country by migratory beekeepers is another factor for *Varroa* spread (Boecking and Genersch, 2008).

Varroa is not a serious pest on its original host *Apis cerana* the Asian honeybee due to its reproduction exclusively limited to drone brood (Rath, 1999). It seems that there is a well-adapted relationship as a result of co-evolution between the parasite and host. However, when novel colonies of western honey bee *Apis mellifera* were introduced to East Asia, *Varroa* also spread to them. The first incidents of spread from *cerana* to *mellifera* was observed in Japan in 1957, 80 years after the latter was introduced (Sakai and Okada, 1974). Another contact area was the far east of former Soviet Russia where *mellifera* colonies were infected with *Varroa* from Korea (Crane, 1978). From these routes, *Varroa* spread to all western honey bee colonies in the world except Australia (Rosenkranz *et al.,* 2010). Colonies infected with *Varroa* inevitably collapse within two to three years without proper treatment (Boecking and Genersch, 2008). *Varroa* mite entered Turkey in 1977 and spread to all regions of Turkey in 1984; annual colony loss due to *Varroa* was estimated as 600.000 colonies (Aydın *et al.*, 2007).

There is no absolute effective treatment for *Varroa* infection. Numerous chemicals that are used to control *Varroa* infection can be divided into two groups: miticides with synthetic and persistent active ingredients such as amitraz, flumethrin, fluvalinate and coumaphos; and miticides with natural, non-toxic active ingredients such as thymol, lactic acid, oxalic acid and formic acid (Bogdanov, 2006). Synthetic miticides are fat-soluble and therefore accumulate in the beeswax and propolis (Bogdanov *et al.,* 2006, Wallner, 1999). As the concentrations of these substances increase in the bee wax, their probability of passing through to the honey will also increase. Natural ingredients have lower residue levels compared to synthetic ones (Bogdanov, 2006) but they are also less effective (Imdorf *et al.*, 1999). Another problem is the resistance developed by *Varroa* against those chemicals. Populations resistant to fluvalinate (Milani, 1995; Lodesani *et al.*, 1995), coumaphos (Pettis, 2004) amitraz and flumethrin (Trouiller, 1998, Elzen *et al.*, 1999, Rodriguez-Dehaibes *et al.*, 2005) were reported.

1.1.5 Honey bee Behavior and Pesticides

In honey bee colonies, worker bees forage the landscape for nectar, pollen, water, and propolis (resin). This foraging task can last for minutes to hours. Young bees perform training flights around their colony and practice to navigate themselves using the direction of sunlight and specific landmarks around their colony site. Worker bees return from foraging to the hive inform fellow foragers about the distance, location, and the quality of the food source in a form of ritualistic behavior called the waggle dance. All these show the importance of learning and memory in the honey bee life cycle. (Menzel, 1993; Hammer *et al.,* 1995). Most pesticides are neurotoxins and therefore have the potential to alter learning, memory (Siviter *et al.* 2018), and motor functions of honey bees (Tosi *et al.* 2017).

Pesticides used in agriculture can contaminate foraging honeybees outside of their hives. Beekeepers also apply pesticides directly into the hives to control bee parasites, especially *Varroa*. Indeed, one neonicotinoid type insecticide, imidacloprid is suggested as a significant contributor in colony collapse disorder (Johnson *et al.,* 2010; Lu *et al.,* 2012). Neonicotinoids mimic neurotransmitter acetylcholine and bind to Nicotinic acetylcholine receptors. Continuous stimulation of these receptors by neonicotinoids eventually causes hyper excitation, paralysis, and death (Jeschke *et al.,* 2008). Imidacloprid was reported to disrupt olfactory learning and memory, (Decourtye *et al.*,2004a, b), basic motor functions (Williamson *et al.,* 2014), and foraging activity (Schneider *et al.,* 2012). Synthetic in-hive acaricides used against *Varroa* also has numerous reported detrimental effects on honey bees. Tau fluvalinate and flumethrin are pyrethtorid insecticides and act through the blocking of voltage-gated sodium and calcium channels. This blocking impairs impulse transmission on nerve cells, which leads to paralysis and eventually, death (Davies *et al.,* 2007).Tau fluvalinate was shown to disrupt olfactory learning and memory (Frost *et al.*,2013), cause deficits in locomotor functions (Charreton *et al.*, 2015), increase time spent in food source and decrease bee to bee interactions (Teeters *et al.*, 2012). Tan *et al.* (2013) reported that worker

bees sampled from Asian honey bee (*Apis cerana*) hives treated with flumethrin show reduced olfactory learning performance compared to control hives even after 2 weeks after treatment. Amitraz is an agonist of octopaminergic receptors (Johnson *et al*., 2010). Octopamine is found to be an important modulator in foraging (Barron *et al.*, 2007; Giray *et al.*, 2007), and learning (Agarwal *et al.*, 2011; Behrends and Scheiner, 2012) in honey bees. Therefore, amitraz has the potential to alter or disrupt these processes through its interference on octopaminergic signaling. However, a topically applied sublethal but relatively high doses of amitraz to honey bee workers were found to be not affecting learning and memory (Rix *et al.*, 2016). Coumaphos is an organophosphate pesticide that acts as an inhibitor of the enzyme acetylcholinesterase, which is involved in breaking down the neurotransmitter acetylcholine. This causes excessive levels of acetylcholine which leads to increased excitation, paralysis, and death (Dahlgren, 2014). Sublethal doses of coumaphos were reported to have a modest impairment of olfactory learning (Williamson *et al.*, 2013a). Visual observation also showed that bees exposed to sublethal doses of coumaphos displayed decreased walking and increased self-grooming behavior, had more difficulty to turn themselves when fell over, and had abdominal spasms which are not seen in the control group (Williamson *et al.*, 2013b). Perizin is an insecticide produced by Bayer Company for control of *Varroa* infestation in honeybee colonies. It contains 3.2% coumaphos as an active ingredient. Bevk *et al.* (2012) determined that an acute Perizin dose containing 5µg of coumaphos significantly reduced food transfer between worker bees. Cizelj *et al.* (2016) also observed the downregulation of immune-related genes in colonies treated with Perizin.

1.1.6 Aim of the Study

In this study I tried to provide information on the following questions the following questions:

1. How does sublethal doses of coumaphos exposure affects locomotor activity?

2. How does sublethal comaphos exposure affects aversive and appetitive learning in honeybees?

3. Does the levels of gene expression and enzyme activity involved in coumaphos action and metabolism change?

4. Are there differences among honeybee subspecies of Turkey in response to coumaphos administration?

5. Can we make use of the information obtained in order to develop strategies to prevent colony losses related?

CHAPTER 2

MATERIALS AND METHODS

2.1 Subspecies Analysis

Our study includes the following subspecies maintained in our apiary (Figure 2.1) located near the Biology Department of Middle East Technical University: *A. m. caucasica* (from Borçka, Artvin Province in North East Anatolia), *A. m. carnica* (from Kırklareli province in Thrace Region) and *A. m. syriaca* (from Arsuz, Hatay province in South Anatolia). Authenticities of these subspecies were checked by microsatellite analysis (Bodur *et al*., 2007; Ivgin-Tunca, 2009) each year against hybridization. Our electric shock avoidance and activity monitoring assays were performed using these colonies.

Our *syriaca* colonies were lost in the winter 2016- 2017 period and therefore, new *syriaca* colonies were obtained from Samandağ, Hatay in 2017 spring. PER and AChE activity and gene expression assays were done using these colonies.

Due to financial problems, geometric morphometry (Kandemir, Kence & Kence, 2005) was utilized to assess the authenticity of the new *syriaca* colonies. Left wings were collected from worker bees from each subspecies. Sample sizes were 20, 20, and 30 for *caucasica*, *carnica* and *syriaca* respectively. Photos of the wings placed between two glass slides were taken by a digital camera (LAS EZ) system coupled with a stereomicroscope (LEICA 8AP0). 20 landmarks determined in each photo (Figure 2.2) are digitized into .tps files which include landmark coordinates, by TpsDig version 2.2 software. Then, Morpho-J version 1.06d software was utilized to process landmark coordinates and carry out geometric morphometry analysis (Klingenberg, 2011). The mahalanobis distances and their significance

determined by T square statistic with 10,000 permutations were 7.36 ($p<0.0001$), 7. 35 ($p<0.0001$), and 6.56 ($p<0.0001$), for *caucasic*a-*carnica*, c*aucasica*-new *syriaca* and *carnica*-new *syriaca* comparisons (Figure 2.3).

Figure 2.1. Locations of the common apiary and colony sources.

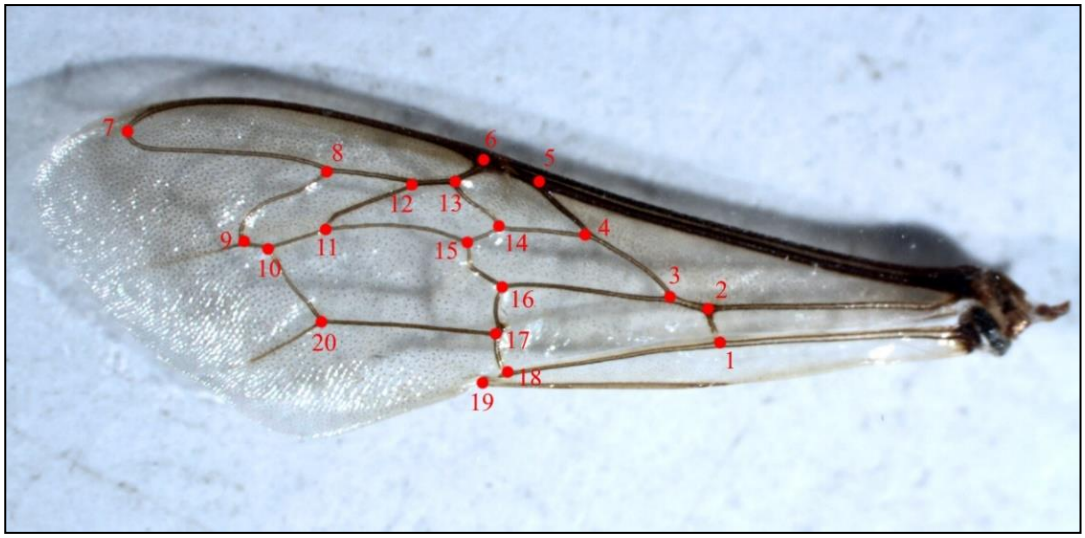

Figure 2.2. Landmark positions on a honey bee wing.

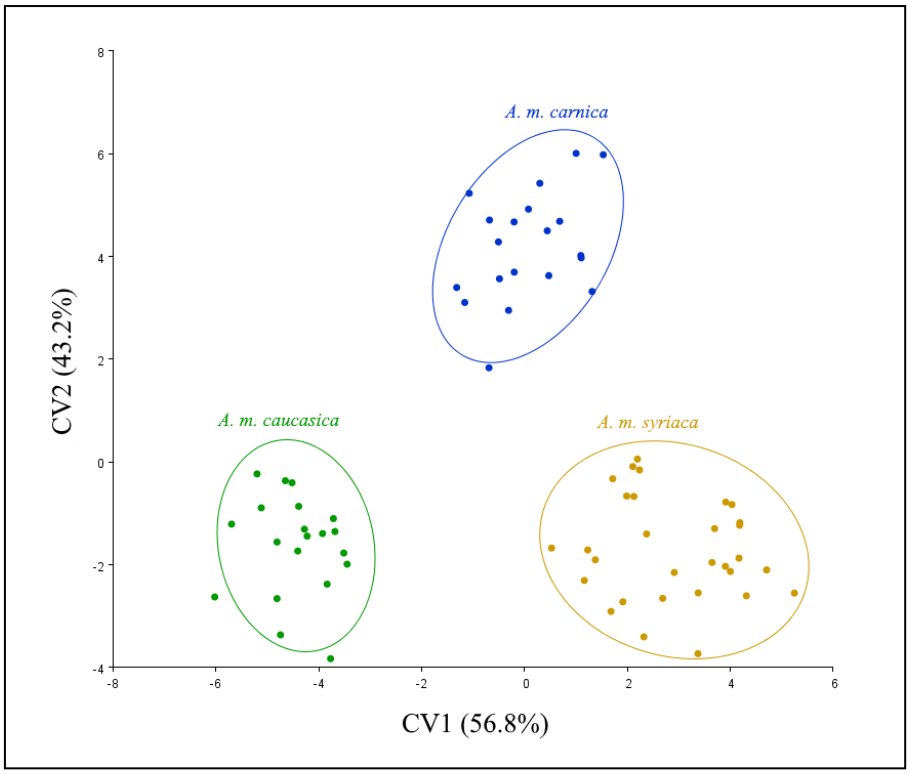

Figure 2.3. Canonical variate analysis (CVA) based on the landmarks on the wing of the honey bee. 56.8% of the variation is represented by CV1 while 43.2% of the variation is represented by CV2.

2.2 Sampling and Perizin Administration

Worker bees were sampled from inside of the hives for the experiments. After sampling, all test bees were brought to the laboratory for Perizin administration. Honey bees were separated into four experiment groups for each of the subspecies: control, 1, 2 and 5 µg treatment groups. Each bee in dose groups received a single administration of 10 µl of 50 % sucrose solution, which contains Perizin amounts equal to 1, 2, or 5 µg of coumaphos while bees in the control group received a 10 µl of % 50 sucrose solution only. Administrated doses and their preparation procedures were given in Table 2.1. Only control and 5 µg coumaphos dose groups were used for shock avoidance, proboscis extension conditioning, acetylcholinesterase activity, and gene expression assays. Honey bees prepared for locomotor activity monitoring were immediately put into locomotor activity

monitor while bees prepared for shock avoidance, proboscis extension reflex (PER) and AChE activity analyzes were incubated at 33°C and 55% (±5) humidity condition in an incubator for four hours to let the bees fully metabolize ingested coumaphos (Van Buren *et al*., 1992). Shock avoidance and PER analyzes were performed immediately after the incubation period while other bees were immediately frozen at -80 °C for later to be used in AChE activity and gene expression analysis.

Table 2.1. Preparation procedure for coumaphos treatment groups

Dose groups	**Perizin**	**%50 Sucrose**
5 µg coumaphos	0.032 µl	9.969 µl
2 µg coumaphos	0.063 µl	9.938 µl
1 µg coumaphos	1.563	9.848 µl

2.3 Locomotor Activity (LMA) Monitoring

Activity monitoring was performed using a high-resolution system (Figure 2.4). An incubator was used to house the monitors and to provide suitable environmental conditions (33°C and 55% (±5) humidity) during assays. Each experiment was performed in dark for 24-hour. For each experiment, 4 activity monitors were used, and 30 test bees were monitored in each monitor. Therefore, 10 bees for each study group and 40 bees for each subspecies (*A. m. caucasica*, *A. m. carnica*, and *A. m. syriaca*.) which makes a total of 120 test bees monitored in each experiment. The number of test bees among different doses and different test groups were kept equal for all experiments. Sample sizes for each group and subspecies were given in Appendixes A, B and C.

The working mechanism of the activity monitoring system is simple. Each test bee was placed into a 15ml falcon tube and each tube was placed into a different cell of the monitor. The infrared light sources around each cell detect the activity of the bees found in the tubes. The monitor sends a signal to computer software whenever a bee passes in front of the light sources around its tube. The software records signals from every monitor and every cell separately.

Activity monitors can send signals at different time intervals varying from 1-second to 60-minutes ranges. For all experiments, measurements were made at 1-minute time intervals. Hence, for each experiment and each test bee, 1440 data were recorded.

Figure 2.4.One of the activity monitors used in the experiments. The activity monitor has 32 cells and each cell can take 15ml (dimensions: 17 mm O.D., 120 mm length) falcon tube.

During the experiments, an environment monitor was used in addition to the activity monitors. The environment monitor keeps track of environmental parameters (temperature, humidity, and light) and makes measurements at each minute. The data provided by the environment monitor were checked after each

experiment to detect any undesired change in environmental conditions that might occur during the experiments.

2.4 Proboscis Extension Reflex (PER) Conditioning Analysis

PER conditioning analysis was carried out based on Abramson *et al.,* (2011) with some modifications. Forager honey bees collected from outside of the hives were harnessed to bullet cases (Figure 2.5) and incubated for 24-hour in an incubator at 25°C temperature and 70 % (±5) humidity. Each bee in the treatment group received a single dose of 10 µl sucrose solution containing 5 µg coumaphos while the control group received only 10 µl sucrose solution. Treated bees were kept in an incubator for an additional three hours and then subjected to PER conditioning.

PER conditioning was performed in a ventilated cabin. Sample sizes for each group and subspecies were given in Appendix D. The conditioning process consists of two phases: acquisition and extinction. In the acquisition process, each bee was subjected to an odor for three seconds using a 50 ml syringe containing a piece of filter paper dipped in essential oil containing odor. Immediately after giving odor, a cotton swab dipped in 50 % sucrose solution was touched to the antenna, then to the now extended proboscis for a 3-second feeding as a reward. 12 conditioning trials were performed for each subset of bees with 10-minute intervals. Honey bees that extended its proboscis during odor administration were considered as positive (1) while bees which only extended their proboscis to sucrose feeding were considered as negative (0). Honey bees that did not respond to both odor application and sucrose feeding were discarded from the experiment. In the extinction phase, bees which responded positively to odor administration were used. This time only odor was applied without any sucrose reward. Again 12 conditioning trials were performed for each subset of bees with 10-minute intervals. Bees that extended their proboscis during odor administration, were considered as positive (1) while bees that did not respond to odor were considered negative (0). Briefly, acquisition phase measures how many trials shall a bee be

conditioned to respond to a specific odor while extinction phase measures in how many trials will the conditioning be broken.

Figure 2.5. Honey bees harnessed to bullet cases for PER conditioning analysis.

2.5 Avoidance Conditioning

Avoidance conditioning analysis was performed based on Agarwal et al., (2011) with some modifications. The apparatus used for avoidance conditioning consists of two parts: a lower metal grid placed on a double colored surface for electric shock application and an upper plastic tube with a transparent roof to confine and watch the movement of individual honey bees (Figure 2.6). One bee is placed into the plastic tube and kept in there for 2 minutes for acclimation to the device and then bee is subjected to 6 volts of electric current by binding two electrodes to the corners of the metallic grid. The electric shock area corresponds to the blue area of the surface while the safe zone is a yellow color area. Electric shock treatment lasted for 5 minutes and during this time, the operator watched the back and forward movements of the bee in the tube. Using a chronometer, the numbers of the entrance to the blue electric shock area and the time spent in it were recorded. Results were expressed in mean ± SE and $p < .05$ was considered significant. Sample sizes for each group and subspecies were given in Appendix E.

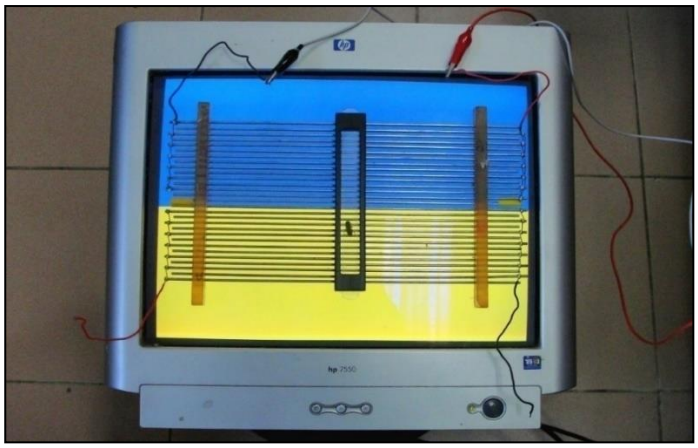

Figure 2.6. Electric shock grid system for avoidance conditioning trials of honey bees.

2.6 AChE Activity Analysis

Brains and midguts of previously frozen control and treatment bees were dissected on dry ice. Sample sizes for each group and subspecies were given in Appendix E. Each brain or midgut was put into a 1.5 ml centrifuge tube. After the addition of molecular biology water (250 µl for brain and 350 µl for midgut), samples were homogenized by microtube pestles. Slurry and particles were removed from each homogenate by filtering through a 5 ml syringe with a small piece of cotton stuffed into. AChE activities were determined by Ellman's Assay (Ellmann *et al.*, 1961). The reaction mixture contained 25 µl of homogenate, 280 µM of 5´- 5´dithiobis-2-nitrobenzoic acid (DTNB), and 0.86 mM of Acetylthiocholine. Total protein contents were measured by Bradford assay and results were expressed as Units/mg protein.

2.7 Gene Expression by Real-time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR (qPCR) technique has been utilized to measure relative expression changes of selected genes. For that, RNAs were extracted from whole bee brain tissues kept in RNA later tissue storage solution at -80^{o}C. For brain tissue RNA's,

no DNase treatment applied since the primers used with brain samples were designed as intron-spanning. Midgut samples were DNase treated with Invitrogen Ambion Turbo DNase kit since primers could not be designed to span introns since some genes were composed of one exon. cDNA synthesis from RNA samples was conducted using the Bio-Rad iScript Reverse Transcription Supermix kit. For qPCR experiments, Life Science's Light Cycler® 480 SYBR Green I Master kit, and Light Cycler® 480 white 96 Multiwell Plates were utilized with Light Cycler® 480 Instrument. The protocol was applied using 2 µl of cDNA, 2 µl of primer mix, 6 µl of nuclease-free water and 10 µl of SYBR Green I dye. Primers were designed via NCBI's Primer-BLAST tool for nicotinic acetylcholine receptor subunit alpha-10 (nAChRa5), muscarinic acetylcholine receptor (mAChR), vesicular acetylcholine receptor (VAChT), GABA receptor subunit beta ($GABA_A$ beta), GABA type B receptor subunit 1 ($GABA_B$ sub1), GABA type B receptor subunit 2 ($GABA_B$ sub2) for brain samples; cytochrome P450 9e2 Q1 (CYP9Q1), cytochrome P450 9e2Q2 (CYP9Q2), cytochrome P450 9e2Q3 (CYP9Q3) for mid-gut samples and; Glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH) and 40S Ribosomal protein S18 (rps18) as internal controls. Gene ID's and primer sequences were given in Table 2.2. We first designed primers for nicotinic acetylcholine receptor alpha 6 subunit (LOC551010) as it was observed to have expression differences in Wang *et al.*, (2013) but the primers did not work in qPCR which may be related to provisional Ref Seq status of this gene. We then designed primers for subunit alpha 10 which has an annotated Ref Seq status and this time qPCR was successful. Two to three technical repeats of qPCR experiments were applied with 6 brain and midgut samples for every 4 groups as *caucasica*-treatment, *syriaca*-treatment, *caucasica*-control, *syriaca*-control. For the analyzes, geometric means of two reference genes, GAPDH and rpS18, were taken and the delta ct method was utilized for calculations of relative expression values.

Table 2.2. Primers for genes.

Gene	ID	Forward primer	Reverse Primer
rps18	5552726	GCAAGATGTCTCTCGTCATTCC	ACCGTCAATATTCGTGCCCA
GAPDH	410122	CTGCACAGACCCGAGTGAAT	CAACAACCTGAGCACCGAAC
mAChR	412299	GAAGGAAACCAAGAAGCGGC	GTCTAACGCTTCATCGCTGG
nAChRa5	408525	TTTGGACGGACCATCACCTG	TGTGGATCGGCGTTGTTGTA
VAChT	725064	GACGTTCCAAAGCTACCCCA	TCTAACCGAGCTGAGACCGA
$GABA_A$ beta	406124	GAACTACGGAGGTCCACCAG	TCAACACTTCGGACACGGAG
$GABA_B$ sub1	113218647	GAGGTTCGTTACCTCCCGAC	GGCTCACACTGGCTGTCAT
$GABA_B$ sub2	410140	GAGACCGAAATTCCCAGGCA	GCCGAAACGGAATGTCGATG
CYP9Q1	410492	ATAGCGAGATGCGTGTACGG	TGTGAACGGGCAGGATCTTC
CYP9Q2	408452	AAACGTGCGTGCTTCTTCAC	GACAATTGGCCGTTGTTGCT
CYP9Q3	408453	CATGCTGTTCGCGATGAAGG	CAGCACGGACAAAAAGTCGG

2.8 Statistical Analysis

The SPSS program was used for statistical analyzes. The normality of data was determined by the Shapiro-Wilk test. In the comparisons of control and treatment groups of each subspecies, Student's t-test was applied for normally distributed data and, log or square root transformation was used if one or both groups were not normally distributed. In case even transformation could not normalize data, both parametric t-test and non-parametric Mann-Whitney U test was applied to ensure the results are robust to normality assumptions. Because each subspecies has its control and treatment groups with unique distributions, p values obtained by

statistical comparison of control and treatment groups of each subspecies cannot be used to make a comparison between the three subspecies. Therefore, treatment groups of each subspecies were normalized to their controls to determine possible differences among subspecies in terms of their response to Perizin administration. This was done by dividing each variable of a subspecies treatment group data to the mean of its respective control group data. This new ratio data was log-transformed and were analyzed to compare the subspecies. One-way ANOVA with post hoc Tukey test is used for normally distributed data. If data of one or more subspecies groups did not fit a normal distribution, both One-way ANOVA and the non-parametric Kruskal-Wallis test with post hoc analysis were applied for statistical validation. In PER assay, odd ratio was used to analyze the differences between proportions of bees, which are either died or became unresponsive to sucrose during experiment trials, in control and treatment groups of subspecies.

CHAPTER 3

RESULTS

3.1 Locomotor Activity

Graphics, which were made from pooled locomotor activity (LMA) data of 475 worker bees in four sets of experiments, were shown in Figure 3.1. The graphics indicate that most visible LMA differences between groups are present in *syriaca* subspecies. Therefore, first 12-hour, 24-hour and second 12-hour periods of total locomotor activities of experimental groups for each subspecies were analyzed.

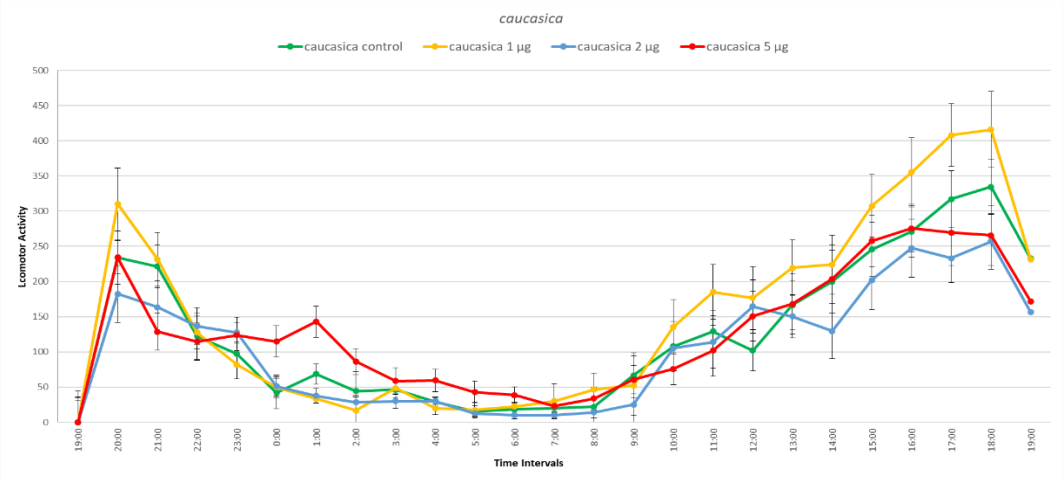

Figure 3.1. Means and standard errors of total locomotor activities of each hour in control and treatment groups of bees.

3.1.1 Analysis of First 12-hour Activity

Statistics of control vs 1 µg comparisons of three honey bee subspecies for 12-hour activities of each individual were given in Tables A.1 to A.4 of appendix A.

Barplot graphic of the data was given in Figure 3.2. Because distributions were not normal and log transformation could not be applied due to the presence of 0 value

in data of *syriaca*, square root transformation was applied which normalized distributions except *syriaca* 1 µg treatment group. Both parametric and non-parametric comparisons failed to show any significance between control and treatment groups of any subspecies ($p > .05$).

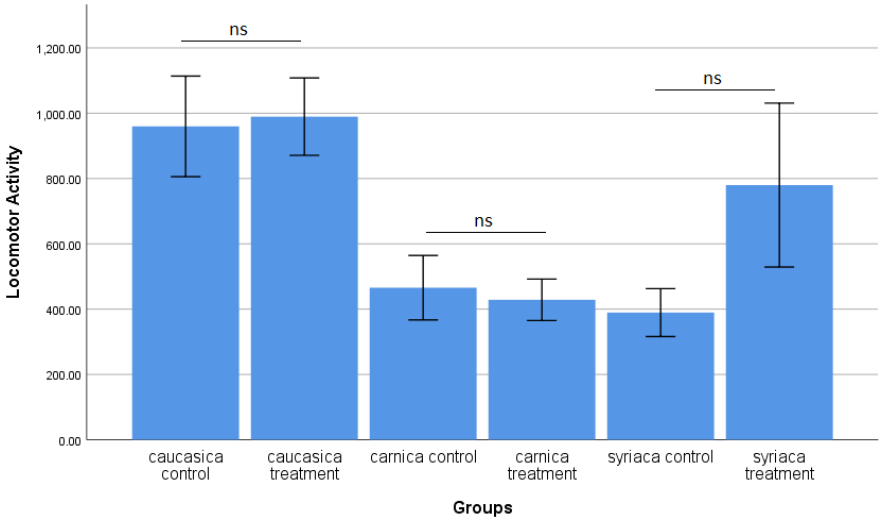

Figure 3.2. Comparison of the effect of 1 µg coumaphos dose in terms of first 12-hour locomotor activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

In control vs 2 µg comparison, (Figure 3.3, Tables A.5 to A.8 in Appendix A) square root transformation normalized all control and treatment groups data except *carnica* treatment group. Therefore, both parametric and non-parametric tests were applied for *carnica*. *syriaca* treatment group showed a significant increase in LMA ($p < .05$) while no significant difference was found in *caucasica* and *carnica* subspecies ($p > .05$) as indicated by Student's t and Mann-Whitney U (for *carnica*) tests.

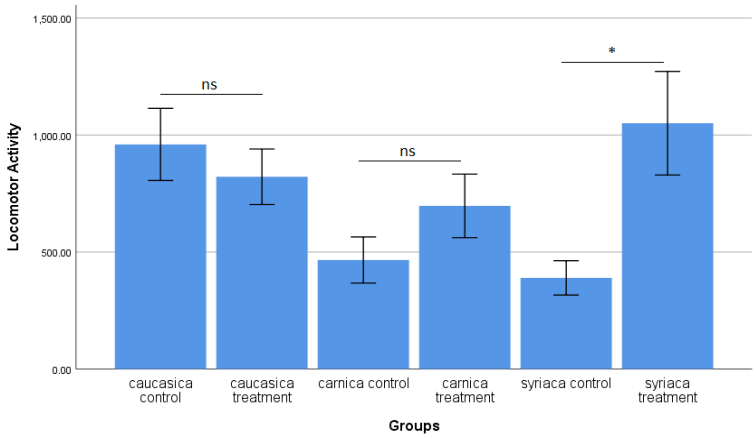

Figure 3.3. Comparison of the effect of 2 µg coumaphos dose in terms of first 12-hour locomotor activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

In control vs 5 µg comparison (Figure 3.4; Tables A.9 to A.11 in Appendix A) square root transformation managed to normalize all data removing the need for a non-parametric test. t-test comparisons indicated a significant increase of LMA in the treatment group of *syriaca* compared to controls ($p < .001$) while a marginally significant ($p = .049$) no significant ($p > .05$) difference was found in *carnica* and *caucasica* subspecies respectively.

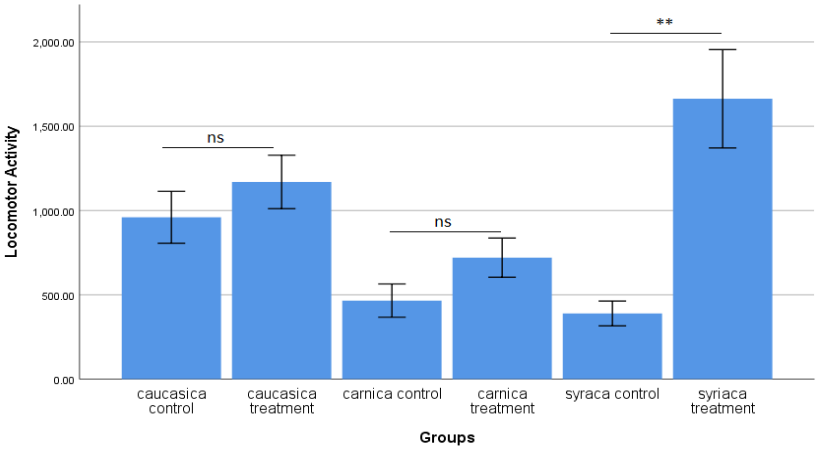

Figure 3.4. Comparison of the effect of 5 µg coumaphos dose in terms of first 12-hour locomotor activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

Because control vs 5 µg treatment group provided normalized results for all three subspecies, normalized treatment data were used the further compare the LMA's. (Figure 3.5; Tables A.12 to A.14 of Appendix A). One-way ANOVA with post hoc comparisons of log-transformed data showed that *syriaca* has a significantly higher LMA ratio compared to *caucasica* and *carnica* ($p < .001$) while the latter two did not significantly differ from each other ($p > .05$).

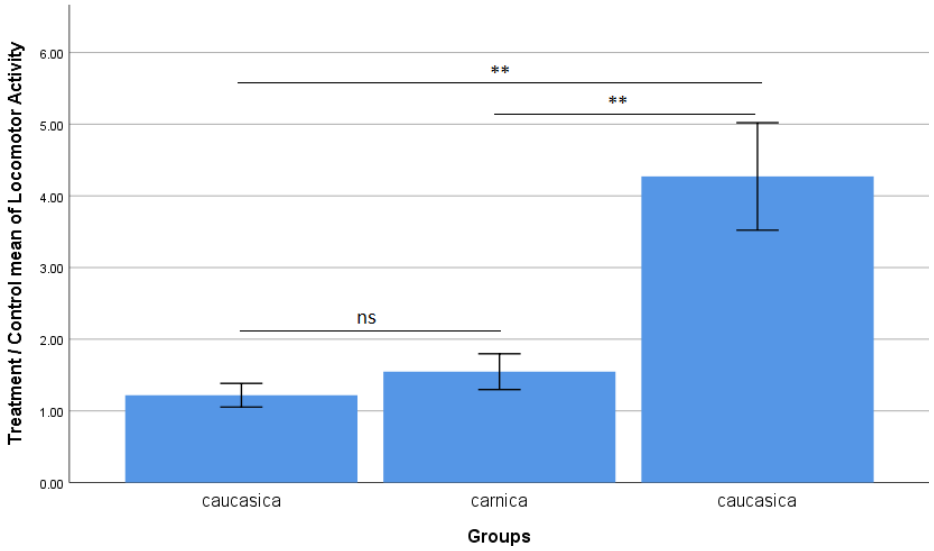

Figure 3.5. The effect of 5 µg coumaphos dose on honeybee races in terms of first 12-hour locomotor activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.1.2 Analysis of 24-Hour Activity

Statistics of control and 1 µg treatment groups for 24-hour analysis of LMA's of each individual were given in Tables B.1 to B.3 of Appendix B.

Control vs 1 µg treatment comparisons of 24-hour data (Figure 3.6) revealed no difference ($p > .05$) between control and treatment groups of all three subspecies after data normalization by square root transformation.

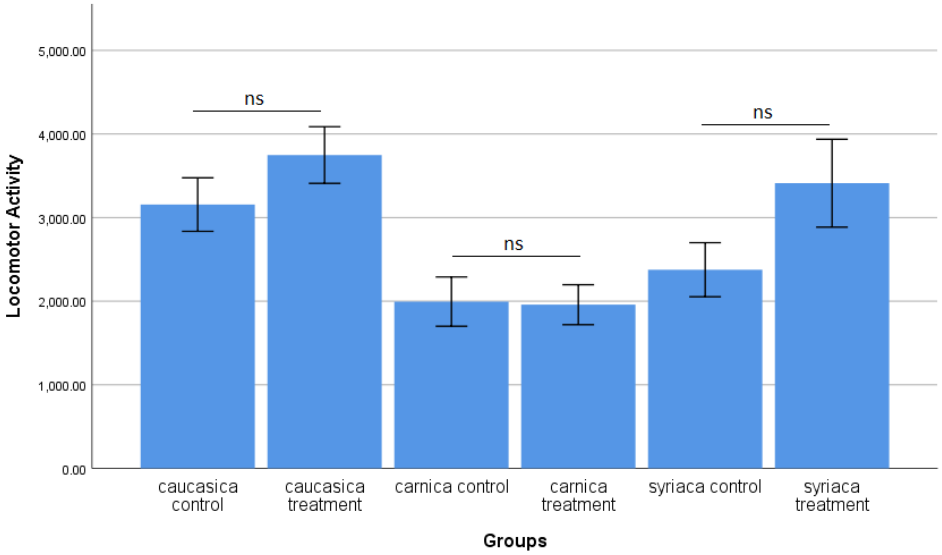

Figure 3.6. Comparison of the effect of 1 µg coumaphos dose in terms of 24-hour locomotor activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

In control vs 2 µg treatment comparisons (Fig 3.7; Tables B.4 to B.8 in Appendix B), log transformation normalized *caucasica* and *carnica* but not *syriaca*. Student's t-test comparisons revealed no significant difference between control and treatment groups of *caucasica* ($p > .05$) significant difference was observed between control and treatment groups of *carnica* and *syriaca* ($p < .05$). Non-parametric Mann-Whitney U test, on the other hand, revealed a marginally significant difference ($p = 0.048$) in *carnica* and no significant difference in *syriaca* ($p > .05$).

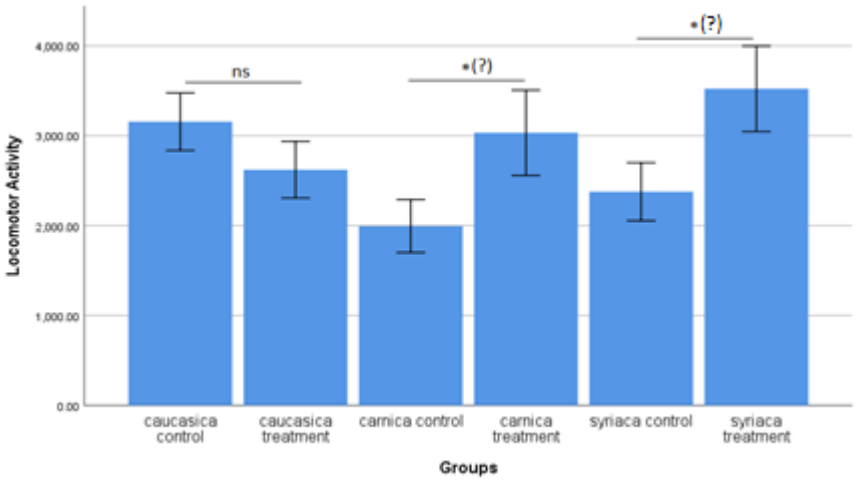

Figure 3.7. Comparison of the effect of 2 µg coumaphos dose in terms of 24-hour locomotor activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

In control vs 5 µg treatment data, (Figure 3.8; Tables B.9 to B.11 of Appendix B) square root transformation normalized all experimental groups. Student's t-test comparisons revealed no significant difference between control and treatment groups of *caucasica* and *carnica* ($p > .05$) while LMA of *syriaca* treatment group was significantly higher ($p < .05$) compared to control group.

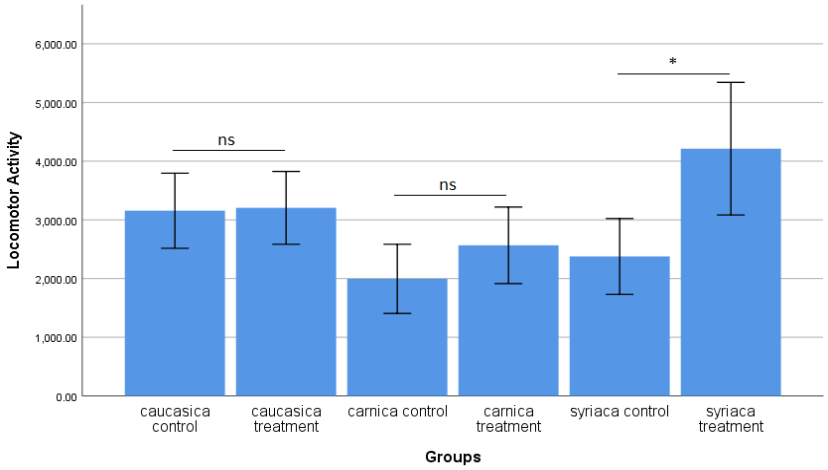

Figure 3.8. Comparison of the effect of 5 µg coumaphos dose in terms of 24-hour locomotor activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

As with the 12-hour of data, normalized control means were used to further compare the effects of coumaphos treatment on subspecies (Figure 3.9; Tables B.12 to B.14 in Appendix B). One-way ANOVA with post hoc comparisons showed that LMA of *syriaca* was significantly increased compared to *caucasica* ($p < .05$) while no significant difference ($p > .05$) was found in *caucasica- carnica* or *carnica-syriaca* comparisons.

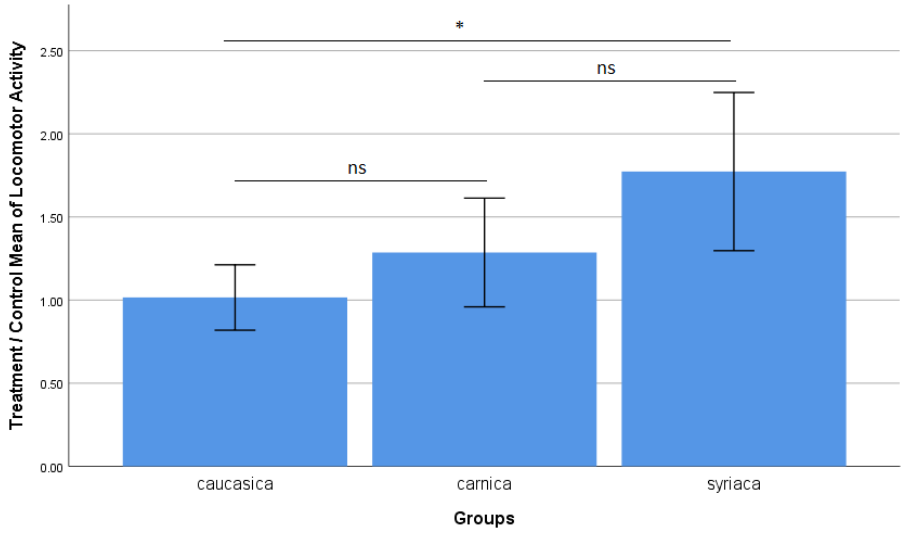

Figure 3.9. The effect of 5 µg coumaphos dose on honeybee races in terms of 24-hour locomotor activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.1.3 Analysis of Second 12-Hour Activity

In control vs 2 µg treatment data (Figure 3.10) of last 12- hour analysis, log and square root transformations failed to normalize *carnica* control and treatment data respectively and therefore, both parametric and non-parametric tests were utilized. No significant difference was found between control and treatment groups of *caucasica* and *syriaca* ($p > .05$) whereas treatment group of *carnica* was significantly increased ($p < .05$) compared to its controls in t-test comparisons. In non- parametric Mann-Whitney U test however, no significant difference ($p > .05$) was found between groups of *carnica* (Tables C.1 to C.4 of Appendix C).

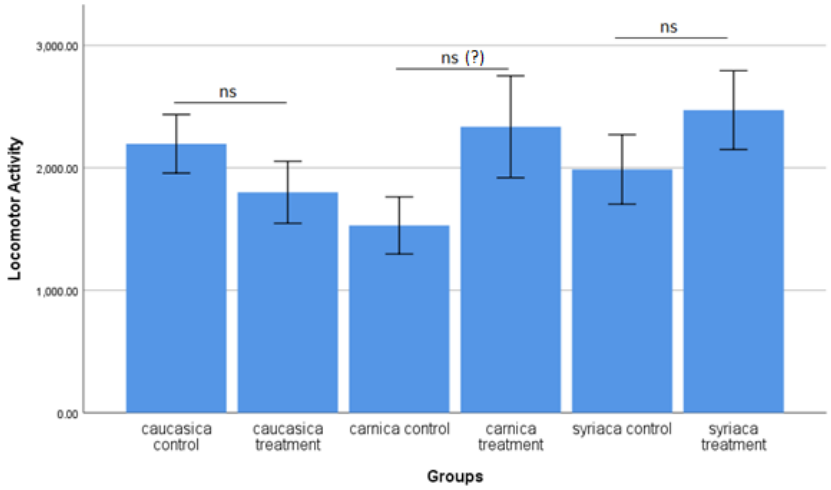

Figure 3.10. Comparison of the effect of 2 µg coumaphos dose in terms of second 12-hour locomotor activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

In control vs 5 µg comparison (Figure 3.11) square root transformation normalized data of all groups. t-test analysis showed no significant difference ($p > .05$) between control and treatment groups of all three subspecies (Tables C.5 to C.7).

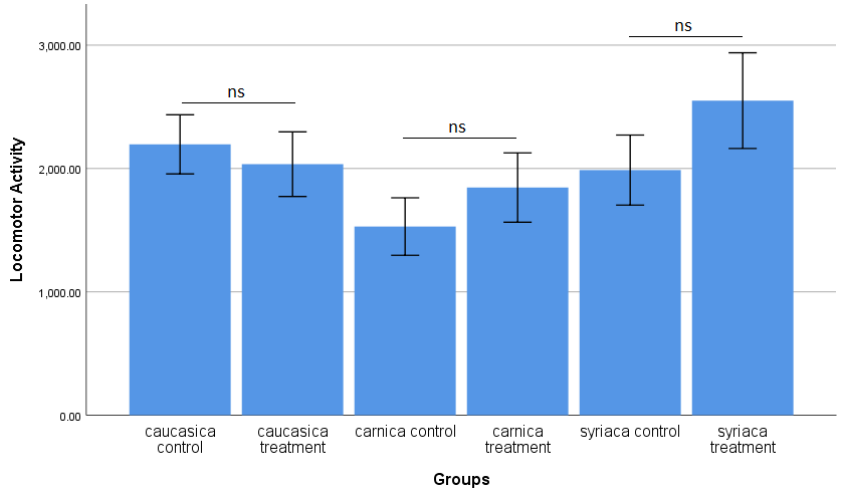

Figure 3.11. Comparison of the effect of 5 µg coumaphos dose in terms of second 12-hour locomotor activity. Data are represented as mean ± standard error. * p < .05, ** p < .001; ns, not significant.

Both parametric and non- parametric comparisons of normalized treatment data (Figure 3.12) also showed no significant difference (p > .05) among subspecies (Tables C.8 to C.11).

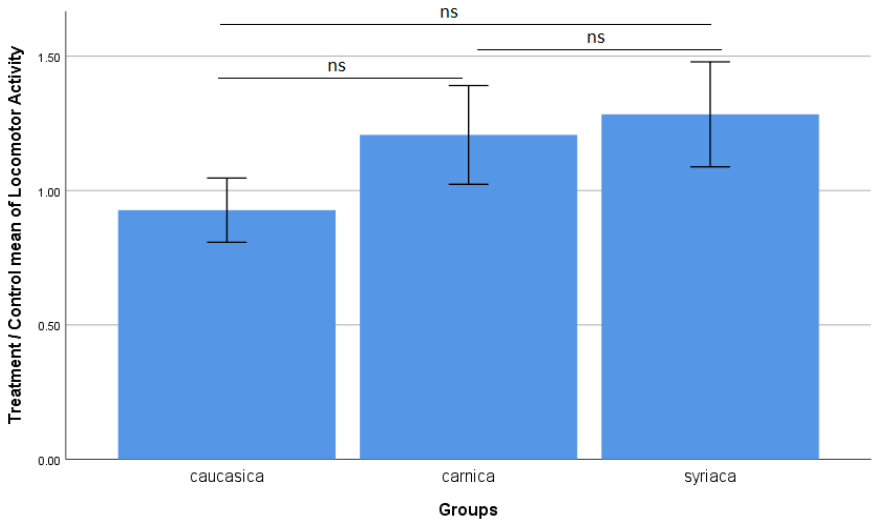

Figure 3.12. The effect of 5 µg coumaphos dose on honeybee races in terms of second 12-hour locomotor activity. Data are represented as mean ± standard error. * p < .05, ** p < .001; ns, not significant.

Monitor experiments were started at 19:00 pm and lasted for 24 hours. First 12 - hour periods can be considered nighttime while second 12-hours periods daytime. Therefore, we also compared and first and second 12-hour periods of control groups among three subspecies to find out if there is a difference between nighttime and daytime activities (Figure 3.13). Square root transformation normalized the data and Student's t-test comparisons showed that locomotor activities in first nighttime 12-hour periods were significantly lower ($p < .001$) than the second daytime 12-hour periods (Tables C.11 to C.13).

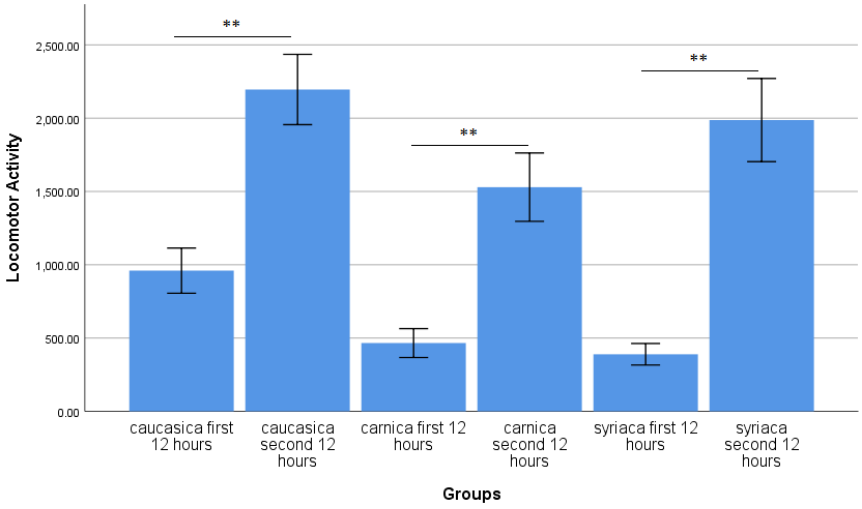

Figure 3.13. Comparison of the first and second 12-hours activity data of control groups in three subspecies. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.2 Proboscis Extension Reflex (PER) Conditioning Analysis

One odor -sandal tree oil - was used in the analyses. Statistical analyses were separately performed on acquisition and extinction data consisting of learning performances (total number of positive responses to odor administration during trials) of healthy individual bees, which did not die or become unresponsive during the experiments.

3.2.1 Acquisition Data Analysis

Statistics of acquisition scores of each individual were given in Tables D.1 to D.4 of Appendix D. Because both log and square root transformations failed to normalize the acquisition data, we used parametric and non-parametric tests together to compare control and treatment groups. In terms of acquisition score (Figure 3.14), no significant difference *(p* > .05) was found between control and treatment groups of *caucasica* and *carnica* by both Student's t and Mann- Whitney U tests. In *syriaca* however, acquisition scores of the treatment group were significantly (*p* < .05) lower than the control group.

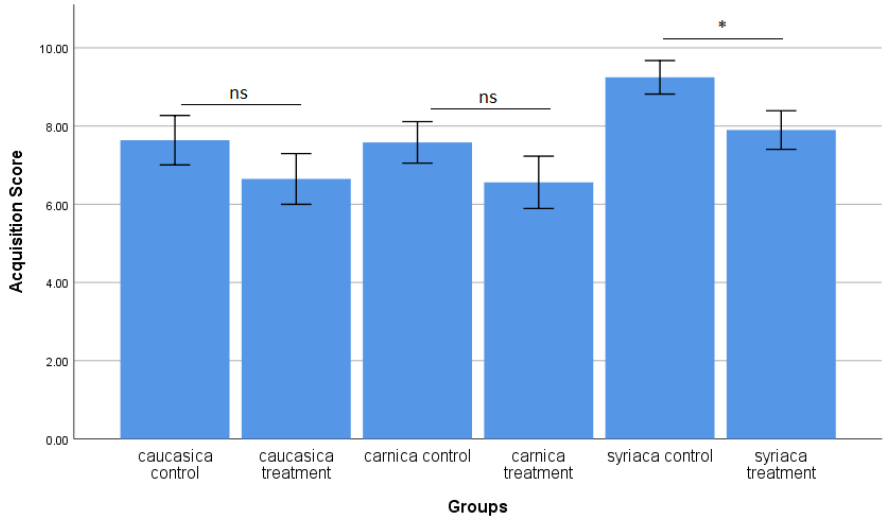

Figure 3.14. The effect of 5 µg coumaphos dose on honeybee races in terms of acquisition phase. Data are represented as mean ± standard error. * *p* < .05, ** *p* < .001; ns, not significant.

On the other hand, a comparison of subspecies groups using normalized treatment data (Figure 3.15) revealed no significant difference *(p* > .05) in the acquisition phase by both one-way ANOVA with post hoc analysis and Kruskal-Wallis with pairwise comparisons (Tables D.5 to D.8 in Appendix D).

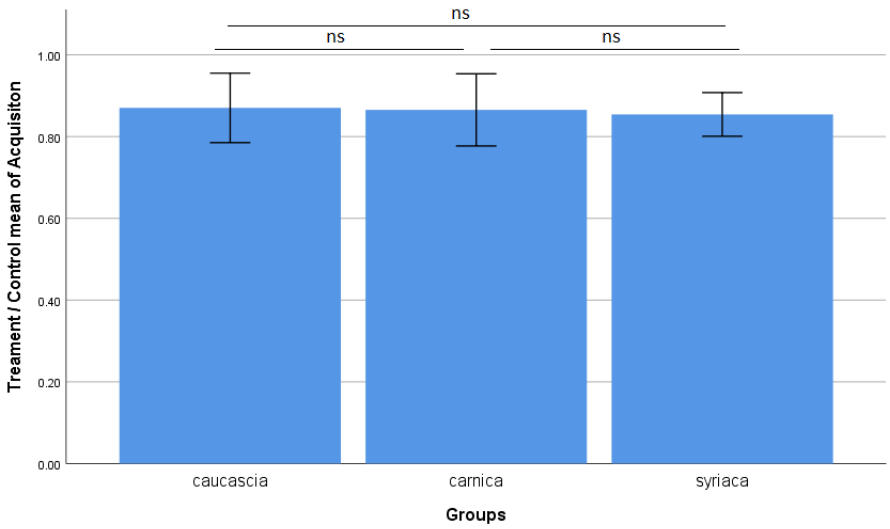

Figure 3.15. Comparison of the effect of coumaphos in acquisition phase. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.2.2 Extinction Data Analysis

As in acquisition data, both log and square root transformations failed to normalize the extinction data (Tables D.9 to D.12 in Appendix D) and therefore, we used parametric and non-parametric tests together to compare experimental groups. No significant difference *($p > .05$)* was found between the control and treatment groups of all three subspecies in the extinction phase (Figure 3.16) by both parametric and non-parametric tests between control and treatment groups.

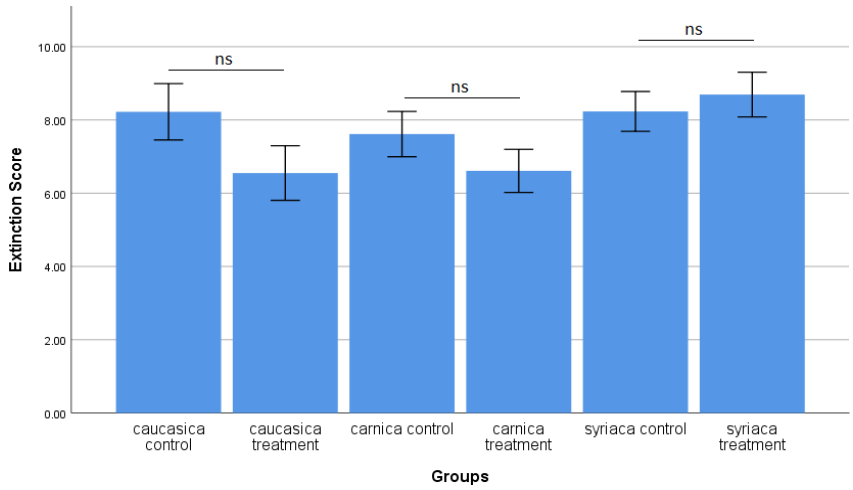

Figure 3.16. The effect of 5 µg coumaphos dose on honeybee races in terms of extinction phase. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

Comparison of subspecies using log-transformed normalized treatment data (Figure 3.17), revealed that however, extinction scores of *syriaca* were significantly increased ($p < .05$) compared to *caucasica* as indicated by both one way ANOVA and Kruskal-Wallis post hoc comparisons while no significant difference ($p > .05$) was present between latter two subspecies in extinction phase by both parametric and non-parametric tests (Tables D.13 to D.16 in Appendix D).

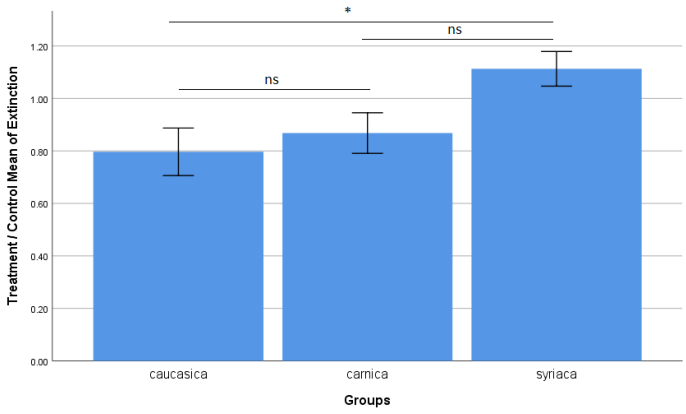

Figure 3.17. The effect of 5 µg coumaphos dose on honeybee races in terms of extinction phase. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.2.3 Dead and No Response Ratio

Because some bees were observed to be dead or became unresponsive to both sugar and odor in the acquisition phase, proportions of these bees to the experimental groups were also shown in Table 3.1. According to the table, treatment groups of all subspecies had higher proportions of dead and unresponsive bees compared to controls while this difference was considerably higher in *syriaca* compared to *caucasica* or *carnica*.

Groups	Dead and No response (%)	Difference	Odd Ratio*	S.E**
caucasica control	10.00	25.85	5.03	0.60
caucasica treatment	35.85			
carnica control	4.92	18.90	6.04	1.05
carnica treatment	23.81			
syriaca control	6.67	43.33	14.00	0.56
syriaca treatment	50.00			

Table 3.1. Percentage of bees that died or became unresponsive to sugar stimuli among experimental groups in Proboscis extension (PER) assay. * Odd ratio of treatment/control. ** Standard error of odd ratio.

3.3 Shock Avoidance Analysis

Two variables were analyzed in the first analysis: Time spent (seconds) in the electric shock area during the 5 minutes of experiment period (duration) and the number of passes of the moving bee between shock area and safe area (movement).

3.3.1 Duration Analysis

Statistics of duration data were given in Tables E.1 to E.4 in Appendix E. Log and square root transformations failed to normalize *syriaca* control and treatment

groups simultaneously and therefore both t-test and Mann-Whitney U tests were applied to that subspecies. In terms of duration data, the barplot in Figure 3.18 indicates that treatment groups of all three subspecies showed an increase in time spent in the shock area. A t-test comparison of log-transformed data showed that this increase was barely ($p = 0.049$) significant in *caucasica* while in *carnica* and *syriaca*, both t-test and Mann-Whitney U test comparisons of square root transformed data showed a highly significant ($p < .001$) increase in duration time compared to controls.

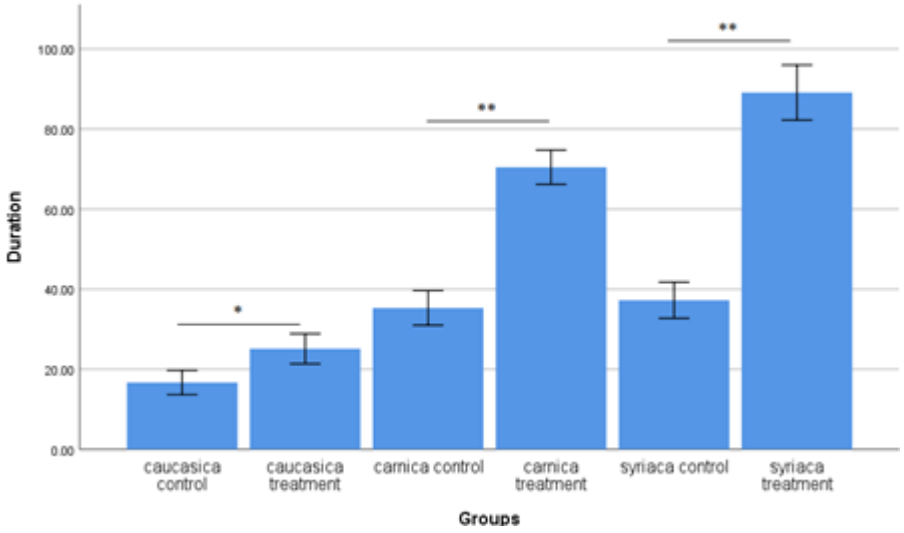

Figure 3.18. The effect of 5 µg coumaphos dose on honeybee races in terms of duration. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

Log-transformed normalized treatment data of three subspecies (Figure 3.19); Tables E.6 to E.9 in Appendix E) were not also fully normal and therefore analyzed by one-way ANOVA and as well as non-parametric Kruskal-Wallis tests. Results indicated a significant difference in terms of duration between groups ($p<0.001$). Post hoc pairwise comparisons of these tests also showed that the duration ratio of *caucasica* was significantly lower from both *carnica* and *syriaca* ($p< .05$) while the latter two did not significantly differ from each other ($p > .05$).

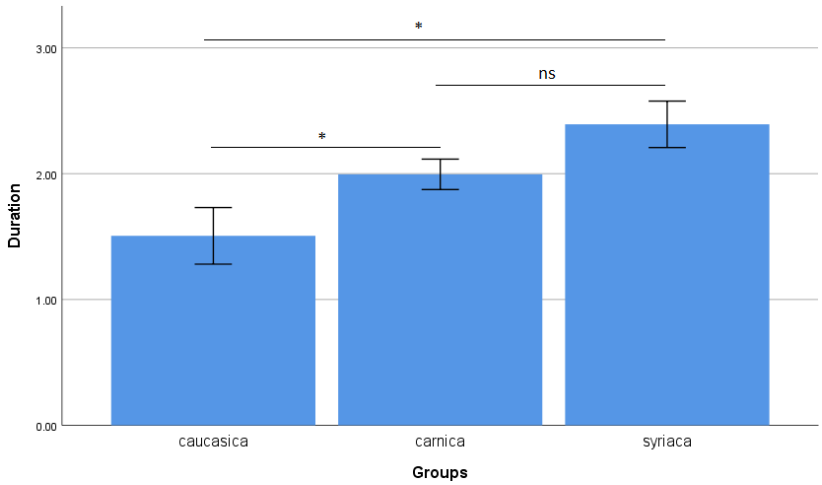

Figure 3.19. The effect of 5 µg coumaphos dose on honeybee races in terms of duration. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.3.2 Movement Analysis

Statistics of movement data were given in Tables E.1 to E.5 in Appendix E. Log and square root transformations could not normalize treatment group of *caucasica* and therefore both t-test and Mann-Whitney U tests are utilized for that subspecies. In terms of movement, (Figure 3.20), both t-test and Mann Whitney U test comparisons showed a significant decrease ($p < .001$) in the treatment group of *caucasica* compared to controls. No significant difference was found between groups of *syriaca* ($p > .05$) on the other hand.

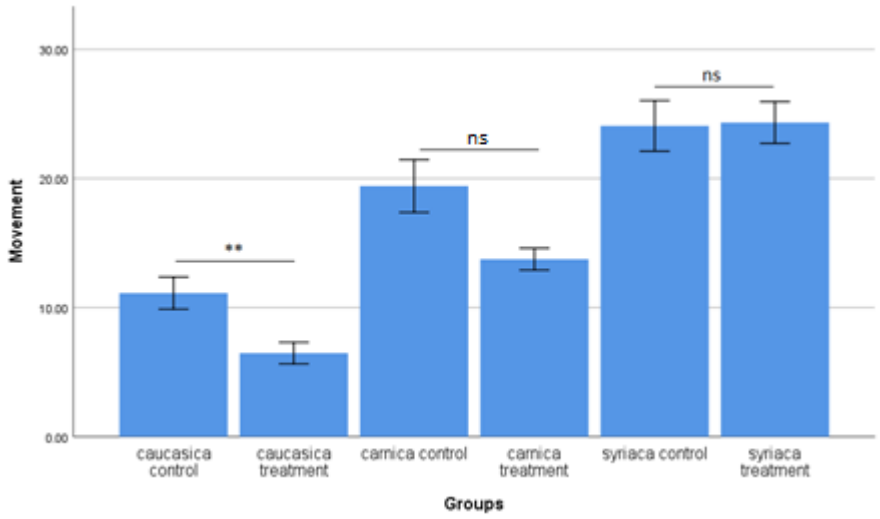

Figure 3.20. The effect of 5 µg coumaphos dose on honeybee races in terms of movement. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

Both parametric and non-parametric tests were used in the comparison of log-transformed normalized treatment movement data of subspecies, (Figure 3.21; Tables E.6 to E.9 in Appendix E) as log transformation could not normalize all of them. One-way ANOVA with post hoc comparisons as well as Kruskal-Wallis with pairwise comparisons showed that all three subspecies significantly differ from each other in terms of movement ($p < .001$ for *caucasica-syriaca*; $p < .05$ for other two comparisons) with *caucasica* having the lowest movement while *syriaca* scored highest.

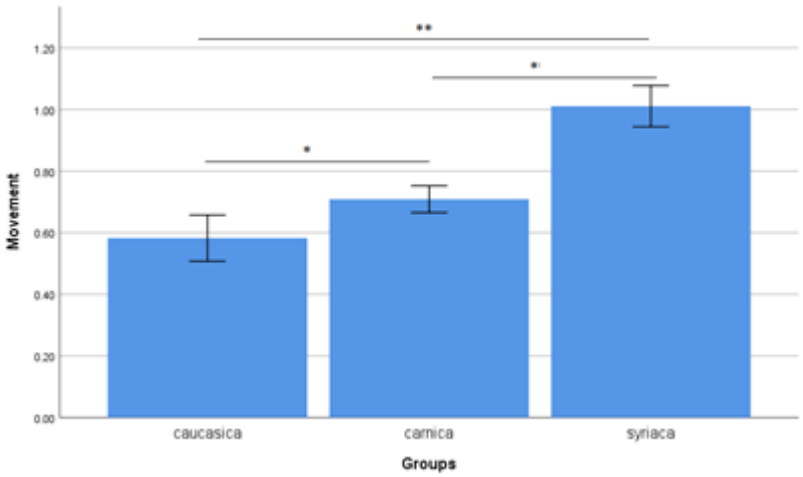

Figure 3.21. The effect of 5 µg coumaphos dose on honeybee races in terms of duration. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.4 Acetylcholinesterase (AChE) Activity Assay

3.4.1 Brain AChE Activity

Control vs treatment data of the subspecies was normally distributed and therefore only t-test was applied. Brain AChE activities of treatment groups of *caucasica* and *syriaca* groups seemed to be slightly decreased while *carnica* treatment group was lightly increased compared to control groups (Figure 3.22). However, t-test comparisons revealed that these differences were not significant ($p > .05$; Tables F.1 to F.3 in Appendix F).

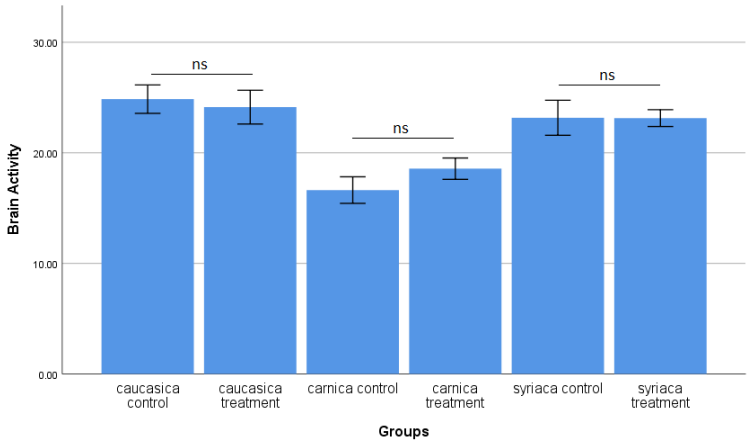

Figure 3.22. The effect of 5 µg coumaphos dose on honeybee races in terms of brain AChE activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

Distributions were normal in the log-transformed normalized treatment data of three subspecies and therefore only one-way ANOVA was applied. The brain activity of *carnica* seemed to be slightly higher than the other two according to the graphic (Figure 3.23; Tables F.4 to F.6 in Appendix F). However, one-way ANOVA and subsequent post hoc comparisons of the log-transformed data revealed no significant difference between groups ($p > .05$).

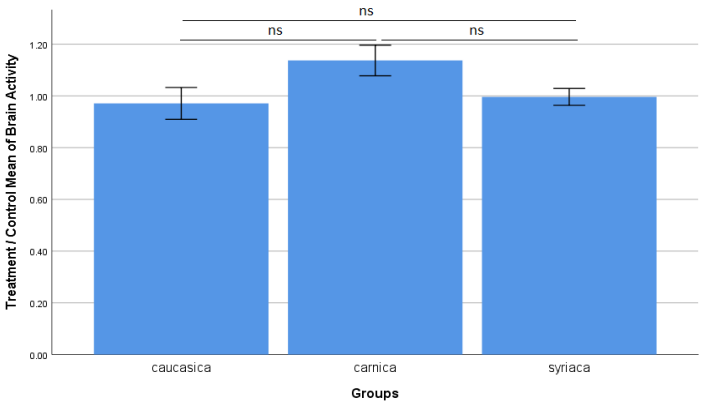

Figure 3.23. The effect of 5 µg coumaphos dose on honeybee races in terms of brain AChE activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.4.1 Midgut AChE Activity

Statistics of midgut AChE activity data can be found at Tables F.7 to F.9 in Appendix F. Because treatment data of *syriaca* was marginally different from normal distribution (p = .050 in Shapiro-Wilk Test) control and treatment data of these subspecies were further normalized by log transformation. Data from other experimental groups were normally distributed. t-test comparisons showed that AChE activities of treatment groups were significantly lower (p < .001) compared to controls in all three subspecies (Figure 3.24).

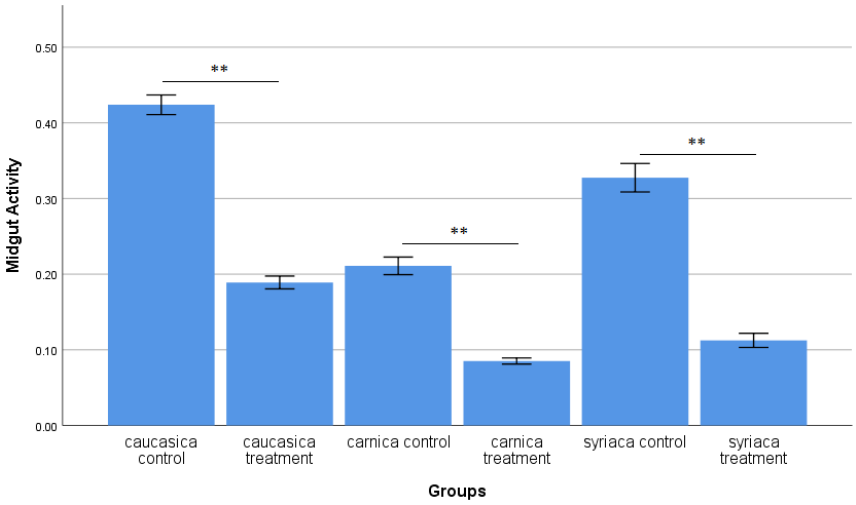

Figure 3.24. The effect of 5 µg coumaphos dose on honeybee races in terms of midgut AChE activity. Data are represented as mean ± standard error. * p < .05, ** p < .001; ns, not significant.

In the comparison of normalized treatment data of three subspecies, log transformation normalized all data and therefore only one-way ANOVA was applied (Tables F.10 to F.12 in Appendix F). Lowest AChE activity was observed in *syriaca* which was followed by *carnica* and *syriaca* (Figure 3.25). One-way ANOVA with post hoc analysis showed that AChE activities of *syriaca* treatment group were significantly (p < .05) lower than *caucasica* while no significant differences were found in other comparisons of subspecies (p > .05).

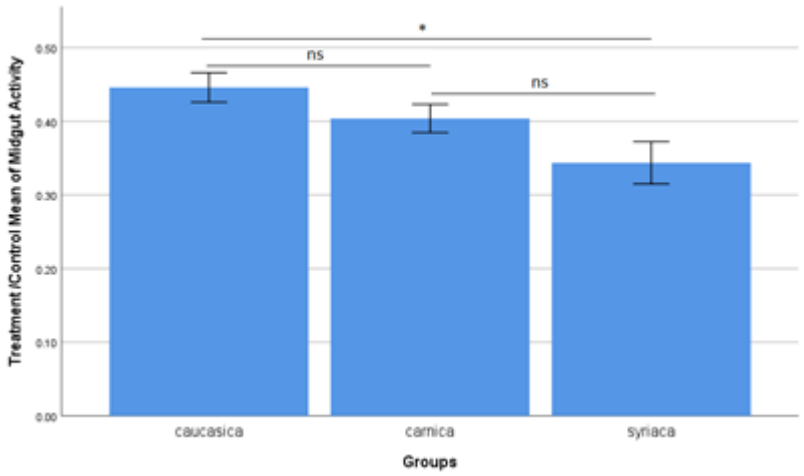

Figure 3.25. The effect of 5 µg coumaphos dose on honeybee races in terms of midgut AChE activity. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.5 Quantitative Real-Time PCR (Q-RT PCR) Assay

3.5.1 Brain Gene Expression

Statistics of brain gene expression data is presented in Table G.1 to G.3 in Appendix G. The *caucasica* control group data of NAChRa5 gene did not fit the normal distribution and therefore log transformation was applied to the control and treatment group of these subspecies which normalized the data. Students t-test comparison showed no significant difference ($p > .05$) between control and treatment groups of *caucasica* and *syriaca* in any gene analyzed except a marginally significant ($p = .051$) decrease in $GABA_B$ sub1 expression of *caucasica* treatment group compared to controls (Figure 3.26).

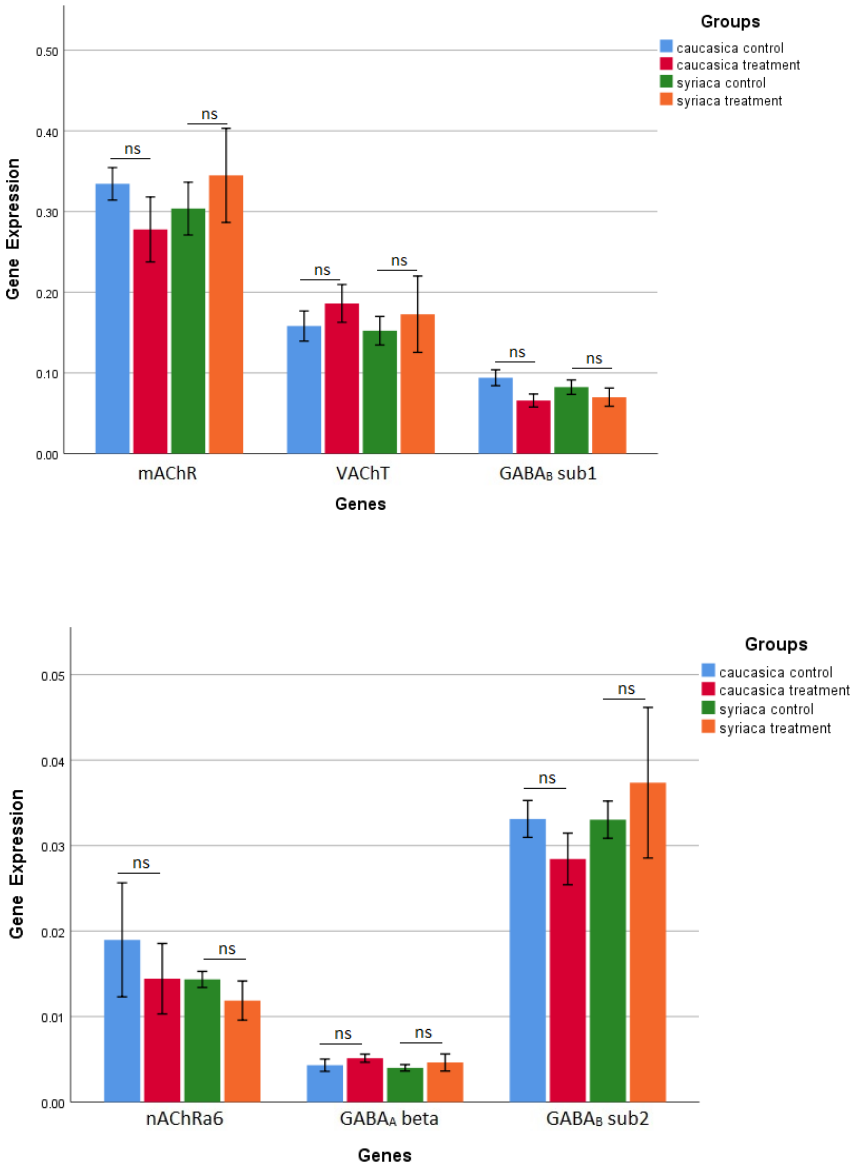

Figure 3.26. The effect of 5 µg coumaphos dose on honeybee races in terms of brain gene expressions of six genes. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

In log-transformed normalized treatment data (Tables G.4 to G.6 in Appendix G), *syriaca* group of $GABA_A$ beta expression was not normally distributed and therefore both t-test and Mann Whitney U tests were utilized for this locus. Parametric and non-parametric comparisons of *caucasica* and *syriaca* also showed no significant difference ($P > .05$) in any of the gene investigated (Figure 3.27).

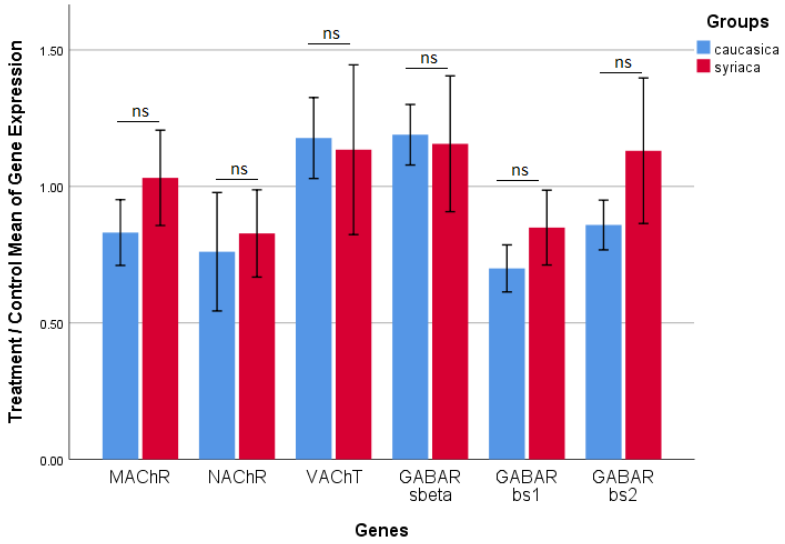

Figure 3.27. The effect of 5 µg coumaphos dose on honeybee races in terms of brain gene expressions of six genes. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

3.5.2 Midgut Gene Expression

Statistics of midgut gene expression data are presented at Tables G.7 to G.10 in Appendix G. Data of all groups and genes were normally distributed except control and treatment groups of *syriaca* in CYP9Q3 gene. Control group of *syriaca* in CYP9Q1 gene were also slightly significant ($p = 0.06$). Log transformation however normalized these groups. both parametric and non-parametric comparisons showed significant difference ($p < .05$) in CYP9Q1 gene of *syriaca* while no significant difference ($p > .05$) was found control and treatment groups of subspecies in other genes analyzed (Figure 3.28).

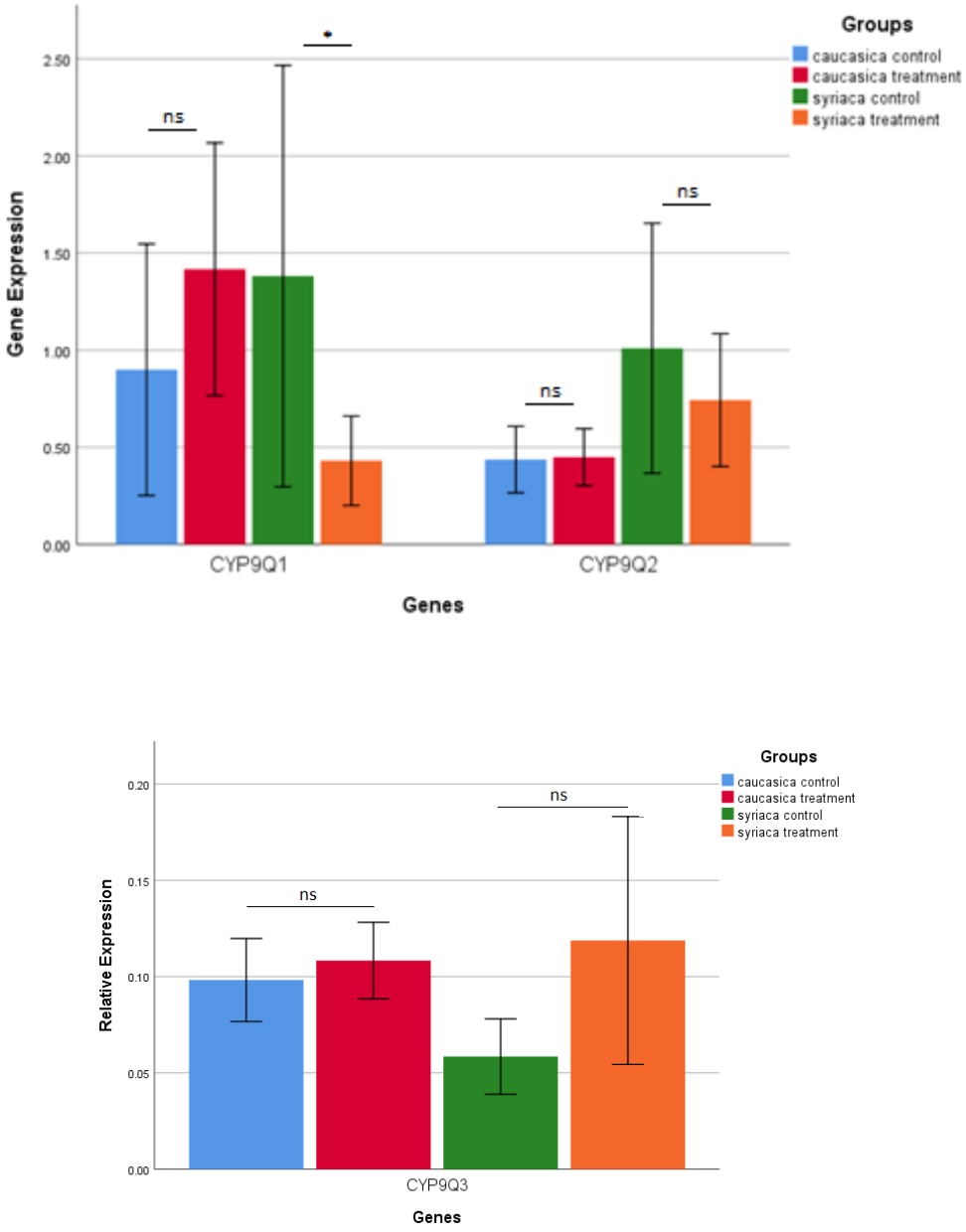

Figure 3.28. The effect of 5 µg coumaphos dose on honeybee races in terms of midgut gene expressions of three genes. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

In normalized treatment data comparisons, a significant difference ($p < .05$) was observed between *caucasica* and *syriaca* in the CYP9Q1 gene (Figure 3.29) while no significant difference ($p > .05$) was observed between subspecies in other two genes (Tables G.11 and G.12 in Appendix G).

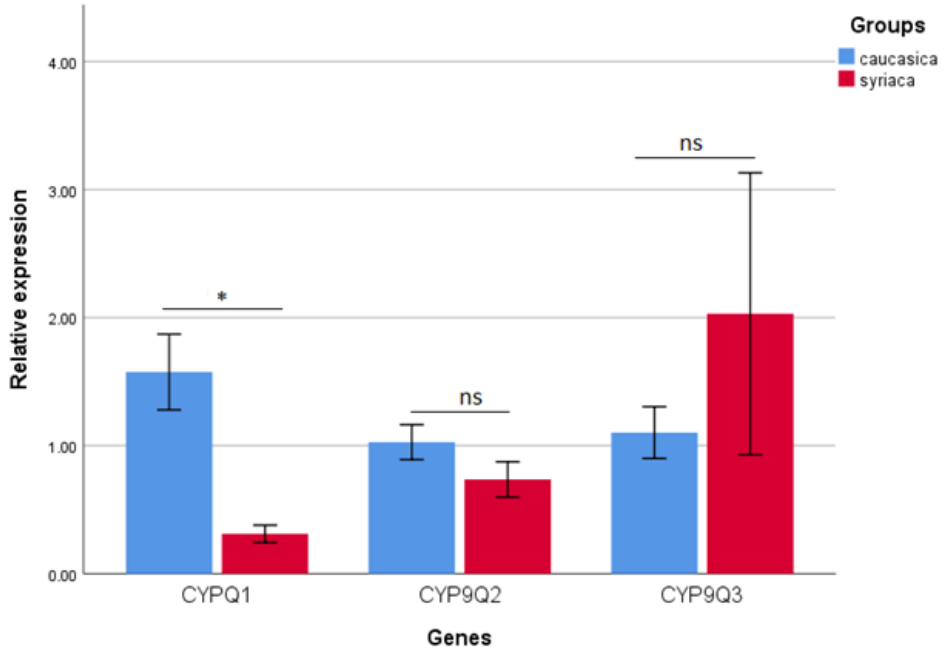

Figure 3.29. The effect of 5 µg coumaphos dose on honeybee races in terms of midgut gene expressions of three genes. Data are represented as mean ± standard error. * $p < .05$, ** $p < .001$; ns, not significant.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

Effects of acaricide Perizin on three different honey bee subspecies distributed different parts of Turkey in terms of behavioral and biochemical aspects were investigated.

4.1.1 Locomotor Activity Assay

Among the studies investigating the effects of pesticides on honey bee locomotor activity, observation of bee motility in a petri dish or another translucent chamber is a common method. Parameters such as the time spent in walking during the assay period or calculated walking distance of the subject bee on a surface divided into squares or grids are used for evaluation (El Hassani *et al.*, 2008a; Williamson *et al.*, 2013b; Bartling *et al.*, 2019). A more advanced method is using video tracking during observation (Charreton *et al.*, 2015; Teeters *et al.*, 2012; Tosi *et al.* 2017). Another system is utilizing special chambers with sensors which gives a signal when subject bees pass through. Such systems are frequently used in studies of circadian rhythms (Bloch *et al.*, 2002; Harano *et al.*, 2007; Giannoni-Guzmán *et al*., 2014). In our study, we used a locomotor activity monitoring system developed by Trikinetics Incorporate. This system, previously used for *Drosophila*, was modified to utilize honey bees and other similar sized insects. Each module of this system included 32 chambers equipped with infrared sensors. A falcon tube with a single live bee can be fitted into each chamber and with multiple modules connected, locomotor activities of up to 3480 individual insects can be measured

(Giannoni-Guzmán *et al*., 2014). Our study is the first one utilizing this system to investigate the effects of pesticides on locomotor activities of honey bees.

The first 12-hour locomotor activity (LMA) data revealed that LMA's of both 2 µg and 5 µg treatment groups of *syriaca* were significantly increased compared to their control groups while no significant difference was observed in *caucasica* and *carnica* in all dose groups. When normalized treatment data of three subspecies were compared, LMA of *syriaca* was also significantly increased compared to *caucasica* and *carnica* while no significant difference was found between the latter two.

In the full 24-hour LMA data, control vs 1 µg coumaphos dose comparisons gave no significant differences in all three subspecies as in 12-hour data indicating that 1µg coumaphos equivalent Perizin dose was not enough to make significant alterations in locomotor activity of honey bee subspecies used in our experiment. LMA's of both *carnica* and *syriaca* 2 µg. treatment groups were significantly increased compared to controls in parametric tests . These increases were marginally significant and non-significant respectively in parametric tests. No significant difference in terms of parametric tests was observed in *caucasica* on the other hand. In 5 µg dose however, the increased LMA observed in *carnica* was reversed and only *syriaca* treatment group's LMA was significantly increased compared to its control. Comparison of normalized 5µg treatment data among three subspecies also revealed that LMA of *syriaca* was significantly increased compared to *caucasica* while no other significant difference was found between subspecies. According to our results, *syriaca* was found to be most susceptible species to Perizin as the total LMA of *syriaca* 2 µg coumaphos treatment group was significantly increased compared to the control group in the initial 12-hour while LMA of 5 µg coumaphos treatment group was significantly increased in both 12-hour and 24-hour analyzes. Parametric comparison showed a significant increase in 2 µg coumaphos treatment group of *carnica* compared to controls in 24-hour of LMA assay however, no significant LMA change was observed in 5 µg dose group interestingly. This phenomenon is difficult to explain. Maybe there is a

late sensitization to low doses of coumaphos in *carnica* which is followed by desensitization in higher doses. On the other hand, non-parametric comparison was barely significant (p = 0.048) so this increase may represent random error. Meanwhile, caucasica appeared to be resistant to LMA related effects of coumaphos as no significant difference was found between all dose groups compared to the control group in both 12 and 24-hour analyzes.

Our locomotor activity monitoring system (LAM) is an adaptation of the *Drosophila* activity monitoring (DAM) system of Trikinetics Inc. (Waltham, MA, USA) which was modified to accommodate honey bees and other similar sized insects (Giannoni-Guzmán *et al*., 2014). Our study is the first in using LAM system to compare the effects of insecticide treatment on locomotor activities of different honey bee subspecies. Williamson *et al.* (2013b) observed behaviors of honey bees (*A. m. mellifera*) individually for 15 minutes in a petri dish after feeding them with 363, 36.3, and 3.6 ppb sucrose solutions of AChE inhibitors coumaphos, chlorpyrifos, aldicarb, and donepezil for 24-hour. They classified observed behaviors as walking, flying, remaining still, falling upside down, grooming, and unusual abdominal spasms and movements. The authors observed that walking behavior, the character associated with our study was slightly decreased by coumaphos, chlorpyrifos, and aldicarb treatment in summer bees but not in winter bees while this decrease was statistically significant only chlorpyrifos, not in coumaphos and aldicarb. On the other hand, grooming behavior was found to be significantly increased in all four pesticide treatment groups of pooled summer and winter bee data. Stürmer *et al.* (2014) treated cockroaches *Phoetallia pallida* with 0.25, 0.5, and 1 µM doses of organophosphate pesticide trichlorfon. They measured grooming activities of control and treatment groups in an open chamber with a video camera on top while locomotor activities were measured by immersing cockroaches in a tank filled with water and recording their swimming activities. Grooming rates were significantly increased in all three dose groups while the swimming rate was significantly increased in 1 µM dose group compared to controls. On the other hand, Fournier-*Level et al.* (2016) investigated combined

effects of temperature and DDT on locomotor activity of three *Drosophila* strains using DAM system of Trikinetics. The authors identified 5 different groups of activity patterns in 23-hour locomotor activity analyzes and they found that increasing DDT dose also increased the frequency of activity group no:5 which is peak activity in the early period and high mortality. Early peak activity is similar to our results of 2 µg and 5 µg coumaphos treatment groups of *syriaca* although we encountered negligible mortality (just three dead bees in all four replicates) in our LMA assays.

Honeybee foragers have a strong diurnal cycle. They forage outside the hive during the day and mostly inactive at night. Young bees performing in hive-tasks do not have a diurnal cycle and their activity/inactivity ratio is similar in day and night. However, as they get older and become closer to foraging age, bees gradually have a diurnal cycle with increasing inactivity at night (Crasheim *et al*., 1996; Moore *et al*., 1998). Toma *et al.* (2000) measured individual locomotor activities of honeybees with different age groups. They observed that newly emerged bees did not show any locomotor activity with a rhythm pattern for a few days and mean age they began to display circadian rhythm was 7-8 days. Giannoni-Guzmán *et al*. (2014) measured individual locomotor activities of honey bee foragers belonging to three races (gentle Africanized bees, *carni*ca and *caucasica*) using a same device Trikinetic device we used. They found that circadian activity of all bee races were less than 24 hours in constant darkness and there was a large amount of activity variation among individuals in all races. Our LMA assays were initiated at 19:00 PM and lasted for 24 hours. Therefore, the first 12-hour period can be accepted as night while second 12-hour period as day. Significantly lower activities were observed in all subspecies in first 12-hour period compared to second. Therefore, our bees may be displaying a circadian rhythmic activity although there is a high amount of individual variation. We used bees collected from combs inside of the hives in our experiment. Forager bees spend most of the day outside of the hive and do not visit comb cells (Crasheim *et al*., 1996). So, our samples almost entirely consisted of pre-forager bees younger than 20 days. This may explain the

individual variation as some of the bees included in the assay may be noticeably young bees which do not show any rhythmic activity as indicated in Toma *et al.* (2000). However, in *syriaca*, coumaphos treatment significantly increased activity in first 12-hour night period while no significant difference was found between control and treatment groups in second 12-hour day period. This may show that coumaphos effect disruption in honeybee diurnal rhythm keeping bees active at night which may be related to increased excitation by AChE inhibition. In *carnica*, second 12 hours LMA activity of 2 µg coumaphos treatment group was significantly increased compared to its control in t-test but not in non-parametric Mann-Whitney U test. No significant difference was observed in 5 µg dose group on the other hand. This situation is similar to 24-hour analysis. Therefore, this LMA increase in 2 µg dose groups of *carnica* in second 12-hour period may be a delayed response to coumaphos or a statistical deviation due to high individual variations in locomotor activities among the groups.

4.1.2 Proboscis Extension Reflex Assay

Antennae are the main chemosensory organs in honey bees. A hungry bee extends its proboscis to feed when its antennae contacted to a food source such as sugar solution. In PER assay, antennae of a harnessed bee are first exposed to an odor and then to a sucrose solution subsequently, the odor is called conditioned stimulus (CS) while sucrose solution is called unconditioned stimulus (US). After single or multiple trials of CS-US pairing, the bee makes an association between them and extends its proboscis when CS was applied (Bitterman *et al.*, 1983). PER responses to CS -US pairings can be recorded as "1" for the positive response to CS and "0" for no response to CS. These binary data can later be analyzed for comparing learning performances among subject bees. After bees are conditioned to respond to CS-US pairings (acquisition phase), another protocol called "extinction" can be applied. In this phase, only CS is applied to break the association between CS and US. In brief, acquisition determines the performance of being conditioned to an

olfactory stimulus associated with a food reward while extinction measures the ability to break this conditioning when the food reward became absent. Bees that are successfully conditioned can be re-tested after a few hours to several days to measure short term or long-term memory retention. (Matsumoto *et al.,* 2012). Honey bees can also be tested for their abilities to discriminate between stimulants by using two odors one of whom is paired with US (CS^+) while other is not (CS^-; Deisig *et al.*, 2001). Since its introduction, PER assay is used in numerous studies about different aspects of learning and memory in honey bees (Giurfa and Sandoz, 2012). PER assays were also used to assess learning and memory differences among Turkish honeybee subspecies (Abramson *et al.*, 2008; Erdem, 2018). PER assay is frequently used to assess the effects of pesticides on olfactory learning memory functions of honey bees (Pham-Deleque *et al.*, 2002).

In our PER assay we used acquisition and extinction paradigm. When acquisition data of control and treatment groups of three subspecies were compared separately, the acquisition score of *syriaca* treatment group was significantly decreased compared to controls while no significant difference was observed in *carnica* and *syriaca*. On the other hand, comparison of normalized treatment data revealed no significant difference between the three subspecies. This shows that although *syriaca* treatment group had a lower acquisition point compared to its control group indicating an impairment in PER conditioning, this difference is not strong enough to manifest itself at the subspecies comparison level.

Means of extinction scores were decreased in treatment groups of *caucasica* and c*arnica* while the extinction score of *syriaca* treatment group showed a slight increase compared to controls. None of these changes were found to be statistically significant although. Comparison of normalized treatment data among subspecies showed a significant elevation of the extinction score of *syriaca* compared to *caucasica*. Increased extinction score in *syriaca* indicates a difficulty in breaking the conditioning when the reward was absent. Therefore, we can conclude that coumaphos treatment caused an impairment of olfactory learning in terms of both conditioning and un-conditioning in *syriaca* but this is not a clear and definitive

impairment as it revealed itself in either control vs. treatment or subspecies comparisons, not both of them.

When percentages of bees that became unresponsive or died during the acquisition phase of PER experimentations were compared between control and treatment groups of subspecies, *syriaca* treatment group had as high as 50% dead or unresponsive individual percentage rate which was followed by *caucasica* (10) and *carnica* (23,81) treatment groups. In terms of control groups, *carnica* appeared to be most resilient to PER experimentation with a percentage of 4,91% dead and unresponsive individuals, followed by *syriaca* (6,67) and *caucasica* (10). It is clear that treatment groups of all subspecies had increased dead and no response ratios compared to their controls and this increase was the greatest in *syriaca* as indicated by both percentage difference and odd ratio parameters. This showed that coumaphos treatment of *syriaca*, greatly affected and dramatically increased dead or unresponsive bees in acquisition phase PER assay whereas other subspecies were not affected to an extent. Because bees that died or became unresponsive during the PER trials were excluded from analysis, this may have caused an artificial selection leaving the most resilient and coumaphos resistant bees for PER analysis. This may be the reason why coumaphos treatment caused large numbers of unresponsive or dead bees in *syriaca* but its detrimental effect on learning scores was less significant.

Weick *et al.* (2002) exposed bees of undefined subspecies to different sublethal concentrations of coumaphos, the active ingredient of Perizin, through cuticular exposure (1 µl droplet of 0.01, 0.1 or 10% coumaphos in acetone) or intracranial injection (1 µl 0.07% coumaphos in hexane). When these bees and their respective controls were subjected to PER assay, no significant difference in terms of acquisition and extinction scores was found between control and treatment groups of both cuticularly exposed and intracranially injected bees in all coumaphos doses. Williamson et al. (2013a) fed A. *mellifera mellifera* worker bees 5 µl sucrose containing 1,81 ng coumaphos and their controls fed with 5µl sucrose only. Then they subjected these bees to PER experiment consisting of 6 trials with inter-trial

intervals of 10 minutes (spaces training) or only 30 seconds (massed training). They found that coumaphos treated group of both massed and spaced training experiments had significantly lower acquisition rates compared to controls. Doses used in these studies were quite small compared to our dose of Perizin solution equivalent of 5 µg coumaphos in 10 µl sucrose solution which was adapted from Bevk *et al.* (2012) but it should be noted that that these studies used laboratory-grade coumaphos with high purity while we used a commercial product containing %3,2 coumaphos as the active ingredient. Urlacher *et al.* (2016) measured olfactory learning and memory with PER assay in honey bees treated with various single acute doses of the organophosphate pesticide chlorpyrifos. PER assay consisted of four to five acquisition trials with a single odor. To test memory retention, bees that were successful in acquisition trials were incubated for 1 hour, then exposed to the odor they are trained with and two additional unfamiliar odors (one was structurally similar to training odor while other was not) and their PER response was recorded. It was shown that chlorpyrifos treatment decreased the acquisition rate of honey bees compared to controls. In addition to this, chlorpyrifos treated bees could not discriminate between familiar and unfamiliar odors showing impairment in memory retention.

4.1.3 Electric Shock Avoidance Assay

Another aspect of associative learning is aversive learning. In this context, the unconditioned stimulus (US) is not a reward but a punishment that causes an aversive behavior. Electric shock is a commonly used US but other stimulus types such as formic acid can also be used (Abramson, 1986; Tedjakumala and Giurfa, 2013). Conditioned stimuli can be either olfactory or visual cues while the measurable response to US can be sting extension reflex (SER) of a harnessed bee, which is a defensive behavior to irritable stimuli (Breed *et al.*, 2004; Vergoz et al.,2007; Mota *et al.*, 2011). Zhang *et al.* (2015) used aversive olfactory conditioning through SER on imidacloprid treated honeybees and found that

Imidacloprid treatment significantly decreased aversive learning and memory. Another system is using free walking bees in a chamber or a maze. Some part of the chamber o maze is equipped with metal grids to give electric shock as punishment. In time, subject bees learn to avert the shock area and spend more time in the "safe" area. This can be used as a variable to compare learning time and ability among individual bees. Visual and olfactory cues are also can be associated with shock and safe areas for conditioning (Agarwal *et al.*, 2011; Dinges *et al.*,2013 Schott *et al.*,2015; Morgane and Giovanni, 2019). Bartling *et al.* (2019) used a chamber system in which shock and safe sides were associated with two different odors and found that sub lathe doses of neonicotinoid insecticide clothianidin significantly decreased aversive conditioning ability in honey bees. Electric shock avoidance (ESA) assay we used in our study was previously used by other researchers (Agarval *et al*., 2011; Dinges *et al*., 2013; Avalos *et al*., 2017), our study is the first that we used the effects of an acaricide in three indigenous honey bee subspecies in terms of electric shock avoidance. Also, these studies mentioned above solely used the time spent on the electric shock of safe zone as an indicator of avoidance behavior while we also used the number of transitions between safe side and electric shock side as an additional variable because a honey bee may repeatedly enter the shock side and immediately return to the safe side. This also can indicate a lack of learning to avoid electric shock, but it may not reveal itself when only time spent on electric shock or safe side is measured. Therefore, we analyzed both duration (time spent in electric shock side) and transition (numbers of transitional movements between safe and shock sides) in our ESA assays of control and treatment groups of three subspecies.

In terms of electric shock avoidance behavior, duration (times spent in electric shock side) was found to be significantly increased in treatment groups of *carnica* and *syriaca* compared to their controls while a barely significant difference in *caucasica* treatment group was observed. When log-transformed normalized treatment data of three subspecies were compared for better evaluation, the duration of carnica and *syriaca* were also increased compared to *caucasica*. This

may indicate that sublethal doses of coumaphos caused an impairment in pain and danger avoidance behaviors of *carnica* and *syriaca* while *caucasica* seemed more resistant to this effect.

When numbers of transitional movements between safe and shock sides were compared among three subspecies, no significant difference was found between control and treatment groups of *carnica* and *syriaca* , while movement number of *caucasica* treatment group was significantly decreased compared to control group. Comparison of log-transformed normalized treatment data among subspecies showed that *caucasica* had the lowest and *syriaca* had highest movement score in the presence of coumaphos while *carnica* was placed between them and all differences between subspecies were significant. This shows that *carnica* and *syriaca* treatment groups not only spend more time in the shock side but also increasingly attempted to leave the safe zone and enter the shock area compared to *caucasica*. In the case of *caucasica* on the other hand, coumaphos administration did not affect the time spent on the shock side but interestingly decreased transition movement number. The decrease in transition number can be due to some desensitization or adaptation effect against Perizin. Urlacher *et al.* (2016) subjected honey bees treated with organophosphate pesticide chlorpyriphos to aversive learning assay based on the association of an odor with an electric shock. Aversive response to electric shock was indicated by sting extension. No significant difference was found between control and chlorpyrifos treated bees in terms of aversive learning scores and 1-hour memory retention. Valvassori *et al.* (2007) measured aversive memory in Wistar rats treated with organophosphate malathion using a grid where a mild electric shock was applied. They found that sublethal single dose of 50, 100, and 150 mg /kg malathion administration significantly reduced aversive memory compared to controls although none of the dose groups caused a significant decrease in brain AChE levels. Levi *et al.* (2008) treated adult mice with 0.5 mg/kg organophosphate insecticide diisopropylfluorophosphate for four consecutive days and measured their aversive learning using a shuttle box system with safe and electric shock areas where electric shock application was

associated with a sound tone. No significant difference was found between the control and treatment groups in terms of avoidance learning. On the other hand, administration of an acute single dose (2 mg/kg) dichlorvos significantly decreased active avoidance in Wistar rats in a remarkably similar two-way electric shock assay (Savi *et al.,* 2003). To sum up, it can be argued that changes in avoidance related to sublethal doses of organophosphate pesticides show variation according to type and doses of the given pesticide and also, subspecies of the subject animal as we showed in our study.

4.1.4 Acetylcholinesterase Activity Assay

Coumaphos, the active ingredient of Perizin, is an organophosphate pesticide that acts through inhibition of neurotransmitter acetylcholinesterase. Therefore, we measured acetylcholinesterase (AChE) activities in brains and midguts in control and treatment groups of three honey bee subspecies to compare the effects of Perizin among these subspecies. Brain and midgut tissues were selected because the brain is the nexus of the central nervous system and behavioral responses while midgut is the center of pesticide metabolism in honey bees (Mao *et al.*, 2011). We used Ellmann's assay to measure AChE activities. This method uses acetylthiocholine as substrate and 5´- 5´dithiobis-2- nitrobenzoic acid (DTNB) as an indicator. AChE cleaves acetylthiocholine into acetate and thiocholine. Sulfhydryl (thiol) groups of Thiocholine react with DTNB, producing a yellow color of which intensity can be measured spectrophotometrically (Ellman *et al.*, 1961). It is a relatively simple, rapid, and economical assay frequently being used in studies of organophosphate toxicity (Worek *et al.*, 2012).

In Acetylcholinesterase activity measurements of brain tissues, AChE activities of *carnica* treatment group were slightly increased while *caucasica* and *syriaca* treatment groups showed slightly decreased AChE activities compared to their control groups. However, none of these differences were found to be statistically significant. A comparison of normalized treatment data also showed no significant

difference between subspecies. In midgut tissues however, AChE activities of treatment groups of all subspecies were significantly decreased compared to controls. A comparison of normalized treatment data also revealed that the AChE activity of *syriaca* was significantly decreased compared to *caucasica* while no significant differences were found between *caucasica* and *carnica*. These results showed that the behavior-altering effects of coumaphos did not reveal themselves in brain tissue but in midgut tissue. Coumaphos decreased midgut AChE activities of all subspecies while this decrease was more intense in *syriaca*. Lienau *et al.* (1990) observed that treatment of juvenile swarm colonies with Perizin did not inhibit brain AChE activities of the bees. Weick *et al.* (2002) measured AChE levels in brains of honey bees exposed to coumaphos through cuticles after they concluded PER experiments on them. They found no significant difference between treatment and control groups. It should be noted that these authors also observed no significant difference between control and treatment groups in terms of both acquisition and extinction ratios in PER assays. Williamson *et al.* (2013b) also did not observe a significant, dose-dependent AChE activity inhibition in brains and guts of honey bees (*A. m. mellifera*) exposed to sublethal doses of coumaphos although the same coumaphos doses caused a significant alteration in some behavioral aspects of such as walking, grooming, abdominal spasms and being unable to recover when fallen over. The authors also tested oxon metabolite of coumaphos and observed significant, dose-dependent AChE inhibition in both brain and midgut tissues. Coumaphos belongs to the thiophosphate branch of organophosphorus insecticides and in vivo produced phosphate ester or oxon metabolites of thiophosphates are more potent inhibitors of AChE compared to their parent compounds (Fukuto, 1990). On the contrary, Dahlgren (2014) observed that lethal toxicity of coumaphos was approximately 2-fold more for workers and 4-fold more for queens compared to coumaphos oxon. This author used the topical application of coumaphos and its oxon metabolite while we used direct ingestion in a sucrose solution. This may show that although more potent AChE inhibitor, coumaphos oxon may have a reduced penetration from the cuticle, contributing to

its lower toxicity in topical application compared to its parent compound. More interestingly, Dahlgren (2014) also investigated the metabolism of topically applied coumaphos in worker and queen bees by for 5 days with 24-hour intervals using GC/MS and LC/MS. She found a decrease in coumaphos and an increase in an unknown metabolite throughout the time and surprisingly, coumaphos oxon was not determined in any of the time intervals. The author concluded that honey bees metabolized coumaphos into possibly less toxic metabolites, not more potent AChE inhibitor coumaphos oxon. Mao et al (2011) revealed that three cytochrome P450's: CYP9Q1, CYP9Q2, and CYP9Q3 are predominantly involved in coumaphos metabolism. Piperonylbutoxide (PBO) is a compound that has inhibitory effects on p450's and it was reported to have a synergistic effect with coumaphos in cattle ticks (Li *et al*., 2003) and honey bees (Johnson *et al*., 2009) indicating the predominant role of p450's in coumaphos metabolism. In our AChE experiments, we used direct ingestion of a single acute Perizin dose equivalent of 5 µg coumaphos in honey bees. Perizin ingestion significantly decreased midgut AChE activities of all three subspecies. This is most likely because midgut is the place where Perizin is stored and metabolized after ingestion. This decrease was more prominent in *syriaca* compared to *carnica* and *caucasica* which may indicate that coumaphos metabolism rate and/or efficiency is lower in *syriaca* than the other two subspecies. In terms of behavioral assays, *syriaca* displayed the highest total locomotor activity in monitoring assay, highest duration and movement scores in ESA assay, highest extinction, and lowest survival scores in PER assay compared to *caucasica* and *carnica*. In AChE activity assay also, the lowest midgut AChE activities were observed in *syriaca* compared to the other two. This may show that the metabolism of coumaphos into less toxic components is slower and/or less efficient in *syriaca* which makes this subspecies more susceptible to the behavior-altering effects of coumaphos. Contrary to midgut results, no significant difference was found in brain AChE activities of all three subspecies in terms of both control vs. treatment group comparisons, and comparisons of log-transformed treatment groups normalized to their control means. Williamson et al. (2013b) also failed to

find any significant AChE inhibition in honey bee brain tissues although the same coumaphos doses significantly altered behavioral patterns of worker honey bees as shown in their assay. This may show that either brain acetylcholinesterase activity levels are poor indicators of behavioral changes in honey bees exposed to sublethal doses of coumaphos or other unknown mechanisms in the brain have roles in responses to behavior-altering effects of coumaphos.

4.1.5 Gene Expression Assay

Gene expression studies are essential to further reveal the mechanisms of possible behavioral and biochemical variations in response to coumaphos administration among honey bee subspecies. Boncristiani *et al.* (2012) measured several expressions of several genes in worker bees sampled from beehives each treated with several commercial acaricides including one with coumaphos as the active ingredient. Two immune genes and one development and one protein kinase-based detoxification genes were found to be down-regulated while one cytochrome p459 detoxification gene was found to be up-regulated by coumaphos treatment. Gregorc *et al.* (2018) measured expressions of several antioxidant genes in worker honey bees fed with sugar patties containing different amounts of coumaphos, imidacloprid, and a mixture of two. Coumaphos and coumaphos + imidacloprid administrations significantly decreased expressions of five antioxidant genes. Gene expression studies using RT-qPCR were carried out in brains and midguts of *caucasica* and *syriaca* worker samples. Because coumaphos is an AChE inhibitor, six target genes related to cholinergic metabolism and learning behavior were measured in brain samples. These genes are muscarinic acetylcholine receptor (mAChR), nicotinic acetylcholine receptor alpha 10 subunit (nAChRa5), Vesicular acetylcholine transporter (VAChT), gamma-aminobutyric acid (GABA) receptor subunit beta ($GABA_A$ beta), GABA type B receptor subunit 1 ($GABA_B$ sub1) and GABA type B receptor subunit 2 ($GABA_B$ sub2). As an AChE inhibitor, coumaphos acts through hyperstimulation of nicotinic and muscarinic acetylcholine

receptors by excessive acetylcholine (Colovic, *et al*., 2013) and therefore these receptors may have a role in behavioral tolerance or sensitivity to sublethal doses of coumaphos. Both nicotinic and muscarinic acetylcholine receptors are known to be involved in learning and memory (Hasselmo et al.,2006; Gauthier *et al.*, 2010; Poulin *et al*., 2010;) GABA is the major neurotransmitter of inhibitor function in the central nervous system (Sivilotti *et al.,* 1991). GABA signaling is also involved in learning, memory, and anxiety (Kalueff and Nutt, 1996; Collinson *et al.*, 2002). There are two types of GABA receptors: type A receptors ($GABA_A$) are ionotropic receptors of Cys-loop ligand-gated receptor superfamily which also includes nicotinic acetylcholine receptors. Induction of these receptors opens an integral ion channel that permits chloride ions to flow into the postsynaptic neuron, polarizing it and inhibiting a new action potential. Type B receptors ($GABA_B$) on the other hand, are G protein-coupled receptors and inhibit neurotransmission by mediating either calcium or potassium channels through secondary neurotransmitters. $GABA_A$ receptors are more numerous and provide fast, immediate inhibition while $GABA_B$ receptors have slow but long-lasting inhibitory effects (Jazvinšćak and Vlainić, 2015). The vesicular acetylcholine (VAChT) transporter is a neurotransmitter transporter responsible for carrying acetylcholine into secretary organelles of neurons to be released in time of synaptic transmission (Erickson and Varequi, 2000). This makes VAChT an important component of the cholinergic transmission system. In midgut samples, we chose to analyze the expressions of CYP9Q1, CYP9Q2, and CYP9Q genes. The midgut is the center of xenobiotic metabolism in the honey bees and these three cytochrome p450 oxidase genes are found to be the genes thathave a major role in coumaphos metabolism (Mao *et al.*, 2011). Piperonylbutoxide (PBO) is a compound that has inhibitory effects on p450's and it was reported to have a synergistic effect with coumaphos in cattle ticks (Li *et al.*, 2003) and honey bees (Johnson *et al.*, 2009) indicating the predominant role of p450's in coumaphos metabolism.

In our brain gene expression studies, no difference was found between control and treatment groups of *caucasica* and *syriaca* in any genes. Only a marginally significant decrease ($P = 0.051$) was found in GABA type B receptor subunit1 expression of the treatment group of *caucasica* compared to its controls but this did

not reflect itself in normalized treatment data comparisons. The lack of significant differences in brain gene expression is similar to brain AChE activity studies discussed above. Sivam et al. (1983) measured brain GABA and muscarinic receptor numbers in rats by using specific ligands after acute and chronic administration of AChE inhibitor diisopropylfluorophosphate (DFP). The authors found that both acute chronic DFP administration significantly decreased brain AChE levels, increased GABA receptor numbers but did not affect muscarinic receptors. The authors also concluded that the increase in GABA receptors may be a metabolic response to counter the increase in excitatory cholinergic activity due to AChE inhibition. Chronic administration of three cholinesterase inhibitors was found to increase NAChR levels in rat brains (Reid and Sabbagh; 2008). On the other hand, Shao *et al.* (2013) showed the topical application of paraoxon and chlorpyrifos to house flies showed a positive correlation between AChE inhibition and NAChR activation. The authors also found that the application of nine organophosphate and carbamate insecticides with varying chemical structures showed a positive correlation between poisoning signs and NAChR activation, indicating the importance of nicotinic receptors in AChE inhibitor toxicity of insects. These receptors are also involved in learning, memory and locomotion. Raccuglia and Mueller (2020) observed that injection of a GABA A receptor agonist before olfactory conditioning trials significantly impaired memory forming in honey bees. Gauthier (2010) injected three NAChR antagonists mecamylamine, α-bungarotoxin, and methylylcaconitine to honey bee brains before PER trials and observed that mecamylamine prevented one trial PER learning while α-bungarotoxin and methylylcaconitine decreased long term memory performances. Wang *et al.* (2013) compared gene expression differences between honey bees subjected to olfactory PER conditioning and untrained bees by tag-based digital gene expression (DGE) method. They found expression differences in Nicotinic acetylcholine receptor alpha 6 subunit (nAChRa6), muscarinic acetylcholine receptor (mACHR), and GABA receptor subunit beta ($GABA_A$ beta). Mustard *et al.* (2019) also found that activation of $GABA_A$ and $GABA_B$ receptors through

agonists decreased walking behavior of honeybees in an observation chamber. We did not observe any significant differences in brain AChE activity levels between control and treatment groups of our three subspecies. Therefore, it can be suggested that behavior altering effects of coumaphos on honeybees may be related to synaptic receptors and transporters related to cholinergic system. However, gene expression studies in brain samples could not reveal any significant difference in any selected gene of *caucasica* and *syriaca* in terms of both control vs treatment and normalized treatment comparisons. This can be attributed to two reasons: sublethal doses of coumaphos may be causing behavioral alterations through a different pathway, not related to cholinergic or any other system involving the gene we investigated or, the gene expression changes caused by sublethal doses coumaphos are more subtle than the detection sensitivity of RT-PCR method.

In our midgut samples, a significant decrease was found between treatment groups of *syriaca* compared to controls in CYP9Q1 whereas no significant difference was found in other two. Comparisons of normalized treatment data also displayed a significant decrease in CYP9Q1 expression in *syriaca* compared to *caucasica* while no significant difference was observed in other genes. Boncristiani *et al.* (2012) investigated gene expressions of three cytochrome p450 variants (Cyp4G11, CYP306a1, CYP6AS14) in samples collected from Hawaiian beehives (subspecies not specified) treated with a commercial coumaphos based acaricide found significantly decreased expression of CYP306a1 gene compared to control hives. On the other hand, Al Naggar *et al.* (2015) found no significant differences in the expression of these three cyp450 variants in honeybees exposed to sublethal doses of four organophosphate pesticides (Diazinon, Malathion, Profenos, and Chlorpyrifos) separately. These studies and our results show that specific cyp450 genes can be negatively affected by coumaphos. Dahlgren (2014) argued that coumaphos is broken down into less toxic metabolites by honey bee detoxification mechanisms. Consistent with this statement, the decrease of CYP9Q1 expression in coumaphos treatment group of *syriaca* compared to *caucasica*, indicates that coumaphos related inhibition of CYP9Q1 may lead to a decrease in the metabolic

detoxification of coumaphos which may cause increased coumaphos exposure to the central nervous system in *syriaca* and this can be a basis to explain the increased locomotor activity and also decreased learning and memory performance observed in *syriaca* compared to other subspecies in this study.

4.1.6 Overall Discussion of Results and Suggestions for Further Studies

In our study, *caucasica* appeared to be most resistant to the effects of coumaphos administration in terms of behavioral, biochemical, and genetic aspects, while *syriaca* was observed as the most susceptible to its effects. On the other hand, *carnica* showed intermediate values. There may be several reasons for this, such as differences in detoxification metabolism, sensitivity to the target site, penetration barriers, and increased or reduced activation rates are among the mechanisms that cause pesticide tolerance or sensitivity (Dahlgren, 2014). In midgut samples of *syriaca*, the coumaphos related decrease in AChE activity coupled with a decrease in the expression of the CYP9Q1 gene, which is one of three cytochrome p450 genes predominantly involved in coumaphos metabolism, strongly indicates that this subspecies may have slower and/or less effective metabolism of coumaphos into less toxic metabolites compared to *caucasica* and *carnica*, which leads to more amounts of coumaphos reaching into the central nervous system, causing the behavioral alterations observed. On the opposite side, *caucasica* may have much faster and/or efficient metabolism of coumaphos which results in relative resistance to its sublethal effects on the central nervous system. finally, *carnica* may have a relatively moderate metabolism speed or efficiency among *caucasica* and *syriaca* which are at the extreme sides of the spectrum. On the contrary, no significant difference was observed in both brain AChE activities and brain expression levels of the genes investigated between control and treatment groups as well as normalized treatment data of all three subspecies. This indicates that the differences found in behavioral assays as well as in midgut metabolism between three subspecies did not reflect themselves in brain experiments. There may be two

possible explanations for this phenomenon. First, our biochemical and genetic assays may not be sensitive enough to determine the coumaphos related subtle changes in AChE activity and expressions of selected genes. The quantitative RT-PCR can accurately determine 23% and greater differences in gene expression levels among groups (Gentle *et al.*, 2001). Therefore, difference ratios lower than that threshold may not have been accurately detected in our experiments. The Ellman procedure we utilized in our AChE activity experiments is an indirect colorimetric measurement method based on the reaction of DTNB with thiol groups formed by AChE mediated cleavage of acetylthiocholine. This assay has a limited sensitivity with a threshold of 3 µM and DTNB has a possibility of reacting with free thiol groups of proteins as well as a tendency to undergo hydrolysis under reaction conditions (Gorun *et al.*, 1978; Hansen *et al.*, 2007). Thus, limited assay sensitivity may explain why coumaphos related changes were observed in behavioral assays but not in biochemical and genetic markers measured in the brain. The second possibility is that the effects of sublethal doses of coumaphos on honey bee behavior may be related to neurotransmitter mechanisms different than the Acetylcholine and GABA systems investigated in our biochemical and genetic assays. Williamson et al. (2013b) found increased AChE gene expression in brain and gut tissues of honey bees treated with acute sublethal doses of coumaphos while no significant change was found in brain AChE activities revealing the possibility that the AChE inhibition effect of coumaphos may have been counterbalanced by increased AChE synthesis. However, the authors also observed behavioral alterations and impairments in coumaphos treated bees. This may indicate that the behavioral effects of coumaphos may be initiated by mechanisms different than acetylcholine system. Other neurotransmitter systems which have roles in honey bee learning and memory, are glutamate and biogenic amine (dopamine and octopamine) systems. Glutamate is an excitatory neurotransmitter also present in the insect central nervous system (Gauthier and Grünewald, 2011). Vesicular glutamate transporter (VGLUT) is responsible for the transformation of glutamate to synaptic vesicles to be released. A gene encoding this transporter

which is also found in honey bees (Leboulle, 2011). Another transporter for glutamate (excitatory amino acid transporter, Am-EAAT) was also identified in honey bees (Kucharski *et al.*, 2000). Like GABA system, glutamate receptors are also divided into ionotropic and metabotropic receptors. Ionotropic receptors are ligand-gated ion channels providing rapid synaptic transmission. Three types of them were defined and named in the vertebrates according to their specific agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (Ka) receptors. NMDA is permeable to Ca^+ while the other two are more permeable to Na^+ and K^+ ions (Leboulle, 2011, Rousseaux, 2008). Three genes (nmdar1, nmdar2, nmdar3) are found to express NMDA receptor subunits in honey bees (Honey bee Genome Consortium, 2006). nmdar1 gene and its encoding subunit AmNR1 were identified and characterized in honey bees (Zannat *et al.*, 2006). Metabotropic receptors instead, mediate modulatory synaptic activities. These are G protein-coupled receptors acting indirectly through secondary messengers some of which also mediate ion channels (Rousseaux, 2008). Two metabolic glutamate receptors were (AmGluRA and AmGluRB) are described in honey bees (Funada *et al.*, 2004). There are also glutamate-gated chloride channels (GluCls) present in invertebrates that act as inhibitory receptors. (Raymond and Satelle, 2002). One gene, Amel_GluCl encoding these receptors were identified in honey bee (Jones and Satelle, 2006). Glutamate is shown to be an important aspect of learning and memory. Locatelli *et al.* (2005) showed that the release of glutamate in the honey bee brain before olfactory training provided a stable long term memory. Kucharski *et al.* (2007) found that injecting bees with both agonists and antagonists of AmGluRA receptor 1 hour before olfactory training impaired long-term memory, while injections 1-hour post-training and 1 hour before memory testing had no effects. Injection of a GluCL agonist before olfactory learning trials were shown to impair long term memory in bees (El Hassani *et al.*, 2008b). All these studies show the importance of glutamate and its receptors on honey bee behavior.

In addition to glutamate, biogenic amines are molecules acting as important neuromodulators in the nervous system. They consist of dopamine, serotonin, octopamine, tyramine, and histamine (Gauthier and Grunewald, 2011). All biogenic amine receptors defined in invertebrates are G protein-coupled receptors. There are three dopamine (AmDOP1, AmDOP2, and AmDOP3), two serotonin (Am5-HT_7 and Am5-HT_{1A}) one octopamine (AmOA1) and one tyramine (AmTYR1) receptors defined in honey bees (Gauthier and Grunewald, 2011; Scheiner *et al.*, 2006). Dopamine has a role in the locomotion activity of honey bees. Virgin queens were found to have increased dopamine levels compared to mating queens and these increased levels found to be correlated with higher locomotor activity in virgin queens (Harano *et al.*, 2005, 2008). Dopamine injection decreased acquisition rates of olfactory learning in honey bees in both treatments of 3 minutes before and 15 minutes after training assay (Mercer and Menzel 1982). Octopamine is also involved in learning and memory. Octopamine was found to increase the rate of PER response to a stimulus not associated with reward (Mercer and Menzel 1982). Silencing of octopamine receptor expression by siRNA treatment impaired olfactory learning and memory (Farooqui *et al.*, 2013). Menzel *et al.* (1999) treated honey bees of *carnica* subspecies with reserpine which depleted biogenic amines in the brain and subjected to PER conditioning. Authors found that reserpine impaired conditioning, sensitization and memory retrieval. Octopamine application to reserpine treated bees ameliorated conditioning but not sensitization and memory retrieval. On the other hand, dopamine injection reversed the decreased PER latency caused by reserpine, indicating its positive effect on motor patterns. Agarwal *et al.* (2011) put dopamine or octopamine treated honey bees to an electric shock avoidance assay very similar to ours and found that dopamine decreased the time spent on the shock side while octopamine increased it. Knowledge of the behavioral effects of other biogenic amines is relatively limited. Histamine application was found to inhibit odor caused responses in honeybee antennal lobes (Sachse *et al.*, 2006). Serotonin injection on the other hand, impaired olfactory conditioning (Menzel *et al.*, 1999).

Sloley and Owen (1982) did not find significant changes in dopamine and serotonin levels in one cricket (*Acheta pennsylvanicus*) and one cockroach (*Periplaneta americana*) when they were exposed to organophosphate pesticide dicrotophos. On the other hand, Stürmer *et al.* (2014) observed increased walking and grooming behavior in adult male *Phoetalia pallida* roaches treated with organophosphorus insecticide trichlorfon and found that the increase in grooming behavior was reversed by administration of a dopamine receptor blocker. Idriss *et al.* (1986) also showed that organophosphate and carbamate insecticides initiate action potentials in glutamatergic neuromuscular junctions in muscle tissues of *Locusta migratoria*, indicating glutamate synapses as action sites for AChE inhibitor insecticides.

In conclusion, further studies should be performed on potential involvements of these non-cholinergic neurotransmitter mechanisms in the behavioral effects of sublethal coumaphos doses in honey bees. Especially dopamine and octopamine systems are important due to their roles in locomotor activity, appetitive, and aversive learning processes. Either gene expressions of neurotransmitters and their receptors can be measured in coumaphos treated bees or various agonists and antagonists of neurotransmitters can be administered with coumaphos to bees before learning and behavior assays.

Whatever the underlying mechanisms on the variation in the behavioral response to sub-lethal doses Perizin and its active metabolite coumaphos in different three native honey bee subspecies are, they should have been related to evolutionary, environmental and/or adaptive differences among these subspecies. *A. m. syriaca* is native of southern Anatolia with an arid climate characterized by long hot summers and short winters. The presence of predatory wasp species in their native habitat also forced *syriaca* bees to have specific adaptations such as aggressive behavior, increased hive defense and reduced foraging activity *A. m. caucasica* on the other hand is native to the northeast Anatolia with east Black Sea coast which is characterized by a heavily forested temperate climate area and a relatively short, three month foraging season coupled with a long winter and more sequential

blooming of flowers. It is also known for having a very calm and gentle behavior and easy handling contrast to *syriaca* (Ruttner, 1988; Çakmak *et al.*, 1998, 2011; Zakour and Bienefeld, 2014). *A. m. carnica* is native to the Thrace region and it also has a gentle behavior (Kaftanoğlu, 2001). *carnica* also belongs to a different genetic lineage (C) from *caucasica* and *syriaca* (Whitfield, 2006). Stationary honeybee colonies from Thrace region also form a separate cluster from the rest of the Anatolian populations, supporting this theory (Tunca, 2009, Kükrer, 2013).

In addition to its gentle behavior, *caucasica* has a low swarming tendency, high honey yield, high propolis collection ability but also an increased tendency to hive robbing. *carnica is* also gentle with high honey yield, low propolis collection and hive robbing behavior but it has a high swarming ability as a downside (Kaftanoğlu, 2001). *caucasica* queens are the most preferred type by Turkish beekeepers and queen breeders with a percentage of 60.7%. *carnica* comes second (21.4 %) followed *by anatoliaca* with 17.9 % (Karaca and Karaman, 2018). *carnica* colonies are also found to be relatively more aggressive compared to *caucasica* colonies in Turkey (Güler, 1995). Kence *et al*. (2013) compared colony performances and *Varroa* mite infestation and defensive behaviors of Turkish honey bee subspecies in a common garden. *syriaca* colonies were characterized with reduced honey storage with high brood production, *carnica* hives had large honey storage, high number of brood combs and high population numbers. *caucasica* on the other hand, had increased honey storage and large brood area. *carnica* and *syriaca* also had lower varroa infestation levels and higher rate of hygienic behavior, which is removal of larvae infested with *Varroa*, compared to *caucasica*.

Therefore, it can be said that *caucasica* and *syriaca* are at the opposite ends in terms of life history, habitat preference, distribution, and behavioral patterns. While *carnica* is placed between them in terms of these properties and their behavioral responses to sub-lethal exposure to coumaphos is also found to be consistent with this situation. This consistency between life history and coumaphos resistance brings about one question: What are the specific adaptive properties which may

have roles in coumaphos susceptibility or resistance in Turkish honey bee subspecies?

Honeybees are exposed to various phytochemicals when foraging for elements such as nectar, pollen or propolis necessary for colony vitality (Johnson *et al*., 2012). *caucasica* is adapted to long winters and short foraging season of its native habitat, which causes a tendency to store honey in the combs rather than brood rearing. Therefore, *caucasica* foragers may have been exposed to more xenobiotics during extensive foraging for nectar and, vitality and performance of each honeybee may be relatively more important for the colony due to low amount of brood rearing. All these traits may force *caucasica* bees to develop a more robust cyp450 based xenobiotic metabolism to cope with harmful effect of chemicals encountered during foraging. In *syriaca* on the other hand, long hot summers and short winters of the native habitat and, predatory pressure of wasp species, cause reduced foraging activity and a tendency to carry out brood rearing more extensively in combs compared to honey storage. These traits may mitigate the selection pressure for a strong cyp450 metabolism in *syriaca* due to lower exposure of phytochemicals during foraging and relatively lower importance of the well-being of individual bees for the colony due to extensive brood rearing activity. *carnica* bees finally, display both high honey production and high brood rearing abilities. So although there may be an increased selection pressure for more effective xenobiotic metabolism due to high exposure to phytochemicals, this pressure may have been moderated by increased brood rearing which increases population turnover and lower the relative importance of individual bees for viability of the entire colony.

Another important aspect that may be role in the effects of coumaphos on honeybee behavior is plasticity in learning. In our electric shock assay, normalized treatment data analysis showed that duration parameter of *caucasica* treatment group were significantly decreased compared to *syriaca* treatment group. In terms of movement score on the other hand, a significant grading occurred within subspecies as *caucasica* had the lowest movement score and syriaca had the

highest while *carnica* is ranked between them. This shows that *syriaca* treatment group not only spent more time in the electric shock area but also made more attempts to enter the shock area compared to *caucasica*. Erdem (2018) also found that *caucasica* bees had significantly better learning score in terms of both duration and movement parameters compared to *carnica* and *syriaca* in a same electric shock assay protocol used in this study. Çakmak *et al.* (2010) performed experiments on foraging behaviors of these three subspecies using artificial flowers with different colors containing different concentrations of sucrose solutions as reward. Their results showed that *caucasica* bees had lower fidelity to a specific flower color compared to *carnica* and *syriaca*. In terms of flowers with variable sucrose concentrations, *syriaca* bees revealed a specialist pattern and constantly visited the same color of flowers regardless of the reward variability. *caucasica* and *carnica* on the other hand displayed a generalist behavior and had a tendency for switching to flowers of different colors when a more attractive reward was present. These results indicate that *caucasica* has a more flexible and adaptable learning ability while *syriaca* has a rigid and somehow obsessive learning behavior. This may explain the opposite performances of *caucasica* and *syriaca* in the ESA assay. These contrasts in learning behavior between *caucasica* and *syriaca* can be attributed to the following factors: *syriaca* is endemic to a habitat where bee wolves (*Philanthus Triangulum*) and oriental wasps (*Vespa orientalis*) are common while these predatory species are not frequently encountered in the endemic region of *caucasica*. Wasps prey on bees at hive entrances while bee volves attack bees on flowers (Çakmak and Wells, 2001). A strong fidelity to one food source color or type in *syriaca* therefore, removes the tendency to look for alternative food source types, which decreases the foraging time as well as the risk of predation. *caucasica* on the other hand, is not under such a strong predation pressure. Besides, its native habitats are mountainous regions with long winters and short summers. This climate necessitates a large honey storage for overwintering and may lead *caucasica* to a more generalized and flexible learning behavior which includes switching to different flower types with higher nectar contents when they become

available. Also, Agarwal *et al*. (2011) used an ESA protocol remarkably similar to our study and found that bees treated with dopamine spent less time in the shock side while octopamine-treated bees spent more time in the shock side. This further emphasizes the importance of these two biogenic amines in learning ability and plasticity and indicates their possible roles in behavioral effects of coumaphos.

All these lead to a hypothesis that honey bees with similar behavioral and ecological traits to the subspecies we investigated in this study may have similar behavioral responses to sublethal doses of pesticides. To validate this hypothesis, further studies comparing the behavioral effects should be further tested for more organophosphates and also other types of pesticides on Turkish and other honey bee subspecies.

4.2 CONCLUSION

In conclusion, susceptibility to acute sublethal ingestion of acaricide Perizin differed among three native Turkish honey bee subspecies in terms of behavioral, biochemical, and genetic parameters. Our behavioral assays showed that *caucasica* is quite resistant to behavioral effects of acute sublethal coumaphos administration as no significant change was observed in all three behavioral assays (locomotor activity, electric shock avoidance, and olfactory learning). *syriaca* on the other hand appeared to be highly susceptible to the effects of coumaphos as significant behavioral changes were observed in *syriaca* treatment group specimens of all three assays. *carnica* finally, had a moderate susceptibility, putting it between opposite ends of *caucasica* and *syriaca*. Enzyme activity and gene expression analysis indicated that differences detoxification rates of Perizin's active ingredient coumaphos have a role in tolerance to behavioral effects of acute Perizin however, the exact mechanism responsible for behavioral changes caused by sublethal coumaphos exposures needs to be investigated. Further studies should include more precise assay methods as well as other neurotransmitter systems especially dopamine – octopamine system. We did not encounter any other study in the

literature comparing the effects of acaricides on different honey bee subspecies. Therefore, we think that our study will give way to a new area of comparative research in honey bees which will include different subspecies, different xenobiotics, and, different behavioral, biochemical, and genetic assays. Also, our study with further studies may help identification and selective breeding of honey bee subspecies resistant to sub-lethal effects of pesticides used in beekeeping or agriculture, which will benefit Turkish and international beekeeping.

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APPENDICES

A. Statistical Tables of 12-Hour Locomotor Activity Analysis

Table A.1. Means, sample sizes, sd's and se's of control vs 1µg coumaphos treatment groups of 12-hour analysis of activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica* control	Mean	959.67	154.078
		Sample Size	24	
		Std. Deviation	754.823	
	caucasica 1 µg treatment	Mean	989.61	118.566
		Sample Size	23	
		Std. Deviation	568.621	
	carnica control	Mean	465.63	98.649
		Sample Size	24	
		Std. Deviation	483.279	
	carnica 1 µg treatment	Mean	428.63	63.489
		Sample Size	24	
		Std. Deviation	311.031	
	syriaca control	Mean	389.38	73.421
		Sample Size	24	
		Std. Deviation	359.688	
	*syriaca*1 µg treatment	Mean	779.96	251.030
		Sample Size	24	
		Median	537.50	
		Std. Deviation	1229.793	

Table A.2. Normality tests of original and square root-transformed data of control vs 1 µg coumaphos treatment data of three subspecies in 12-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	*caucasica* control	0.888	24	0.012
	caucasica treatment	0.962	23	0.509
	carnica control	0.763	24	0.000
	carnica treatment	0.913	24	0.040
	syriaca control	0.890	24	0.013
	syriaca treatment	0.551	24	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of LMA	*caucasica* control	0.955	24	0.343
	caucasica treatment	0.987	23	0.985
	carnica control	0.924	24	0.072
	carnica treatment	0.957	24	0.372
	syriaca control	0.967	24	0.583
	syriaca treatment	0.877	24	0.007

Table A.3. Student's t-test comparisons of square root-transformed control vs 1 µg coumaphos data of three subspecies in 12-hour analysis of activity monitoring assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	1.189	0.281	-0.425	45	0.673	-1.335	3.140	-7.659	4.989
	Equal variances not assumed			-0.427	43.640	0.671	-1.335	3.125	-7.635	4.964

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.286	0.595	0.024	46	0.981	0.062	2.549	-5.070	5.193
	Equal variances not assumed			0.024	43.657	0.981	0.062	2.549	-5.077	5.200

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	2.119	0.152	-1.428	46	0.160	-5.560	3.894	-13.398	2.279
	Equal variances not assumed			-1.428	37.380	0.162	-5.560	3.894	-13.447	2.328

Table A.4. Mann-Whitney U test comparisons of control vs 1 µg coumaphos data of *syriaca* subspecies in 12-hour analysis of activity monitoring assay.

syriaca	
Test Statistic	356.500
Standardized Test Statistic	1.412
Standard Error	48.496
Significance	0.158

Table A.5. Means, sample sizes, sd's and se's of control vs 2 µg coumaphos treatment groups of 12-hour analysis of activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica* control	Mean	959.67	154.078
		Sample Size	24	
		Std. Deviation	754.823	
	caucasica 2 µg treatment	Mean	821.48	118.736
		Sample Size	23	
		Std. Deviation	569.438	
	carnica control	Mean	465.63	98.649
		Sample Size	24	
		Std. Deviation	483.279	
	carnica 2 µg treatment	Mean	697.08	135.756
		Sample Size	24	
		Std. Deviation	665.066	
	syriaca control	Mean	389.38	73.421
		Sample Size	24	
		Std. Deviation	359.688	
	syriaca 2 µg treatment	Mean	1050.42	221.379
		Sample Size	24	
		Std. Deviation	1084.531	

Table A.6. Normality tests of original and square root-transformed data of control vs 2 µg coumaphos treatment data of three subspecies in 12-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	*caucasica* control	0.888	24	0.012
	caucasica treatment	0.890	23	0.016
	carnica control	0.763	24	0.000
	carnica treatment	0.808	24	0.000
	syriaca control	0.890	24	0.013
	syriaca treatment	0.774	24	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of LMA	*caucasica* control	0.955	24	0.343
	caucasica treatment	0.960	23	0.469
	carnica control	0.924	24	0.072
	carnica treatment	0.899	24	0.020
	syriaca control	0.967	24	0.583
	syriaca treatment	0.935	24	0.128

Table A.7. Student's t-test comparisons of square root-transformed control vs 2 µg coumaphos data of three subspecies in 12-hour analysis of activity monitoring assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	1.131	0.293	0.565	45	0.575	1.819	3.219	-4.664	8.303
	Equal variances not assumed			0.567	44.410	0.573	1.819	3.208	-4.644	8.283

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	1.383	0.246	-1.447	46	0.155	-4.492	3.105	-10.742	1.758
	Equal variances not assumed			-1.447	44.712	0.155	-4.492	3.105	-10.746	1.763

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	1.687	0.200	-3.130	46	0.003	-11.519	3.680	-18.926	-4.112
	Equal variances not assumed			-3.130	39.161	0.003	-11.519	3.680	-18.961	-4.077

Table A.8. Mann-Whitney U test comparisons of control vs 2 µg coumaphos data of *carnica* in 12-hour analysis of activity monitoring assay.

carnica	
Test Statistic	36.500
Standardized Test Statistic	1.206
Standard Error	48.493
Significance	0.228

Table A.9. Means, sample sizes, sd's and se's of control vs 5 µg coumaphos treatment groups of 12-hour analysis of activity monitoring assay.

Groups		Statistic	Std. Error
caucasica control	Mean	959.67	154.078
	Sample Size	24	
	Std. Deviation	754.823	
caucasica 5 µg treatment	Mean	1169.54	158.659
	Sample Size	24	
	Std. Deviation	777.267	
carnica control	Mean	465.63	98.649
	Sample Size	24	
	Std. Deviation	483.279	
carnica 5 µg treatment	Mean	720.33	116.399
	Sample Size	24	
	Std. Deviation	570.237	
syriaca control	Mean	389.38	73.421
	Sample Size	24	
	Std. Deviation	359.688	
syriaca 5 µg treatment	Mean	1663.00	291.770
	Sample Size	21	
	Std. Deviation	1337.056	

Table A.10. Normality tests of original and square root-transformed data of control vs 5µg coumaphos treatment data of three subspecies in 12-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	caucasica control	0.888	24	0.012
	caucasica treatment	0.839	24	0.001
	carnica control	0.763	24	0.000
	carnica treatment	0.843	24	0.002
	syriaca control	0.890	24	0.013
	syriaca treatment	0.842	21	0.003

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of LMA	*caucasica* control	0.955	24	0.343
	caucasica treatment	0.945	24	0.211
	carnica control	0.924	24	0.072
	carnica treatment	0.966	24	0.566
	syriaca control	0.967	24	0.583
	syriaca treatment	0.934	21	0.168

Table A.11. Student's t-test comparisons of square root-transformed control vs 5 µg coumaphos treatment data of three subspecies in 12-hour analysis of activity monitoring assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.639	0.428	-1.187	46	0.241	-3.855	3.249	-10.395	2.685
	Equal variances not assumed			-1.187	45.460	0.242	-3.855	3.249	-10.397	2.687

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.029	0.866	-2.022	46	0.049	-5.729	2.832	-11.430	-0.027
	Equal variances not assumed			-2.022	46.000	0.049	-5.729	2.832	-11.430	-0.027

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	3.354	0.074	-5.559	43	0.000	-20.755	3.733	-28.284	-13.226
	Equal variances not assumed			-5.405	33.391	0.000	-20.755	3.840	-28.564	-12.947

Table A.12. Means, sample sizes, sd's and se's of 5 µg treatment/control mean data of three subspecies in 12-hour analysis of activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica*	Mean	1.219	.166
		Sample Size	24	
		Std. Deviation	.810	
	carnica	Mean	1.548	.250
		Sample Size	24	
		Std. Deviation	1.225	
	syriaca	Mean	4.271	.750
		Sample Size	21	
		Std. Deviation	3.434	

Table A.13. Normality tests of original and log-transformed data of 5 ug treatment/control mean data of three subspecies in 12-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	caucasica	0.839	24	0.001
	carnica	0.843	24	0.002
	syriaca	0.842	21	0.003

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of LMA	caucasica	0.946	24	0.219
	carnica	0.980	24	0.905
	syriaca	0.973	21	0.798

Table A.14. One-way ANOVA with post hoc comparisons of log-transformed 5 ug treatment/control mean data of three subspecies in 12-hour analysis of activity monitoring assay.

Log of LMA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17.907	2	8.954	15.320	0.000
Within Groups	38.572	66	0.584		
Total	56.479	68			

Log of LMA

Levene Statistic	df1	df2	Sig.
0.622	2	66	0.540

Dependent Variable:

(I) Groups			Mean Difference (I-J)	Std. Error	Sig.	Interval	
						Lower Bound	Upper Bound
Tukey HSD	caucasica	carnica	-0.15035	0.22068	0.775	-0.6795	0.3788
		syriaca	-1,17391*	0.22843	0.000	-1.7216	-0.6262
	carnica	caucasica	0.15035	0.22068	0.775	-0.3788	0.6795
		syriaca	-1,02356*	0.22843	0.000	-1.5713	-0.4758
	syriaca	caucasica	1,17391*	0.22843	0.000	0.6262	1.7216
		carnica	1,02356*	0.22843	0.000	0.4758	1.5713
Bonferroni	caucasica	carnica	-0.15035	0.22068	1.000	-0.6925	0.3918
		syriaca	-1,17391*	0.22843	0.000	-1.7351	-0.6128
	carnica	caucasica	0.15035	0.22068	1.000	-0.3918	0.6925
		syriaca	-1,02356*	0.22843	0.000	-1.5847	-0.4624
	syriaca	caucasica	1,17391*	0.22843	0.000	0.6128	1.7351
		carnica	1,02356*	0.22843	0.000	0.4624	1.5847

*. The mean difference is significant at the 0.05 level.

B. Statistical Tables of 24-Hour Locomotor Activity Analysis

Table B.1. Means, sample sizes, sd's and se's of control vs 1 µg coumaphos treatment groups of 24-hour of activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica* control	Mean	3155.29	319.937
		Sample Size	24	
		Std. Deviation	1567.366	
	caucasica 1 µg treatment	Mean	3748.22	338.502
		Sample Size	23	
		Std. Deviation	1623.396	
	carnica control	Mean	1994.96	294.679
		Sample Size	24	
		Std. Deviation	1443.625	
	carnica 1 µg treatment	Mean	1957.96	239.142
		Sample Size	24	
		Std. Deviation	1171.549	
	syriaca control	Mean	2376.50	323.471
		Sample Size	24	
		Std. Deviation	1584.679	
	syriaca 1 µg treatment	Mean	3411.46	525.724
		Sample Size	24	
		Std. Deviation	2575.512	

Table B.2. Normality tests of original and square root-transformed data of control vs 1 µg coumaphos treatment data of three subspecies in 24-hour analysis of activity monitoring assay.

	groups	Shapiro-Wilk		
		Statistic	df	Sig.
LMA	*caucasica* control	0.927	24	0.083
	caucasica treatment	0.893	23	0.018
	carnica control	0.838	24	0.001
	carnica treatment	0.913	24	0.041
	syriaca control	0.942	24	0.179
	syriaca treatment	0.792	24	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of LMA	*caucasica* control	0.964	24	0.519
	caucasica treatment	0.932	23	0.120
	carnica control	0.919	24	0.057
	carnica treatment	0.973	24	0.729
	syriaca control	0.973	24	0.735
	syriaca treatment	0.931	24	0.103

Table B.3. Student's t-test comparisons of square root-transformed control vs 1 µg coumaphos treatment data of three subspecies in 24-hour analysis of activity monitoring assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.242	0.625	-1.414	45	0.164	-5.454	3.856	-13.220	2.312
	Equal variances not assumed			-1.417	44.887	0.163	-5.454	3.848	-13.205	2.297

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.403	0.529	-0.071	46	0.944	-0.287	4.042	-8.423	7.849
	Equal variances not assumed			-0.071	44.807	0.944	-0.287	4.042	-8.429	7.854

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.006	0.939	-1.784	46	0.081	-9.477	5.311	-20.168	1.215
	Equal variances not assumed			-1.784	45.338	0.081	-9.477	5.311	-20.172	1.219

Table B.4. Means, sample sizes, sd's and se's of control vs 2 µg coumaphos treatment groups of 24-hour analysis of activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica* control	Mean	3155.29	319.937
		Sample Size	24	
		Std. Deviation	1567.366	
	caucasica 2 µg treatment	Mean	2621.43	315.225
		Sample Size	23	
		Std. Deviation	1511.765	
	carnica control	Mean	1994.96	294.679
		Sample Size	24	
		Std. Deviation	1443.625	
	carnica 2 µg treatment	Mean	3032.33	473.926
		Sample Size	24	
		Std. Deviation	2321.752	
	syriaca control	Mean	2376.50	323.471
		Sample Size	24	
		Std. Deviation	1584.679	
	syriaca 2 µg treatment	Mean	3521.54	475.608
		Sample Size	24	
		Std. Deviation	2329.995	

Table B.5. Normality tests of original and log-transformed data of control vs 2 µg coumaphos treatment data of three subspecies in 24-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	*caucasica* control	0.927	24	0.083
	caucasica treatment	0.819	23	0.001
	carnica control	0.838	24	0.001
	carnica treatment	0.725	24	0.000
	syriaca control	0.942	24	0.179
	syriaca treatment	0.774	24	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of LMA	*caucasica* control	0.955	24	0.349
	caucasica treatment	0.945	23	0.229
	carnica control	0.966	24	0.579
	carnica treatment	0.965	24	0.548
	syriaca control	0.897	24	0.018
	syriaca treatment	0.940	24	0.160

Table B.6. Student's t-test comparisons of log-transformed control vs 2 µg coumaphos treatment data of three subspecies in 24-hour analysis of activity monitoring assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Log of LMA	Equal variances assumed	0.054	0.817	1.271	45	0.210	0.191	0.150	-0.112	0.493
	Equal variances not assumed			1.272	44.992	0.210	0.191	0.150	-0.111	0.492

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Log of LMA	Equal variances assumed	0.160	0.691	-2.333	46	0.024	-0.451	0.193	-0.839	-0.062
	Equal variances not assumed			-2.333	45.506	0.024	-0.451	0.193	-0.839	-0.062

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Log of LMA	Equal variances assumed	3.175	0.081	-2.388	46	0.021	-0.529	0.222	-0.975	-0.083
	Equal variances not assumed			-2.388	38.208	0.022	-0.529	0.222	-0.978	-0.081

Table B.7. Mann-Whitney U test comparisons of control vs 2 µg coumaphos treatment data of *carnica* subspecies in 24-hour analysis of activity monitoring assay.

carnica	
Test Statistic	384.000
Standardized Test Statistic	1.979
Standard Error	48.497
Significance	0.048

Table B.8. Mann-Whitney U test comparisons of control vs 2 µg coumaphos treatment data of *syriaca* subspecies in 24-hour analysis of activity monitoring assay.

syriaca	
Test Statistic	376.500
Standardized Test Statistic	1.815
Standard Error	48.497
Significance	0.070

Table B.9. Means, sample sizes, sd's and se's of control vs 5 µg coumaphos treatment groups of 24-hour activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica* control	Mean	3155.29	319.937
		Sample Size	24	
		Std. Deviation	1567.366	
	caucasica 5 µg treatment	Mean	3204.58	310.074
		Sample Size	24	
		Std. Deviation	1519.046	
	carnica control	Mean	1994.96	294.679
		Sample Size	24	
		Std. Deviation	1443.625	
	carnica 5 µg treatment	Mean	2565.88	326.220
		Sample Size	24	
		Std. Deviation	1598.145	
	syriaca control	Mean	2376.50	323.471
		Sample Size	24	
		Std. Deviation	1584.679	
	syriaca 5 µg treatment	Mean	4213.19	565.141
		Sample Size	21	
		Std. Deviation	2589.802	

Table B.10. Normality tests of original and square root-transformed data of control vs 5 µg coumaphos treatment data of three subspecies in 24-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	*caucasica* control	0.927	24	0.083
	caucasica treatment	0.934	24	0.119
	carnica control	0.838	24	0.001
	carnica treatment	0.906	24	0.029
	syriaca control	0.942	24	0.179
	syriaca treatment	0.893	21	0.025

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of LMA	*caucasica* control	0.964	24	0.519
	caucasica treatment	0.958	24	0.406
	carnica control	0.919	24	0.057
	carnica treatment	0.972	24	0.704
	syriaca control	0.973	24	0.735
	syriaca treatment	0.964	21	0.606

Table B.11. Student's t-test comparisons of original and square root-transformed control vs 5 µg coumaphos treatment data of three subspecies in 24-hour analysis of activity monitoring assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
LMA	Equal variances assumed	0.158	0.693	-0.111	46	0.912	-49.292	445.540	-946.117	847.533
	Equal variances not assumed			-0.111	45.955	0.912	-49.292	445.540	-946.140	847.557

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.019	0.892	-1.405	46	0.167	-6.195	4.410	-15.073	2.682
	Equal variances not assumed			-1.405	45.976	0.167	-6.195	4.410	-15.073	2.682

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.152	0.698	-3.060	43	0.004	-16.498	5.392	-27.372	-5.624
	Equal variances not assumed			-3.041	40.877	0.004	-16.498	5.426	-27.457	-5.539

Table B.12. Means, sample sizes, sd's and se's of 5 µg treatment/control mean data of three subspecies in 24-hour activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica*	Mean	1.016	.0983
		Sample Size	24	
		Std. Deviation	.482	
	carnica	Mean	1.287	.164
		Sample Size	24	
		Std. Deviation	.802	
	syriaca	Mean	1.773	.238
		Sample Size	21	
		Std. Deviation	1.090	

Table B.13. Normality tests of original and square root-transformed data of 5 ug treatment/control mean data of three subspecies in 24-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	*caucasica*	0.934	24	0.119
	carnica	0.906	24	0.029
	syriaca	0.893	21	0.025

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of LMA	*caucasica*	0.951	24	0.281
	carnica	0.946	24	0.224
	syriaca	0.978	21	0.888

Table B.14. One-way ANOVA with post hoc comparisons of log-transformed 5 ug treatment/control mean data of three subspecies 24-hour activity monitoring assay.

Log of LMA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.931	2	1.466	3.920	0.025
Within Groups	24.673	66	0.374		
Total	27.604	68			

Log of LMA

Levene Statistic	df1	df2	Sig.
0.494	2	66	0.613

Multiple Comparisons

Dependent Variable:

(I) Groups			Mean Difference (I-J)	Std. Error	Sig.	Interval	
						Lower Bound	Upper Bound
Tukey HSD	caucasica	carnica	-0.15386	0.17650	0.660	-0.5771	0.2693
		syriaca	-,50260*	0.18269	0.021	-0.9406	-0.0646
	carnica	caucasica	0.15386	0.17650	0.660	-0.2693	0.5771
		syriaca	-0.34874	0.18269	0.144	-0.7868	0.0893
	syriaca	caucasica	,50260*	0.18269	0.021	0.0646	0.9406
		carnica	0.34874	0.18269	0.144	-0.0893	0.7868
Bonferroni	caucasica	carnica	-0.15386	0.17650	1.000	-0.5874	0.2797
		syriaca	-,50260*	0.18269	0.023	-0.9514	-0.0538
	carnica	caucasica	0.15386	0.17650	1.000	-0.2797	0.5874
		syriaca	-0.34874	0.18269	0.182	-0.7975	0.1001
	syriaca	caucasica	,50260*	0.18269	0.023	0.0538	0.9514
		carnica	0.34874	0.18269	0.182	-0.1001	0.7975

*. The mean difference is significant at the 0.05 level.

C. Statistical Tables of Second 12-hour Locomotor Activity Analysis

Table C.1. Means, sample sizes, sd's and se's of control vs 2 µg coumaphos treatment groups of second 12-hour of activity monitoring assay.

Groups		Statistic	Std. Error
caucasica control	Mean	2195.63	239.753
	Sample Size	24	
	Std. Deviation	1174.544	
caucasica 2 µg treatment	Mean	1799.96	253.469
	Sample Size	23	
	Std. Deviation	1215.595	
carnica control	Mean	1529.33	232.979
	Sample Size	24	
	Std. Deviation	1141.361	
carnica 2 µg treatment	Mean	2335.25	415.951
	Sample Size	24	
	Std. Deviation	2037.737	
syriaca control	Mean	1987.13	283.806
	Sample Size	24	
	Std. Deviation	1390.358	
syriaca 2 µg treatment	Mean	2471.13	321.884
	Sample Size	24	
	Std. Deviation	1576.904	

Table C.2. Normality tests of original and square root-transformed data of control vs 2 µg coumaphos treatment data of three subspecies in second 12-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	*caucasica* control	0.903	24	0.024
	caucasica treatment	0.784	23	0.000
	carnica control	0.874	24	0.006
	carnica treatment	0.697	24	0.000
	syriaca control	0.936	24	0.132
	syriaca treatment	0.864	24	0.004

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of LMA	*caucasica* control	0.971	24	0.684
	caucasica treatment	0.948	23	0.261
	carnica control	0.974	24	0.758
	carnica treatment	0.972	24	0.724
	syriaca control	0.880	24	0.008
	syriaca treatment	0.982	24	0.932

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of LMA	*caucasica* control	0.957	24	0.383
	caucasica treatment	0.877	23	0.009
	carnica control	0.945	24	0.213
	carnica treatment	0.871	24	0.006
	syriaca control	0.969	24	0.645
	syriaca treatment	0.946	24	0.223

Table C.3. Student's t-test comparisons of square root-transformed control vs 2 µg coumaphos data of three subspecies in second 12-hour analysis of activity monitoring assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Log of LMA	Equal variances assumed	0.103	0.750	1.449	45	0.154	0.234	0.161	-0.091	0.559
	Equal variances not assumed			1.447	44.603	0.155	0.234	0.162	-0.092	0.560

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Log of LMA	Equal variances assumed	0.160	0.691	-2.333	46	0.024	-0.451	0.193	-0.839	-0.062
	Equal variances not assumed			-2.333	45.506	0.024	-0.451	0.193	-0.839	-0.062

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.912	0.344	-1.351	46	0.183	-6.178	4.572	-15.382	3.025
	Equal variances not assumed			-1.351	45.157	0.183	-6.178	4.572	-15.386	3.030

Table C.4. Mann-Whitney U t-test comparisons of control vs 2 µg coumaphos data of *carnica* subspecies in second 12-hour analysis of activity monitoring assay.

carnica	
Test Statistic	356.500
Standardized Test Statistic	1.412
Standard Error	48.497
Significance	0.066

Table C.5. Means, sample sizes, sd's and se's of control vs 5 µg coumaphos treatment groups of second 12-hour of activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica* control	Mean	2195.63	239.753
		Sample Size	24	
		Std. Deviation	1174.544	
	caucasica 5 µg treatment	Mean	2035.04	262.168
		Sample Size	23	
		Std. Deviation	1284.358	
	carnica control	Mean	1529.33	232.979
		Sample Size	24	
		Std. Deviation	1141.361	
	carnica 5 µg treatment	Mean	1845.54	281.068
		Sample Size	24	
		Std. Deviation	1376.949	
	syriaca control	Mean	1987.13	283.806
		Sample Size	24	
		Std. Deviation	1390.358	
	syriaca 5 µg treatment	Mean	2550.19	388.126
		Sample Size	21	
		Std. Deviation	1778.616	

Table C.6. Normality tests of original and square root-transformed data of control vs 5 µg coumaphos treatment data of three subspecies in 12-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	*caucasica* control	0.903	24	0.024
	caucasica treatment	0.908	24	0.032
	carnica control	0.874	24	0.006
	carnica treatment	0.798	24	0.000
	syriaca control	0.936	24	0.132
	syriaca treatment	0.902	21	0.038

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of LMA	*caucasica* control	0.957	24	0.383
	caucasica treatment	0.960	24	0.436
	carnica control	0.945	24	0.213
	carnica treatment	0.943	24	0.195
	syriaca control	0.969	24	0.645
	syriaca treatment	0.960	21	0.513

Table C.7. Student's t-test comparisons of square root-transformed control vs 5 µg coumaphos data of three subspecies in 12-hour analysis of activity monitoring assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.614	0.437	0.629	46	0.532	2.381	3.783	-5.234	9.995
	Equal variances not assumed			0.629	44.977	0.532	2.381	3.783	-5.239	10.000

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.231	0.633	-0.962	46	0.341	-3.958	4.113	-12.236	4.320
	Equal variances not assumed			-0.962	45.965	0.341	-3.958	4.113	-12.237	4.320

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.007	0.934	-1.060	43	0.295	-5.642	5.322	-16.376	5.091
	Equal variances not assumed			-1.052	40.587	0.299	-5.642	5.362	-16.474	5.189

Table C.8. Means, sample sizes, sd's and se's of 5 ug treatment/control mean data of second 12-hour activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica*	Mean	0.93	0.119
		Sample Size	24	
		Std. Deviation	0.585	
	carnica	Mean	1.21	0.184
		Sample Size	24	
		Std. Deviation	0.900	
	syriaca	Mean	1.28	0.195
		Sample Size	21	
		Std. Deviation	0.895	

Table C.9. Normality tests of original and square root-transformed data of 5 ug treatment/control mean data of three subspecies in second 12-hour analysis of activity monitoring assay.

		Shapiro-Wilk		
Groups		Statistic	df	Sig.
Activity	*caucasica*	0.908	24	0.032
	carnica	0.798	24	0.000
	syriaca	0.902	21	0.038

		Shapiro-Wilk		
Groups		Statistic	df	Sig.
Log of Activity	*caucasica*	0.955	24	0.347
	carnica	0.959	24	0.416
	syriaca	0.565	21	0.000

Table C.10. One-way ANOVA with post hoc comparisons of log-transformed 5 ug treatment/control mean data of three subspecies of second 12-hour activity monitoring assay.

Log of LMA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.776	2	0.388	0.289	0.750
Within Groups	88.576	66	1.342		
Total	89.352	68			

Log of Extinction

Levene Statistic	df1	df2	Sig.
1.522	2	66	0.226

Multiple Comparisons

Groups			Mean Difference (I-J)	Std. Error	Sig.	Interval Lower Bound	Interval Upper Bound
Tukey HSD	*caucasica*	*carnica*	-0.23047	0.33442	0.771	-1.0323	0.5714
		syriaca	-0.01782	0.34616	0.999	-0.8478	0.8122
	carnica	*caucasica*	0.23047	0.33442	0.771	-0.5714	1.0323
		syriaca	0.21265	0.34616	0.813	-0.6173	1.0426
	syriaca	*caucasica*	0.01782	0.34616	0.999	-0.8122	0.8478
		carnica	-0.21265	0.34616	0.813	-1.0426	0.6173
Bonferroni	*caucasica*	*carnica*	-0.23047	0.33442	1.000	-1.0520	0.5911
		syriaca	-0.01782	0.34616	1.000	-0.8682	0.8325
	carnica	*caucasica*	0.23047	0.33442	1.000	-0.5911	1.0520
		syriaca	0.21265	0.34616	1.000	-0.6377	1.0630
	syriaca	*caucasica*	0.01782	0.34616	1.000	-0.8325	0.8682
		carnica	-0.21265	0.34616	1.000	-1.0630	0.6377

Table C.11. Kruskal-Wallis analysis of log-transformed 5 ug treatment/control mean data of three subspecies of second 12-hour activity monitoring assay.

LMA	
Test Statistic	2.900
Degrees of Freedom	2
Significance	0.235

Table C.12. Means, sample sizes, sd's and se's of control groups in first vs second 12-hour of activity monitoring assay.

	Groups		Statistic	Std. Error
LMA	*caucasica* first 12-hour control group	Mean	959.67	154.078
		Sample Size	24	
		Std. Deviation	754.823	
	caucasica second 12-hour control group	Mean	2195.63	239.753
		Sample Size	23	
		Std. Deviation	1174.544	
	carnica first 12-hour control group	Mean	465.63	98.649
		Sample Size	24	
		Std. Deviation	483.279	
	carnica second 12-hour control group	Mean	1529.33	232.979
		Sample Size	24	
		Std. Deviation	1141.361	
	syriaca first 12-hour control group	Mean	389.38	73.421
		Sample Size	24	
		Std. Deviation	359.688	
	syriaca second 12-hour control group	Mean	1987.13	283.806
		Sample Size	21	
		Std. Deviation	1390.358	

Table C.13. Normality tests of original and square root-transformed data control groups of three subspecies in first vs. second 12-hour analysis of activity monitoring assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
LMA	*caucasica* first 12	0.888	24	0.012
	caucasica last 12	0.903	24	0.024
	carnica first 12	0.763	24	0.000
	carnica last 12	0.874	24	0.006
	syriaca first 12	0.890	24	0.013
	syriaca first 12	0.936	24	0.132

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of LMA	*caucasica* first 12	0.955	24	0.343
	caucasica last 12	0.957	24	0.383
	carnica first 12	0.924	24	0.072
	carnica last 12	0.945	24	0.213
	syriaca first 12	0.967	24	0.583
	syriaca first 12	0.969	24	0.645

Table C.14. Student's t-test comparisons of square root-transformed control group data of three subspecies in first vs. second 12-hour analysis of activity monitoring assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	0.000	0.984	-4.811	46	0.000	-16.618	3.454	-23.571	-9.666
	Equal variances not assumed			-4.811	45.984	0.000	-16.618	3.454	-23.571	-9.666

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	3.980	0.052	-4.942	46	0.000	-17.280	3.496	-24.318	-10.242
	Equal variances not assumed			-4.942	41.099	0.000	-17.280	3.496	-24.341	-10.220

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of LMA	Equal variances assumed	7.528	0.009	-6.063	46	0.000	-24.115	3.978	-32.122	-16.108
	Equal variances not assumed			-6.063	36.746	0.000	-24.115	3.978	-32.177	-16.054

D. Statistical Tables of Proboscis Extension Reflex Analysis

Table D.1. Means, sample sizes, sd's and se's of control vs 5 µg coumaphos treatment groups acquisition data in PER assay.

	Groups		Statistic	Std. Error
Acquisition	*caucasica* control	Mean	7.649	.63265
		Sample Size	36	
		Std. Deviation	3.796	
	caucasica treatment	Mean	6.658	.64843
		Sample Size	34	
		Std. Deviation	3.78099	
	carnica control	Mean	7.5918	.53099
		Sample Size	55	
		Std. Deviation	3.93790	
	carnica treatment	Mean	6.5710	.66904
		Sample Size	41	
		Std. Deviation	4.28397	
	syriaca control	Mean	9.2556	.42940
		Sample Size	57	
		Std. Deviation	3.24192	
	syriaca treatment	Mean	7.9080	.49336
		Sample Size	49	
		Std. Deviation	3.45353	

Table D.2. Normality tests of original, log and square root-transformed control vs 5 µg coumaphos treatment acquisition data of three subspecies in PER assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Acquisition	*caucasica* control	0.741	36	0.000
	caucasica treatment	0.889	34	0.002
	carnica control	0.787	55	0.000
	carnica treatment	0.819	41	0.000
	syriaca control	0.607	57	0.000
	syriaca treatment	0.828	49	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of Acquisition	*caucasica* control	0.513	36	0.000
	caucasica treatment	0.577	34	0.000
	carnica control	0.529	55	0.000
	carnica treatment	0.613	41	0.000
	syriaca control	0.377	57	0.000
	syriaca treatment	0.464	49	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of Acquisition	*caucasica* control	0.669	36	0.000
	caucasica treatment	0.829	34	0.000
	carnica control	0.672	55	0.000
	carnica treatment	0.742	41	0.000
	syriaca control	0.492	57	0.000
	syriaca treatment	0.676	49	0.000

Table D.3. Student's t-test comparisons of log and square root-transformed control vs 5 µg coumaphos treatment acquisition data of three subspecies in PER assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Acquisition	Equal variances assumed	0.200	0.656	1.095	68	0.278	0.992	0.906	-0.816	2.800
	Equal variances not assumed			1.095	67.802	0.277	0.992	0.906	-0.816	2.800

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Acquisition	Equal variances assumed	1.505	0.223	1.210	94	0.229	1.021	0.844	-0.654	2.696
	Equal variances not assumed			1.195	82.125	0.235	1.021	0.854	-0.678	2.720

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Acquisition	Equal variances assumed	1.934	0.167	2.070	104	0.041	1.348	0.651	0.057	2.638
	Equal variances not assumed			2.060	99.384	0.042	1.348	0.654	0.050	2.645

Table D.4. Mann-Whitney U test comparison of square root-transformed control vs 5 µg coumaphos treatment acquisition data of three subspecies in PER assay.

caucasica	
Test Statistic	522.500
Standard Error	84.022
Standardized Test Statistic	-1.065
Significance	0.287

carnica	
Test Statistic	973.500
Standard Error	133.181
Standardized Test Statistic	-1.156
Significance	0,248

syriaca	
Test Statistic	990.000
Standard Error	152.508
Standardized Test Statistic	2.665
Significance	0.08

Table D.5. Means, sample sizes, sd's and se's of 5 µg treatment/control mean acquisition data of three subspecies in PER assay.

Descriptives

	Groups		Statistic	Std. Error
Acquisition	*caucasica*	Mean	.871	.085
		Sample Size	34	
		Std. Deviation	.495	
	carnica	Mean	.866	.089
		Sample Size	41	
		Std. Deviation	.566	
	syriaca	Mean	.856	.054
		Sample Size	49	
		Std. Deviation	.374	

Table D.6. Normality tests of original and log-transformed 5 µg treatment/control mean acquisition data of three subspecies in PER assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Acquisition	*caucasica*	0.889	34	0.002
	carnica	0.819	41	0.000
	syriaca	0.828	49	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of Acquisition	*caucasica*	0.568	34	0.000
	carnica	0.609	41	0.000
	syriaca	0.463	49	0.000

Table D.7. One-way ANOVA with post hoc comparisons of log-transformed 5 µg treatment/control mean acquisition data of three subspecies in PER assay.

Log of Acquisition

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.193	2	7.097	1.237	0.294
Within Groups	693.992	121	5.735		
Total	708.185	123			

Test of Homogeneity of Variances

Log of Acquisition

Levene Statistic	df1	df2	Sig.
6.475	2	121	0.002

Dependent Log of Acquisition

(I) Groups			Mean Difference (I-J)	Std. Error	Sig.	Interval Lower Bound	Interval Upper Bound
Tamhane	caucasica	carnica	0.71408	0.57581	0.524	-0.6941	2.1222
		syriaca	-0.00853	0.46030	1.000	-1.1335	1.1164
	carnica	caucasica	-0.71408	0.57581	0.524	-2.1222	0.6941
		syriaca	-0.72261	0.54850	0.472	-2.0639	0.6187
	syriaca	caucasica	0.00853	0.46030	1.000	-1.1164	1.1335
		carnica	0.72261	0.54850	0.472	-0.6187	2.0639
Dunnett T3	caucasica	carnica	0.71408	0.57581	0.520	-0.6928	2.1210
		syriaca	-0.00853	0.46030	1.000	-1.1325	1.1154
	carnica	caucasica	-0.71408	0.57581	0.520	-2.1210	0.6928
		syriaca	-0.72261	0.54850	0.469	-2.0628	0.6175
	syriaca	caucasica	0.00853	0.46030	1.000	-1.1154	1.1325
		carnica	0.72261	0.54850	0.469	-0.6175	2.0628

Table D.8. Kruskal-Wallis analysis of 5µg treatment /control mean acquisition data of three subspecies in PER assay.

Acquisition	
Test Statistic	1.134
Degrees of Freedom	2
Significance	0.567

Table D.9. Means, sample sizes, sd's and se's of control vs 5 µg coumaphos treatment groups of extinction data in PER assay.

	Groups		Statistic	Std. Error
Extinction	*caucasica* control	Mean	8.233	.769
		Sample Size	27	
		Std. Deviation	3.994	
	caucasica treatment	Mean	6.560	.745
		Sample Size	20	
		Std. Deviation	3.332	
	carnica control	Mean	7.623	.618
		Sample Size	31	
		Std. Deviation	3.442	
	carnica treatment	Mean	6.6189	.589
		Sample Size	23	
		Std. Deviation	2.825	
	syriaca control	Mean	8.243	.544
		Sample Size	43	
		Std. Deviation	3.565	
	syriaca treatment	Mean	9.173	.543
		Sample Size	37	
		Std. Deviation	3.304	

Table D.10. Normality tests of original, log and square root-transformed control vs 5 µg coumaphos treatment extinction data of three subspecies in PER assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Extinction	*caucasica* control	0.828	27	0.000
	caucasica treatment	0.952	20	0.402
	carnica control	0.926	31	0.033
	carnica treatment	0.950	23	0.298
	syriaca control	0.896	43	0.001
	syriaca treatment	0.813	37	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of Extinction	*caucasica* control	0.805	27	0.000
	caucasica treatment	0.894	20	0.032
	carnica control	0.851	31	0.001
	carnica treatment	0.923	23	0.077
	syriaca control	0.836	43	0.000
	syriaca treatment	0.726	37	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. Of Extinction	*caucasica* control	0.830	27	0.000
	caucasica treatment	0.941	20	0.249
	carnica control	0.908	31	0.011
	carnica treatment	0.958	23	0.416
	syriaca control	0.882	43	0.000
	syriaca treatment	0.781	37	0.000

Table D.11. Student's t-test comparisons of control vs 5 µg coumaphos treatment extinction data of three subspecies in PER assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Difference Lower	Interval of the Difference Upper
Extinction	Equal variances assumed	2.624	0.112	1.520	45	0.135	1.672	1.100	-0.543	3.888
	Equal variances not assumed			1.562	44.296	0.125	1.672	1.070	-0.485	3.829

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Difference Lower	Interval of the Difference Upper
Extinction	Equal variances assumed	1.778	0.188	1.142	52	0.259	1.004	0.879	-0.760	2.769
	Equal variances not assumed			1.176	51.415	0.245	1.004	0.854	-0.709	2.718

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Difference Lower	Interval of the Difference Upper
Extinction	Equal variances assumed	1.214	0.274	-1.203	78	0.233	-0.930	0.773	-2.468	0.609
	Equal variances not assumed			-1.210	77.547	0.230	-0.930	0.768	-2.460	0.600

Table D.12. Mann-Whitney U test comparisons of control vs 5 µg coumaphos treatment extinction data of three subspecies in PER assay.

caucasica	
Test Statistic	190.500
Standard Error	45.996
Standardized Test Statistic	-1.728
Significance	0.084

carnica	
Test Statistic	284.500
Standard Error	56.841
Standardized Test Statistic	-1.267
Significance	0.205

syriaca	
Test Statistic	917.500
Standard Error	101.388
Standardized Test Statistic	1.203
Significance	0.229

Table D.13. Means, sample sizes, sd's and se's of 5 µg treatment/control mean extinction data of three subspecies in PER assay.

	Groups		Statistic	Std. Error
Extinction	*caucasica*	Mean	.797	.091
		Sample Size	20	
		Variance	.164	
		Std. Deviation	.405	
	carnica	Mean	.868	.078
		Sample Size	23	
		Variance	.138	
		Std. Deviation	.371	
	syriaca	Mean	1.113	.066
		Sample Size	37	
		Variance	.161	
		Std. Deviation	.402	

Table D.14. Normality tests of original and log-transformed 5 µg treatment/control mean extinction data of three subspecies in PER assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Extinction	*caucasica*	0.952	20	0.402
	carnica	0.950	23	0.298
	syriaca	0.813	37	0.000

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of Extinction	*caucasica*	0.894	20	0.032
	carnica	0.923	23	0.077
	syriaca	0.725	37	0.000

Table D.15. One-way ANOVA with post hoc comparisons of log-transformed 5 µg treatment/control mean extinction data of three subspecies in PER assay.

Log of Extinction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.336	2	1.168	3.731	0.028
Within Groups	24.110	77	0.313		
Total	26.447	79			

Test of Homogeneity of Variances

Log of Extinction

Levene Statistic	df1	df2	Sig.
1.760	2	77	0.179

Log of Extinction

(I) Groups			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	caucasica	carnica	-0.16046	0.17109	0.618	-0.5693	0.2484
		syriaca	-,40775*	0.15530	0.028	-0.7789	-0.0366
	carnica	caucasica	0.16046	0.17109	0.618	-0.2484	0.5693
		syriaca	-0.24729	0.14858	0.225	-0.6024	0.1078
	syriaca	caucasica	,40775*	0.15530	0.028	0.0366	0.7789
		carnica	0.24729	0.14858	0.225	-0.1078	0.6024
Bonferroni	caucasica	carnica	-0.16046	0.17109	1.000	-0.5792	0.2583
		syriaca	-,40775*	0.15530	0.031	-0.7878	-0.0277
	carnica	caucasica	0.16046	0.17109	1.000	-0.2583	0.5792
		syriaca	-0.24729	0.14858	0.300	-0.6109	0.1164
	syriaca	caucasica	,40775*	0.15530	0.031	0.0277	0.7878
		carnica	0.24729	0.14858	0.300	-0.1164	0.6109

Table D.16. Kruskal-Wallis analysis of 5µg treatment /control mean extinction data of three subspecies in PER assay.

Extinction	
Test Statistic	8.117
Degrees of Freedom	2
Significance	0.017

Sample1 -Sample 2	Test statistic	Standard Error	Std. Test Statistic	Significance	Adjusted Significance
caucasica-carnica	-1.959	7.072	-0.277	0.782	1.000
caucasica-syriaca	-15.755	6.420	-2.454	0.014	0.042
Carnica-syriaca	-13.797	6.142	-2.456	0.025	0.074

E. Statistical Tables of Electric Shock Avoidance Analysis

Table E.1. Means, sample sizes, sd's and se's of control vs 5 µg coumaphos treatment groups of duration and movement data in ESA assay.

	Groups		Statistic	Std. Error
Duration	*caucasica* control	Mean	16.683	3.020
		Sample Size	32	
		Std. Deviation	17.085	
	caucasica treatment	Mean	25.129	3.750
		Sample Size	36	
		Std. Deviation	22.503	
	carnica control	Mean	35.313	4.339
		Sample Size	39	
		Std. Deviation	27.102	
	carnica treatment	Mean	70.456	4.274
		Sample Size	42	
		Std. Deviation	30.825	
	syriaca control	Mean	37.2490	4.510
		Sample Size	39	
		Std. Deviation	28.165	
	syriaca treatment	Mean	89.110	6.877
		Sample Size	30	
		Std. Deviation	37.667	
Movement	*caucasica* control	Mean	11.125	1.2458
		Sample Size	32	
		Std. Deviation	7.047	
	caucasica treatment	Mean	6.472	.8343
		Sample Size	36	
		Std. Deviation	5.005	
	carnica control	Mean	19.410	2.0430
		Sample size	39	
		Std. Deviation	12.758	
	carnica treatment	Mean	13.750	.8482
		Sample Size	52	

	Std. Deviation	6.116	
syriaca control	Mean	24.077	1.959
	Sample size	39	
	Std. Deviation	12.237	
syriaca treatment	Mean	24.333	1.614
	Sample Size	30	
	Std. Deviation	8.844	

Table E.2. Normality tests of original, log and square root-transformed data control vs 5 µg coumaphos treatment data of three subspecies in ESA assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Duration	*caucasica* control	0.721	32	0.000
	caucasica treatment	0.806	36	0.000
	carnica control	0.907	39	0.004
	carnica treatment	0.976	52	0.384
	syriaca control	0.833	39	0.000
	syriaca treatment	0.958	30	0.273
movement	*caucasica* control	0.806	32	0.000
	caucasica treatment	0.742	36	0.000
	carnica control	0.878	39	0.001
	carnica treatment	0.957	52	0.058
	syriaca control	0.937	39	0.030
	syriaca treatment	0.977	30	0.754

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of Duration	*caucasica* control	0.979	32	0.784
	caucasica treatment	0.970	36	0.415
	carnica control	0.931	39	0.019
	carnica treatment	0.925	52	0.003
	syriaca control	0.987	39	0.920
	syriaca treatment	0.812	30	0.000
Log of Movement	*caucasica* control	0.960	32	0.271
	caucasica treatment	0.932	36	0.030
	carnica control	0.962	39	0.215
	carnica treatment	0.960	52	0.079
	syriaca control	0.952	39	0.098
	syriaca treatment	0.888	30	0.004

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Sqrt. of Duration	*caucasica* control	0.912	32	0.013
	caucasica treatment	0.909	36	0.006
	carnica control	0.943	39	0.049
	carnica treatment	0.979	52	0.470
	syriaca control	0.944	39	0.054
	syriaca treatment	0.920	30	0.026
Sqrt. of Movement	*caucasica* control	0.914	32	0.014
	caucasica treatment	0.855	36	0.000
	carnica control	0.936	39	0.028
	carnica treatment	0.976	52	0.364
	syriaca control	0.964	39	0.249
	syriaca treatment	0.956	30	0.238

Table E.3. Student's t-test comparisons of log and square root-transformed control vs 5 µg coumaphos treatment data of three subspecies in ESA assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Log of Duration	Equal variances assumed	0.116	0.735	-2.007	66	0.049	-0.444	0.221	-0.886	-0.002
	Equal variances not assumed			-2.001	64.184	0.050	-0.444	0.222	-0.888	-0.001
Log of Movement	Equal variances assumed	0.353	0.554	4.147	66	0.000	0.593	0.143	0.308	0.879
	Equal variances not assumed			4.172	65.983	0.000	0.593	0.142	0.309	0.877

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of Duration	Equal variances assumed	3.715	0.057	-6.006	89	0.000	-2.716	0.452	-3.614	-1.817
	Equal variances not assumed			-5.829	71.683	0.000	-2.716	0.466	-3.644	-1.787
Log of Movement	Equal variances assumed	5.802	0.018	1.972	89	0.052	0.239	0.121	-0.002	0.481
	Equal variances not assumed			1.885	66.141	0.064	0.239	0.127	-0.014	0.493

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Sqrt. of Duration	Equal variances assumed	0.193	0.662	-6.469	67	0.000	-3.425	0.529	-4.482	-2.368
	Equal variances not assumed			-6.393	59.518	0.000	-3.425	0.536	-4.497	-2.353
Sqrt. of Movement	Equal variances assumed	4.836	0.031	-0.330	67	0.742	-0.091	0.277	-0.644	0.461
	Equal variances not assumed			-0.342	66.993	0.734	-0.091	0.268	-0.626	0.443

Table E.4. Mann-Whitney U test comparison of control vs 5 µg coumaphos treatment duration data of *syriaca* subspecies in ESA assay.

Syriaca Duration	
Test Statistic	1008.00
Standard Error	82.613
Standardized Test Statistic	5.120
Significance	0.000

Table E.5. Mann-Whitney U test comparison of control vs 5 µg coumaphos treatment movement data of *caucasica* subspecies in ESA assay.

caucasica Movement	
Test Statistic	269.500
Standard Error	80.909
Standardized Test Statistic	-3.788
Significance	0.000

Table E.6. Means, sample sizes, sd's and se's of 5 µg treatment/control mean data of three subspecies in ESA assay.

	Groups		Statistic	Std. Error
Duration	*caucasica*	Mean	1.507	.225
		Sample Size	36	
		Std. Deviation	1.349	
	carnica	Mean	1.996	.122
		Sample Size	52	
		Std. Deviation	.873	
	syriaca	Mean	2.393	.185
		Sample Size	30	
		Std. Deviation	1.012	
Movement	*caucasica*	Mean	.582	.075
		Sample Size	36	
		Std. Deviation	.450	
	carnica	Mean	.709	.044
		Sample Size	52	
		Std. Deviation	.316	
	syriaca	Mean	1.011	.068
		Sample Size	30	
		Std. Deviation	.368	

Table E.7. Normality tests of original and log-transformed 5 µg treatment/control mean data of three subspecies in ESA assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Duration	*caucasica*	0.806	36	0.000
	carnica	0.976	52	0.384
	syriaca	0.958	30	0.273
Movement	*caucasica*	0.742	36	0.000
	carnica	0.957	52	0.058
	syriaca	0.977	30	0.754

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of Duration	*caucasica*	0.970	36	0.415
	carnica	0.925	52	0.003
	syriaca	0.812	30	0.000
Log of Movement	*caucasica*	0.932	36	0.030
	carnica	0.960	52	0.079
	syriaca	0.888	30	0.004

Table E.8. One-way ANOVA with post hoc comparisons of log-transformed 5 µg treatment/control mean data of three subspecies in ESA assay.

		Sum of Squares	df	Mean Square	F	Sig.
Log of Duration	Between Groups	9.263	2	4.632	9.909	0.000
	Within Groups	53.752	115	0.467		
	Total	63.015	117			
Log of Movement	Between Groups	7.483	2	3.741	13.658	0.000
	Within Groups	31.502	115	0.274		
	Total	38.984	117			

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Log of Duration	5.526	2	115	0.005
Log of Movement	2.518	2	115	0.085

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	Interval Lower Bound	Interval Upper Bound
Log of Duration	Tamhane	caucasica	carnica	-,53518*	0.16550	0.006	-0.9434	-0.1269
			syriaca	-,69267*	0.18833	0.001	-1.1547	-0.2307
		carnica	caucasica	,53518*	0.16550	0.006	0.1269	0.9434
			syriaca	-0.15749	0.13739	0.590	-0.4964	0.1814
		syriaca	caucasica	,69267*	0.18833	0.001	0.2307	1.1547
			carnica	0.15749	0.13739	0.590	-0.1814	0.4964
	Dunnett T3	caucasica	carnica	-,53518*	0.16550	0.006	-0.9429	-0.1274
			syriaca	-,69267*	0.18833	0.001	-1.1542	-0.2311
		carnica	caucasica	,53518*	0.16550	0.006	0.1274	0.9429
			syriaca	-0.15749	0.13739	0.585	-0.4960	0.1810
		syriaca	caucasica	,69267*	0.18833	0.001	0.2311	1.1542
			carnica	0.15749	0.13739	0.585	-0.1810	0.4960
Log of Movement	Tukey HSD	caucasica	carnica	-,29819*	0.11348	0.026	-0.5676	-0.0287
			syriaca	-,67611*	0.12938	0.000	-0.9833	-0.3689
		carnica	caucasica	,29819*	0.11348	0.026	0.0287	0.5676
			syriaca	-,37792*	0.11999	0.006	-0.6628	-0.0930
		syriaca	caucasica	,67611*	0.12938	0.000	0.3689	0.9833
			carnica	,37792*	0.11999	0.006	0.0930	0.6628
	Bonferroni	caucasica	carnica	-,29819*	0.11348	0.029	-0.5739	-0.0225
			syriaca	-,67611*	0.12938	0.000	-0.9904	-0.3618
		carnica	caucasica	,29819*	0.11348	0.029	0.0225	0.5739
			syriaca	-,37792*	0.11999	0.006	-0.6694	-0.0864
		syriaca	caucasica	,67611*	0.12938	0.000	0.3618	0.9904
			carnica	,37792*	0.11999	0.006	0.0864	0.6694

*. The mean difference is significant at the 0.05 level.

Table E.9. Kruskal-Wallis analysis and pairwise comparisons with Bonferroni correction of 5 µg treatment/control mean duration and movement data of three subspecies in ESA assay.

Duration	
Test Statistic	15.489
Degrees of Freedom	2
Significance	0.000

Sample1 -Sample 2	Test statistic	Standard Error	Std. Test Statistic	Significance	Adjusted Significance
caucasica-carnica	20.699	7.417	-2.791	0.005	0.016
caucasica-syriaca	32.300	8.456	-3.820	0.000	0.000
carnica-syriaca	11.601	7.843	-1.479	0.139	0.417

Movement	
Test Statistic	26.412
Degrees of Freedom	2
Significance	0.000

Sample1 -Sample 2	Test statistic	Standard Error	Std. Test Statistic	Significance	Adjusted Significance
caucasica-carnica	-18.947	7.413	-2.556	0.011	0.032
caucasica-syriaca	-43.422	8.452	-5.138	0.000	0.000
carnica-syriaca	-24.476	7.838	-3.123	0.002	0.005

F. Statistical Tables of Acetylcholinesterase Activity Analysis

Table F.1. Means, sample sizes, sd's and se's of control vs 5 µg coumaphos treatment groups of brain activity data in AChE activity assay.

	Groups		Statistic	Std. Error
Activity	*caucasica* control	Mean	24.85	1.285
		Sample Size	24	
		Std. Deviation	6.296	
	caucasica treatment	Mean	24.13	1.530
		Sample Size	25	
		Std. Deviation	7.648	
	carnica control	Mean	16.33	1.144
		Sample Size	13	
		Std. Deviation	4.128	
	carnica treatment	Mean	18.57	.965
		Sample Size	14	
		Std. Deviation	3.614	
	syriaca control	Mean	23.228	1.515
		Sample Size	23	
		Std. Deviation	7.269	
	syriaca treatment	Mean	23.140	.759
		Sample Size	21	
		Std. Deviation	3.479	

Table F.2. Normality tests of brain control vs 5 µg coumaphos treatment data of three subspecies in AChE activity assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Activity	*caucasica* control	0.973	24	0.732
	caucasica treatment	0.936	25	0.117
	carnica control	0.950	13	0.605
	carnica treatment	0.966	14	0.819
	syriaca control	0.952	23	0.328
	syriaca treatment	0.973	21	0.797

Table F.3. Student's t-test comparisons of brain control vs 5 µg coumaphos treatment data of three subspecies in AChE activity assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Activity	Equal variances assumed	0.343	0.561	0.360	47	0.720	0.723	2.006	-3.313	4.758
	Equal variances not assumed			0.362	45.952	0.719	0.723	1.998	-3.299	4.744

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Activity	Equal variances assumed	0.464	0.502	-1.500	25	0.146	-2.236	1.490	-5.305	0.833
	Equal variances not assumed			-1.493	23.957	0.149	-2.236	1.498	-5.328	0.856

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Activity	Equal variances assumed	6.244	0.016	0.050	42	0.960	0.088	1.745	-3.435	3.610
	Equal variances not assumed			0.052	32.197	0.959	0.088	1.695	-3.364	3.540

Table F.4. Means, sample sizes, sd's and se's of 5 µg treatment/control mean of brain activity data of three subspecies in AChE activity assay.

	groups		Statistic	Std. Error
Activity	*caucasica*	Mean	.971	.0616
		Sample Size	25	
		Std. Deviation	.308	
	carnica	Mean	1.137	.0592
		Sample Size	14	
		Std. Deviation	.222	
	syriaca	Mean	.997	.0327
		Sample Size	21	
		Std. Deviation	.150	

Table F.5. Normality tests of original and log-transformed brain 5 µg treatment/ control mean data of three subspecies in AChE activity assay.

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Activity	*caucasica*	0.936	25	0.117
	carnica	0.966	14	0.819
	syriaca	0.973	21	0.797

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
Log of Activity	*caucasica*	0.988	25	0.990
	carnica	0.955	14	0.645
	syriaca	0.937	21	0.187

Table F.6. One-way ANOVA with post hoc comparisons of log-transformed 5 µg treatment/control mean brain activity data of three subspecies in AChE activity assay.

LogActivity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.306	2	0.153	2.678	0.077
Within Groups	3.256	57	0.057		
Total	3.562	59			

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
3.339	2	58	0.042

Groups			Mean Difference (I-J)	Std. Error	Sig.	Interval Lower Bound	Interval Upper Bound
Tamhane	*caucasica*	*carnica*	-0.18442	0.08121	0.085	-0.3878	0.0190
		syriaca	-0.05904	0.07009	0.789	-0.2342	0.1161
	carnica	*caucasica*	0.18442	0.08121	0.085	-0.0190	0.3878
		syriaca	0.12538	0.06430	0.178	-0.0399	0.2906
	syriaca	*caucasica*	0.05904	0.07009	0.789	-0.1161	0.2342
		carnica	-0.12538	0.06430	0.178	-0.2906	0.0399
Dunnett T3	*caucasica*	*carnica*	-0.18442	0.08121	0.084	-0.3874	0.0186
		syriaca	-0.05904	0.07009	0.784	-0.2338	0.1158
	carnica	*caucasica*	0.18442	0.08121	0.084	-0.0186	0.3874
		syriaca	0.12538	0.06430	0.174	-0.0393	0.2901
	syriaca	*caucasica*	0.05904	0.07009	0.784	-0.1158	0.2338
		carnica	-0.12538	0.06430	0.174	-0.2901	0.0393

Table F.7. Means, sample sizes, sd's and se's of control vs 5 µg coumaphos treatment groups of midgut activity data in AChE activity assay.

	Groups		Statistic	Std. Error
Activity	*caucasica* control	Mean	.424	.0130
		Sample Size	25	
		Std. Deviation	.0650	
	caucasica treatment	Mean	.189	.009
		Sample Size	25	
		Std. Deviation	.043	
	carnica control	Mean	.210	.0116
		Sample Size	12	
		Std. Deviation	.0403	
	carnica treatment	Mean	.0852	.004
		Sample Size	15	
		Std. Deviation	.0156	
	syriaca control	Mean	.328	.0189
		Sample Size	23	
		Std. Deviation	.0903	
	syriaca treatment	Mean	.113	.009
		Sample Size	21	
		Std. Deviation	.0431	

Table F.8. Normality tests of midgut control vs 5 µg coumaphos treatment data of three subspecies and log-transformed data of *syriaca* in AChE activity assay.

		Shapiro-Wilk		
Groups		Statistic	df	Sig.
Activity	*caucasica* control	0.982	25	0.927
	caucasica treatment	0.982	25	0.922
	carnica control	0.946	12	0.573
	carnica treatment	0.950	15	0.517
	syriaca control	0.978	23	0.867
	syriaca treatment	0.908	21	0.050

Groups		Shapiro-Wilk		
		Statistic	df	Sig.
	syriaca control	0.963	23	0.531
	syriaca treatment	0.983	21	0.961

Table F.9. Student's t-test comparisons of midgut control vs 5 µg coumaphos treatment data of three subspecies in AChE activity assay.

caucasica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Activity	Equal variances assumed	4.685	0.035	15.144	48	0.000	0.235	0.016	0.204	0.266
	Equal variances not assumed			15.144	41.324	0.000	0.235	0.016	0.204	0.266

carnica		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Activity	Equal variances assumed	8.269	0.008	11.125	25	0.000	0.126	0.011	0.102	0.149
	Equal variances not assumed			10.211	13.658	0.000	0.126	0.012	0.099	0.152

syriaca		Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Interval of the Lower	Interval of the Upper
Log of Activity	Equal variances assumed	1.211	0.277	11.006	42	0.000	1.094	0.099	0.893	1.294
	Equal variances not assumed			10.894	38.298	0.000	1.094	0.100	0.891	1.297

Table F.10. Means, sample sizes, sd's and se's of 5 µg treatment/control mean of midgut activity data of three subspecies in AChE activity assay.

	Groups		Statistic	Std. Error
Activity	*caucasica*	Mean	.45	.020
		Sample Size	25	
		Std. Deviation	.100	
	carnica	Mean	.403	.019
		Sample Size	15	
		Std. Deviation	.074	
	syriaca	Mean	.35	.028
		Sample Size	21	
		Std. Deviation	.131	

Table F.11. Normality tests of original and log-transformed midgut 5 µg treatment/ control mean data of three subspecies in AChE activity assay.

		Shapiro-Wilk		
Groups		Statistic	df	Sig.
Activity	*caucasica*	0.982	25	0.922
	carnica	0.950	15	0.517
	syriaca	0.908	21	0.050

		Shapiro-Wilk		
Groups		Statistic	df	Sig.
Log of Activity	*caucasica*	0.951	25	0.265
	carnica	0.950	15	0.528
	syriaca	0.983	21	0.961

Table F.12. One-way ANOVA with post hoc comparisons of log-transformed 5 µg treatment/control mean midgut activity data of three subspecies in AChE activity assay.

Log of Activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.041	2	0.520	6.594	0.003
Within Groups	4.576	58	0.079		
Total	5.617	60			

Test of Homogeneity of Variances

Log of Extinction

Levene Statistic	df1	df2	Sig.
3.339	2	58	0.042

Log of Activity

(I) Groups			Mean Difference (I-J)	Std. Error	Sig.	Interval Lower Bound	Interval Upper Bound
Tamhane	caucasica	carnica	0.08835	0.06842	0.498	-0.0831	0.2598
		syriaca	,29819*	0.09340	0.009	0.0635	0.5328
	carnica	caucasica	-0.08835	0.06842	0.498	-0.2598	0.0831
		syriaca	0.20984	0.09322	0.092	-0.0253	0.4450
	syriaca	caucasica	-,29819*	0.09340	0.009	-0.5328	-0.0635
		carnica	-0.20984	0.09322	0.092	-0.4450	0.0253
Dunnett T3	caucasica	carnica	0.08835	0.06842	0.491	-0.0828	0.2595
		syriaca	,29819*	0.09340	0.009	0.0640	0.5323
	carnica	caucasica	-0.08835	0.06842	0.491	-0.2595	0.0828
		syriaca	0.20984	0.09322	0.090	-0.0247	0.4444
	syriaca	caucasica	-,29819*	0.09340	0.009	-0.5323	-0.0640
		carnica	-0.20984	0.09322	0.090	-0.4444	0.0247

*. The mean difference is significant at the 0.05 level.

G. Statistical Tables of Quantitative Real-Time PCR Analysis

Table G.1. Means, sample sizes, sd's and se's of control vs 5 µg coumaphos treatment groups of the qRT-PCR assay.

Caucasica	Genes					
Control	NAChRa5	MAChR	VAChT	$GABA_A$ beta	$GABA_B$ sub1	$GABA_B$ sub2
Mean	0.019	0.334	0.158	0.004	0.094	0.033
Sample Size	6	6	6	6	6	6
Std. Dev.	0.016	0.049	0.045	0.001	0.024	0.005
Std. Err.	0.007	0.020	0.018	0.001	0.009	0.002
Treatment						
Mean	0.014	0.277	0.186	0.005	0.065	0.028
Sample Size	6	6	6	6	6	6
Std. Dev.	0.010	0.098	0.057	0.001	0.019	0.007
Std. Err.	0.004	0.040	0.023	0.001	0.008	0.003
Syriaca						
Control						
Mean	0.014	0.303	0.152	0.004	0.082	0.033
Sample Size	6	6	6	6	6	6
Std. Dev.	0.002	0.080	0.043	0.0009	0.021	0.005
Std. Err.	0.001	0.032	0.017	0.0001	0.009	0.002
Treatment						
Mean	0.011	0.344	0.172	0.005	0.069	0.037
Sample Size	6	6	6	6	6	6
Std. Dev.	0.005	0.143	0.013	0.003	0.027	0.021
Std. Err.	0.002	0.058	0.047	0.001	0.011	0.008

Table G.2. Normality tests of brain control vs 5 µg coumaphos treatment data of two subspecies in qRT- PCR assay.

			Shapiro-Wilk		
			Statistic	df	Sig.
NAChRa5 Expression	*caucasica*	control	0.818	6	0.02 0.085(log)
		treatment	0.935	6	0.057 0.618(log)
	syriaca	control	0.872	6	0.236
		treatment	0.84	6	0.13
MAChR Expression	*caucasica*	control	0.865	6	0.205
		treatment	0.916	6	0.474
	syriaca	control	0.933	6	0.6
		treatment	0.967	6	0.874
VAChT Expression	*caucasica*	control	0.925	6	0.541
		treatment	0.973	6	0.913
	syriaca	control	0.965	6	0.854
		treatment	0.903	6	0.389
$GABA_A$ beta Expression	*caucasica*	control	0.969	6	0.884
		treatment	0.8	6	0.059
	syriaca	control	0.962	6	0.833
		treatment	0.88	6	0.269
$GABA_B$ sub1 Expression	*caucasica*	control	0.957	6	0.794
		treatment	0.924	6	0.535
	syriaca	control	0.896	6	0.349
		treatment	0.975	6	0.927
$GABA_B$ sub2 Expression	*caucasica*	control	0.982	6	0.963
		treatment	0.871	6	0.229
	syriaca	control	0.939	6	0.652
		treatment	0.94	6	0.658

Table G.3. t-test comparisons of brain control vs 5 µg coumaphos treatment data of two subspecies in q RT- PCR assay.

		t-test				
NAChRa5		t	df	Sig.	Mean diff.	Std. Err
	caucasica	0.579	10	0.575	0.00454	0.00784
	syriaca	0.998	10	0.342	0.00247	0.00248
mAChR	*caucasica*	1.256	10	0.238	0.05654	0.045
	syriaca	-0.616	10	0.552	-0.0412	0.06692
VAChT	*caucasica*	-0.935	10	0.372	-0.0280	0.02998
	syriaca	-0.405	6.369	0.699	-0.0205	0.05054
$GABA_A$ beta	*caucasica*	-0.946	10	0.366	-0.0008	0.00086
	syriaca	-0.587	6.403	0.577	-0.0006	0.00106
$GABA_B$ sub1	*caucasica*	2.214	10	0.051	0.02824	0.01276
	syriaca	0.861	10	0.409	0.01243	0.01442
$GABA_B$ sub2	*caucasica*	1.262	10	0.236	0.00468	0.00371
	syriaca	-0.476	5.61	0.652	-0.0043	0.00907

Table G.4. Normality tests of brain log-transformed 5 µg treatment/control mean data of subspecies in q RT- PCR assay.

		Shapiro-Wilk		
		t	df	Sig.
NAChRa5	*caucasica*	0.935	6	0.618
	syriaca	0.84	6	0.13
mAChR	*caucasica*	0.876	6	0.251
	syriaca	0.956	6	0.785
VAChT	*caucasica*	0.929	6	0.572
	syriaca	0.911	6	0.443
$GABA_A$ beta	*caucasica*	0.783	6	0.041
	syriaca	0.887	6	0.303
$GABA_B$ sub1	*caucasica*	0.93	6	0.583
	syriaca	0.951	6	0.746
$GABA_B$ sub2	*caucasica*	0.877	6	0.255
	syriaca	0.967	6	0.87

Table G.5. Mann Whitney U test comparison of brain expression data of GABA receptor subunit beta gene in 5 µg treatment/control mean data of *caucasica* in q RT- PCR assay.

caucasica $GABA_A$ beta	
Test Statistic	18.000
Standard Error	6.245
Standardized Test Statistic	0.000
Significance	1.000

Table G.6. Student's t-test comparisons of brain 5 µg treatment/control mean data of two subspecies in q RT- PCR assay.

	T test				
	t	df	Sig.	Mean diff.	Std. Err
NAChRa5	-0.374	10	0.716	-0.1272	0.33969
mAChR	-0.945	10	0.367	-0.2005	0.2121
VAChT	0.632	6.937	0.548	0.2171	0.34349
$GABA_A$ beta	0.527	6.785	0.615	0.13996	0.26561
$GABA_B$ sub1	-0.726	10	0.485	-0.1571	0.21651
$GABA_B$ sub2	-0.588	10	0.57	-0.1588	0.27002

Table G.7. Means, sample sizes, sd's and se's of midgut control vs 5 µg coumaphos treatment groups of the qRT-PCR assay.

Caucasica	Genes		
Control	CYP9Q1	CYP9Q2	CYP9Q3
Mean	0.899	0.437	0.098
Sample Size	6	6	6
Std. Dev.	0.647	0.171	0.052
Std. Err.	0.264	0.070	0.021
Treatment			
Mean	1.416	0.448	0.108
Sample Size	6	6	6
Std. Dev.	0.650	0.146	0.048
Std. Err.	0.265	0.060	0.020
Syriaca			
Control			
Mean	1.381	1.009	0.058
Sample Size	6	6	6
Std. Dev.	1.08	0.643	0.047
Std. Err.	0.442	0.026	0.020
Treatment			
Mean	0.430	0.743	0.118
Sample Size	6	6	6
Std. Dev.	0.229	0.342	0.157
Std. Err.	0.093	0.014	0.064

Table G.8. Normality tests of midgut control vs 5 µg coumaphos treatment data of two subspecies in q RT- PCR assay.

			Shapiro-Wilk		
			Statistic	df	Sig.
CYP9Q1 Expression	*caucasica*	control	0.927	6	0.557
		treatment	0.946	6	0.709
	syriaca	control	0.801	6	0.06 0.127 (log)
		treatment	0.979	6	0.946 0.086 (log)
CYP9Q2 Expression	*caucasica*	control	0.976	6	0.928
		treatment	0.981	6	0.956
	syriaca	control	0.91	6	0.434
		treatment	0.899	6	0.37
CYP9Q3 Expression	*caucasica*	control	0.886	6	0.299
		treatment	0.939	6	0.653
	syriaca	control	0.782	6	0.632 (log)
		treatment	0.65	6	0.406 (log)

Table G.9. Student's t- test comparisons of midgut control vs 5 µg coumaphos treatment data of two subspecies in q RT- PCR assay.

		T test				
		t	df	Sig.	Mean diff.	Std. Err
CYP9Q1	*caucasica*	-1.381	10	0.197	-0.5177	0.37474
	syriaca	2.101	5.448	0.031	0.95097	0.45269
CYP9Q2	*caucasica*	-0.131	10	0.898	-0.0121	0.0919
	syriaca	0.896	10	0.391	0.26673	0.29758
CYP9Q3	*caucasica*	-0.343	10	0.739	-0.0101	0.02931
	syriaca	-0.896	10	0.451	-0.0603	0.06728

Table G.10. Mann-Whitney U test comparison of midgut expression data of CYP9Q1 gene in control vs 5 µg coumaphos treatment data of two subspecies in q RT- PCR assay.

syriaca CYP9Q1	
Test Statistic	3.000
Standard Error	6.245
Standardized Test Statistic	2.402
Significance	0.015

Table G.11. Normality tests of midgut log-transformed 5 µg treatment/control mean data of subspecies in q RT- PCR assay.

		Shapiro-Wilk		
		t	df	Sig.
CYP9Q1	*caucasica*	0.942	6	0.679
	syriaca	0.819	6	0.086
CYP9Q2	*caucasica*	0.981	6	0.957
	syriaca	0.964	6	0.846
CYP9Q3	*caucasica*	0.936	6	0.63
	syriaca	0.905	6	0.406

Table G.12. Student's t-test comparisons of midgut 5 µg treatment/control mean data of two subspecies in q RT- PCR assay.

	t-test				
	t	df	Sig.	Mean diff.	Std. Err
CYP9Q1	4.34	10	0.001	1.73968	0.40083
CYP9Q2	1.639	10	0.132	0.36893	0.22512
CYP9Q3	-0.375	10	0.716	-0.1775	0.47363

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