DIFFERENCES IN LEARNING PERFORMANCE AND RELATED BEHAVIORS ACROSS THREE HONEY BEE SUBSPECIES FROM TURKEY

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ABSTRACT

DIFFERENCES IN LEARNING PERFORMANCE AND RELATED BEHAVIORS ACROSS THREE HONEY BEE SUBSPECIES FROM TURKEY

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In this thesis we studied learning performance in 3 subspecies of honey bee (*Apis mellifera* L.), Carniolan honey bee (*A. m. carnica*), Syrian honey bee (*A. m. syriaca*), and Caucasian honey bee (*A. m. caucasica*). These subspecies are found in remote corners of Turkey and apparently morphologically and genetically diverged from each other. Previous studies have illustrated differences in foraging and defense behavior across these subspecies. Also, numerous examples of behavioral differences across species or subspecies of honey bees, as well as relationships between behavior and learning are found in the scientific literature. Thus, we hypothesize that differences in learning performance may also be found between Syrian, Carniolan, and Caucasian honey bees. To investigate this, we used two discriminant learning assays. One is the Electric Shock Avoidance (ESA) conditioning assay, which uses aversive conditioning with color learning. The other is called the Proboscis Extension Response (PER) conditioning assay and uses appetitive conditioning with odor learning. In addition, to support our results, we monitored daily locomotor activities of honey bees and conducted a starvation study. The results of ESA conditioning assay suggested that the Caucasian honey bee may have higher discriminant learning performance than the Syrian honey bee and the Carniolan honey bee. Meanwhile, the Syrian honey bee

may have impairment in discrimination according to the results of the PER conditioning assay. Overall, these three subspecies appear to have significant differences in learning performance, which we argue may be linked with their natural habitats and foraging behavior.

Keywords: *Apis mellifera*, honey bee, learning, behavior

ÖZ

TÜRKİYE'DEKİ ÜÇ BAL ARISI ALT TÜRÜ ARASINDA ÖĞRENME BAŞARISI VE İLİŞKİLİ DAVRANIŞ FARKLARI

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Bu çalışmada bal arısının (*Apis mellifera* L.) Türkiye'de bulunan 3 alttürü: Trakya arısı (*A. m. carnica*), Suriye arısı (*A. m. syriaca*) ve Kafkas arısı (*A. m. caucasica*) incelenmiştir. Bu alttürler Türkiye'nin birbirine uzak bölgelerinde bulunurlar ve birbirlerinden genetik ve morfolojik özellikler bakımından belirgin olarak farklılaşmışlardır. Yapılan çalışmalar tarlacılık ve savunma davranışı ile de birbirlerinden farklılaştıklarını göstermiştir. Bilimsel literatürde bal arısı türleri veya alttürleri arasındaki davranışsal farklar ve davranışla öğrenme arasında ilişki olduğunu gösteren pek çok çalışma da bulunmaktadır. Bu bilgiler ışığında, Trakya arısı, Suriye arısı ve Kafkas arısı alttürleri arasında öğrenme bakımından da farklılık olabileceği hipotezini ortaya koymaktayız. Bu hipotezimizi test etmek içinse iki farklı ayrıştırmalı öğrenme analizi deneyi kullanılmıştır. Elektrik Şoku Kaçınım koşullanması deneyi kaçınma koşullanması ile renk öğrenimi ilişkisini kullanırken, Proboskis Uzatma Tepkisi deneyi ise koku öğrenimi ile besin koşullanması ilişkisini kullanmaktadır. Bu analizlerden elde edilen sonuçları desteklemek içinse arıların günlük lokomotor aktivitelerini takip edilmiş ve aynı zamanda açlığa dayanıklılık deneyi uygulanmıştır. Çalışmamız sonucunda Kafkas arısının, Suriye ve Trakya arılarına göre Elektrik Şoku Kaçınım koşullanması deneyinde daha yüksek bir ayrıştırmalı öğrenme performansına

sahip olduğu; Suriye arısının ise diğer iki alttüre göre Proboskis Uzatma Tepkisi koşullanması deneyinde daha düşük bir ayrıştırma yetisine sahip olduğu belirlenmiştir. Genel olarak, bu üç alttür arasında öğrenme başarısı bakımından belirgin olarak saptadığımız farklılığın, alttürlerin yaşadıkları habitatlar ve tarlacılık davranışlarıyla ilişkisinin bulunması olasıdır.

Anahtar Kelimeler: *Apis mellifera*, bal arısı, öğrenme, davranış

To my grandparents
Emin Kalkan and Zade "Naile" Kalkan.

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A. m.	*Apis mellifera*
CS	Conditioned Stimulus
CS+	Conditioned Stimulus associated with Unconditioned Stimulus
CS-	Conditioned Stimulus not associated with Unconditioned Stimulus
DAM	*Drosophila* Activity Monitors
ESA	Electric Shock Avoidance
ITI	Intertrial Interval
LAM	Large Activity Monitors
PER	Proboscis Extension Response
SER	Sting Extension Response
US	Unconditioned Stimulus

CHAPTER 1

INTRODUCTION

1.1 Honey Bee

Honey bee has been defined as a venerable animal on the lands where modern-day Turkey is located, which includes Thrace and Asia Minor. In the mythology of Hittites, the resentful god Telipinu, patron of farming, was awakened from his sorrowful sleep by a honey bee and it relieved his anger with honey. There was also a god of beekeeping in Greek mythology called Aristaeus. A silver drachma, an ancient Greek coin, with honey bee obverse was found in the ancient city of Ephesus. Honey bee is also a sacred animal in Islam. A Surah of the Quran was named "The Bees" (an-Naḥl, or سورة النحل, in Arabic). Ages later, honey bee also has a prominent position in the scientific world, especially in agricultural studies and research on behaviour and learning.

The Western honey bee, *Apis mellifera* L., is a eusocial insect in the Hymenoptera order with sawflies, wasps, bees, and ants. The Apidae family includes honey bee genus *Apis* and its relatives like bumblebees, stingless bees, carpenter bees, orchid bees, cuckoo bees (Danforth *et al*., 2013). The *Apis* genus has 3 subgenera, namely *Micrapis*, *Megapis*, and *Apis*. The Western honey bee, *Apis mellifera,* is a diverse taxon with 28 subspecies in all around the world (Michael, 1999). In Turkey, five different subspecies of *Apis mellifera* are found: *A. m. meda, A. m. syriaca, A. m. caucasica, A. m. anatoliaca,* and *A. m. carnica* (Ruttner, 1988; Kandemir *et al*., 2005; Kükrer, 2013). Thus, Turkey is a perfect geographical location to investigate the evolution of honey bees and the emergence of the distinctive characteristics of these subspecies.

It has been suggested that the differentiation among honey bee subspecies was affected by the late Pleistocene glaciation and deglaciation events in the recent tens of thousands of years (Ruttner 1988; Kükrer, 2013). Both genetic drift and local adaptation, because of diverse climatic, topographical and floristic variations available, may have played a role in the differentiation of honey bee subspecies. Due to the dual effect of the local adaptation and genetic drift, morphological differences can be observed across the subspecies such as the differences in the body size, coloration, and the vein pattern of the wings (Ruttner 1988, Nawrocka *et al*., 2017).

In this study we examined 3 subspecies of *Apis mellifera* found in distinct regions of Turkey: the Carniolan honey bee (*A. m. carnica*), the Syrian honey bee (*A. m. syriaca*), and the Caucasian honey bee (*A. m. caucasica*). These subspecies are apparently morphologically and genetically diverged from each other (Kandemir, Kence & Kence, 2000; Kandemir *et al*., 2006), and past studies have illustrated differences in foraging and defense behavior across these subspecies (Ruttner, 1988; Çakmak, Wells & Fıratlı, 1998; Çakmak *et al*., 2010).

1.2 Behavioral Differences in Honey Bee

The earliest observations towards honey bee behavior can be traced back to the Ancient Greeks. In his Book I of *Historia Animalium,* Aristotle defined the honey bee as a social creature, alongside with the man, wasps, ants, and cranes. He had also suspicions about honey bees communicating with one another. In Book IX of *Historia Animalium*, he wrote:

> *On each expedition the bee does not fly from a flower of one kind to a flower of another, but flies from one violet, say, to another violet, and never meddles with another flower until it has got back to the hive; on reaching the hive they throw off their load, and each bee on his return is accompanied by three or four companions. One cannot well tell what is the substance they gather, nor the exact process of their work.*

The suspicion of Aristotle that honey bees were communicating was demonstrated scientifically by Karl von Frisch. In his book *The Dance Language and Orientation of Bees*, he illustrated the patterns of waggle dance of the honey bees as a communication system. In 1973, von Frisch was awarded The Nobel Prize in Physiology or Medicine, along with Konrad Lorenz and Nikolaas Tinbergen for their discoveries concerning organization and elucidation of individual and social behavior patterns ("The Nobel Prize in Physiology or Medicine 1973", 2017).

It is possible that the distinct evolutionary histories of honey bee species or subspecies indicates have not only shaped their morphology and physiology, but also their ethology and behavioral characteristics. Indeed, we have numerous examples on the behavioral differences across honey bee species or subspecies. For instance, it is known that the waggle dance patterns show differences across different honey bee species, such as *Apis florea, Apis cerana*, and *Apis dorsata* (Dyer & Seeley, 1991).

Another behavioral example among *A. mellifera* subspecies involves the defense behavior against *Varroa*, an ectoparasitic mite that originated from Asia. These differences include the grooming dance, nestmate cleaning and group cleaning behavior. The Asiatic honey bee *Apis cerana* has a very effective defense to this parasite. Asiatic honey bee can remove 74% of mites by damaging or killing them by biting (Peng *et al*., 1987). The mean removal ratio of *A. mellifera* subspecies is much lower, only 6% for European honey bee (*A. m. ligustica*), and 39% for Africanized hybrids (Moretto *et al*., 1991). Also, Ruttner and Hanel determined that the removal rate of *Varroa* is between 30% – 70% for the Carniolan honey bee (*A. m. carnica*) (1992).

Differences in the foraging behavior can also be observed in the subspecies of *A. mellifera*. According to research by Fewell and Bertram, African honey bee (*A. m. scutellate*) is more likely to collect pollen, instead of nectar, contrary to the European honey bee (2002).

The level of aggression also shows variation across subspecies. A cross-breeding between subspecies is known to reveal unexpected and interesting aggression

behavior. For instance, the Africanized honey bee, which is also known as the "killer bee", arises from the cross-breeding of the African honey bee (*A. m. scutellate*) and European honey bee subspecies, such as *A. m. ligustica, A. m. iberiensis,* or *A. m. carnica.* This Africanized honey bee has caused the death of many animals, including humans, due to its aggressive defensive behavior and massive stinging response in the Americas (Alaux *et al*., 2009, Schneider, DeGrandi-Hoffman & Smith, 2004, Rodriguez-Lainz, Fritz & McKenna, 1999, Breed, Guzmán-Novoa & Hunt, 2004). In contrast to the Africanized honey bee, European subspecies such as the Carniolan honey bee and Caucasian honey bee are known with their gentle disposition (Winston, 1987). In addition, a research conducted by Guzman-Novoa and Page illustrated that back-crossing Africanized queens with European drones can drastically decrease the defensive behavior (1993).

A unique example to the defensive behavior is observed in Japanese honey bee (*Apis cerana japonica*) against giant hornets (i.e. *Vespa mandarinia japonica)*. *A. c. japonica* is a wild honey bee species native to Japan, while *V. m. japonica* are bee predator species. To defend their hive, Japanese honey bees surround the giant hornet and trap it in a "bee ball". Using this high concentration of honey bees in a small area, the honey bees can increase their core temperature up to 47°C, as well as the CO_2 concentration inside the bee ball by beating their wings. Thus, they can kill the giant hornet in minutes (Sugahara & Sakamoto, 2009). This unique defensive behavior is not observed in European honey bee species.

Behavioral differences have also been reported among the subspecies investigated in this study. For instance, the Syrian honey bee is known with its aggressive behavior and specialized hive defense reaction against wasps (*Vespa spp*.), whereas the Carniolan honey bee and Caucasian honey bee do not show these behaviors, as they lack the high predation pressure caused by the bee predatory wasps (Çakmak, Wells & Fıratlı, 1998; Ruttner,1988). Differences in foraging activity have also been determined across these subspecies. For example, the Syrian honey bee is a specialist, while the Carniolan honey bee and Caucasian honey bee are more generalist species, meaning that they can switch different flower color morphs depending on the rewards

(Çakmak *et al*., 2010). Notably, these behavioral differences could be related to the subspecies' respective habitats. The Syrian honey bee inhabits the subtropical south, which has an arid habitat with mild winters. As a result, it has longer seasonal foraging periods. However, this results in lower diversity in the sources of nectar (Kandemir, Kence & Kence, 2000; Kandemir *et al*., 2006). On the contrary, the Carniolan honey bee is found in temperate north-west of Turkey, and the Caucasian honey bee inhabits the north-east border of Turkey. Hence, their foraging period is restricted with short summers and cold winters, as well as the more sequential blooming of the flowering species. Restricted foraging period and sequential blooming of the flowering species pushes Carniolan honey bee and Caucasian honey bee to maximize the amount of the collected nectar in a limited time from casual nectar sources (Çakmak *et al*., 2010; Perez-Claudio *et al.*, In press).

It is tempting to speculate that these cases of behavioral difference across the three subspecies investigated in this study could be related to their learning performance differences. Below we discuss this possible relationship.

1.3 Relationship Between Behavior and Learning in Honey Bee

The honey bee is equipped with the ability to learn different tasks in nature. Fahrbach & Robinson summarize this feature by saying "foraging worker bees have been demonstrated to orient themselves in space with reference to the location of their nest, they learn both the geographic and temporal resource pattern of their locality, and they also learn to work with many different types of flowers" (1995). As a result, there should be a relationship between learning and behavior.

Gould summarizes the reason why honey bee should have learning abilities from an ethological perspective, by using the concepts of social behavior and communication, orientation and navigation, defense, and food acquisition (1993). Firstly, social signals are generally innate and there is no evidence of effects of learning in the dance language of honey bees could be shown (Gould & Gould, 1988). However, during the

dance, it is shown that the odor, distance, direction, and quality of the food source is learned (Gould, 1993). Secondly, it is known that learning is very crucial for orientation and navigation. Searching flights of forager bees relative to the sun's azimuth and the movement rate of the sun varies with date, latitude, and time of day. Therefore, forager bees should learn the direction and pace of the sun's movement. Additionally, forager bees not only use the sun as a compass but also other physical landmarks on the field (Gould, 1993), which requires spatial learning ability. Third, the honey bee can associate sound with impending shock, which is an auditory-based danger learning (Gould & Towne, 1988). This learning is possibly used to determine wasp attacks (Gould, 1993). In addition, recent studies illustrated that using odorants paired with electric shocks, the honey bee can be conditioned to give response as a defensive reaction, the sting extension reflex (Vergoz *et al*., 2007). Lastly, honey bees generally use a generalist strategy to handle the blossom to maximize their rate of nectar intake. Therefore, they need to learn color, shape, and size of flowers (Gould, 1993).

It is logical to assume that honey bees with a more generalist strategy would need behavioral flexibility to survive, especially in variable floristic environments. Therefore, honey bees need to shift floral choices depending on the blooming periods of different herbs. In such a scenario, a fast learning rate would support behavioral flexibility, because honey bees would need to learn odor, color or shape of flowers in a limited amount of time. An example of behavioral flexibility difference between the Caucasian honey bee and the Syrian honey bee is described in a paper authored by Çakmak *et al*. (2010). Their research showed that the subspecies *A. m. caucasica*, living in a temperate climate, is more likely to switch to a different flower color morph, in contrast to the subtropical subspecies *A. m. syriaca.* The latter does not change flower morph preferences and continues to visit the same flower morph without any sensitivity to variability of reward.

A possible reason why the honey bees has evolved such impressive learning abilities may be due to the instability of information about nectar and pollen relative to a honey

bee's foraging lifetime (Smith *et al*., 2012). All of the above examples illustrate the relationship between behavior and learning in honey bee.

1.4 Aim of the Study

As it was mentioned before, many behavioral differences can be found across honey bee species or subspecies. As we mentioned that previous studies illustratedbehavior and learning abilities are closely bound to each other. Thus, the question becomes whether there is any difference in learning performance found in honey bees or not. We hypothesize that differences in learning performance may be found across the three honey bee subspecies in Turkey, the Syrian honey bee (*A. m. syriaca*), the Carniolan honey bee (*A. m. carnica*), and the Caucasian honey bee (*A. m. caucasica*), given the fact that these subspecies apparently differ from each other in terms of foraging and defense behavior as previously reported (Ruttner,1988; Çakmak, Wells & Fıratlı, 1998; Çakmak *et al*., 2010; Kandemir, Kence & Kence, 2000; Kandemir *et al*., 2006). To investigate that, we used the color learning in Electric Shock Avoidance (ESA) conditioning assay and the odor learning with Proboscis Extension Response (PER) conditioning assay. PER conditioning assay is an appetitive learning study and it may reveal possible learning differences that also may be related to foraging behavior. On the contrary, ESA conditioning assay is a punishment study. These two studies can provide an opportunity to compare possible learning differences across these subspecies through two different aspects. Also, these two assays are well established and frequently used in numerous researches. In addition, to support our results, we monitored daily locomotor activities of honey bees because baseline activity may affect ESA conditioning assay results. We further conducted a starvation study because hunger may have an effect on PER.

1.5 Background on Honey Bee Learning Assays

Honey bee is a perfect animal to study learning, both in the field and in the laboratory because of its sophisticated learning abilities (Smith *et al*., 2012). It is known that the honey bee can learn to differentiate odors, colors, shapes, hierarchies, landmarks, and even time (Gould, 1993). In this study, we conducted plays role in the Proboscis Extension Response (PER) conditioning study and Electric Shock Avoidance (ESA) conditioning assay and odor learning and color learning is of particular importance because odor learning plays role in the PER conditioning study, and color learning is crucial to the ESA conditioning assay. Thus, milestone studies on honey bee learning draw an outline and emphasized the crucial points, so we should take into account of these studies in order to improve our knowledge on honey bee learning.

The pioneering studies about odor learning in honey bees were conducted by von Frisch. He observed free flying foragers and identified that a dancing honey bee not only points out the source of nectar, but also it introduces the odors of the nectar to the foragers. Then, foragers use this odor and spatial cues to reach to the nectar source (Frisch, 1967). After von Frisch, Menzel and colleagues successfully used odors for Proboscis Extension Response (PER) conditioning to discover Conditioned Stimulus (CS) and Unconditioned Stimulus (US) pathways in the honey bee brain (Menzel & Erber, 1978; Hammer & Menzel, 1995). Also, PER conditioning technique has been commonly used in learning studies and bioassays to test the effect of agrochemicals on honey bees. Not only honey bees but also many of the insect species such as bumble bees (*Bombus terrestris*) (Laloi *et al*., 1999), the moths *Heliothis virescens* (Hartlieb, 1996; Skiri *et al*., 2005) and *Spodoptera littoralis* (Fan *et al*., 1997), a butterfly *Agraulis vanillae* (Kroutov *et al*., 1999), and *Drosophila melanogaster* (Chabaud *et al*., 2006) have been subjected to PER conditioning experiments (Abramson, Sokolowski & Wells, 2010).

Other studies from an ecological perspective on odor learning illustrated that odors commonly found in the habitat are learned more easily (Koltermann, 1974), and floral odors are learned faster than most artificial odors (Lindauer 1976; Menzel, 1978).

More recent studies focused on discrimination tasks using odors. In discrimination tasks, honey bees are trained to discriminate between two stimuli: *i)* the conditioned stimulus associated with a positive outcome, which is also known as unconditioned stimulus (US), and *ii)* conditioned stimulus with no outcome (also known as discrimination conditioning). Conditioned stimulus pairing with award is called CS+ and unpairing conditioned stimulus is called CS-, thus subjects learn to give PER to CS+ and no PER to CS-. Also, in those studies, one odor (CS+) is associated with sucrose reward (US) and another one is not (CS-). In these studies, one of the important points is the intensity of these odors. Honey bees cannot easily discriminate low odor concentrations, such as at the 0.02 M level (Fernandez *et al.*, 2009). In addition to the concentration of odor, the exposure time to a particular odor is also important (Wright, Carlton & Smith, 2010). Another study shows that, if one of the odors associated with punishment (i.e. electric shock) reduces the learning rate, using nothing for CS- is shown to result in better results for discrimination studies (Smith, Abramson & Tobin, 1991). Standardization of PER conditioning methods was made possible thanks to these studies. From Menzel to our millennium, many researchers have conducted research to discover the depths of honey bee brains using odor learning (Smith *et al*., 2012).

Our PER conditioning assay is an appetitive conditioning study; strawberry and lemon odors were used as conditioned stimulus (CS), also used in previous research (Wang *et al*., 2013). One odor (CS+) was associated with sucrose reward (US) and another one not (CS-), similarly to previous studies.

Von Frisch was also a pioneer for color learning studies in honey bees. He was the first one to show that honey bees can perceive colors (Frisch, 1914; Menzel, 1985 a). He demonstrated that the honey bee can learn to associate the color blue with sucrose solution, and it can also discriminate other tones of blue. Then, he showed that honey bees are red color blind. Also, he tested variations of colors both at the feeding place and at the hive entrance (Frisch, 1967; Menzel, 1985 b). Later, Menzel and colleagues showed that the rate of odor learning is higher than the rate of color learning. They also demonstrated that there is a learning rate difference across colors. For instance,

the color violet (400-420 nanometer wavelength) is learned the fastest, but bluish green (490 nanometers) is learned the slowest (Menzel & Erber, 1978). Another study illustrated that specific colors under UV wavelengths were being innately chosen by the flower-naive forager honey bees (Giurfa *et al*., 1995).

The honey bee uses color learning for discrimination tasks, similar to odor learning. Giurfa showed this working with free-flying honey bees and using a Y-maze. He used a violet disk with one arm involving a reward such as sucrose solution (CS+), and other arm with no color disc and reward. He found that honey bees can learn to orient themselves according to the violet disk. He also tried adding a blue-colored disk with no reward (CS-), instead of no color, at the end of the arm. He reported that the honey bees were able to increase their rate of correct choices (2004). In a study very similar to Giurfa's, Aurores-Weber and colleagues used quinine as an aversion stimulus for CS- in one arm of the Y-maze and found that aversive reinforcement improves color discrimination learning (2010).

We used color learning in our ESA conditioning assay which is an avoidance conditioning on punishment paradigm. There are two paradigms used for avoidance conditioning in invertebrates: *i)* signaled avoidance paradigm, and *ii)* punishment paradigm (also known as passive avoidance, or place avoidance). The difference between these two paradigms is that animals are trained to avoid the aversive event by responding to a cue in the signaled avoidance paradigm, while the animal avoids the aversive event by not entering a location that produces the aversive event in the punishment paradigm (Agarval *et al*., 2011; Mackintosh, 1974).

Punishment trainings are commonly used for behavioral research in invertebrates. These are often used in maze experiments to enhance the selection of the correct choice. For example, in a study on earthworms, if animals chose the incorrect direction in maze, they were punished by an electric shock (Datta, 1962; Abramson, 1994). In addition, punishment also has been used in insect studies such as cockroach studies through a leg shock (Disterhoft, Haggerty & Corning, 1971; Disterhoft, 1972), or ant studies through vibration (Abramson, 1981).

In early avoidance studies in honey bees, honey bees were confined to a shuttle box with exposure to formic acid as the aversive stimulus (Abramson, 1986). In other studies, punishment was adapted to the free-flying bee situation as well as to the proboscis conditioning situation and shock was associated with odors (Smith, Abramson & Tobin, 1991).

We studied punishment learning performance in honey bees in our ESA experiment. This test has been used by previous researchers (Agarval *et al*., 2011; Dinges *et al*., 2013; Gionnoni-Guzman *et al*., 2014 a; Avalos *et al*., 2017). During this test, individuals were presented two colors in a shuttle box: one color was paired with electric shock and the other was not paired with electric shock. Time spent on the shock side and non-shock side were recorded for determining the differences on learning performance across subspecies.

In early avoidance studies in honey bees, honey bees were confined to a shuttle box with exposure to formic acid as the aversive stimulus (Abramson, 1986). In other studies, punishment was adapted to the free-flying bee situation as well as to the proboscis conditioning situation and shock was associated with odors (Smith, Abramson & Tobin, 1991).

In our ESA experiment, we also studied punishment learning performance of honey bees. This test has been used by previous researchers (Agarval *et al*., 2011; Dinges *et al*., 2013; Gionnoni-Guzman *et al*., 2014 a; Avalos *et al*., 2017). During this test, individuals were presented two colors in a shuttle box: one color was paired with electric shock and the other was not paired with electric shock. Time spent on the shock side and non-shock side were recorded for determining the differences on learning performance across subspecies.

CHAPTER 2

METHOD

2.1 Honey Bees

Honey bee pure lines are maintained in the apiary of Middle East Technical University in Ankara campus. The apiary is over 1 km in nearest direction from the campus border. Honey bee pure lines were obtained from their regions of origin: For the pure line of Carniolan honey bee (*A. m. carnica*) this was Kırklareli, which is in Thrace, for the Syrian honey bee (*A. m. syriaca*) this was Arsuz, Hatay, which is in south Anatolia, and for the Caucasian honey bee (*A. m. caucasica*) this was Borçka, Artvin, which is in north-east Anatolia (Figure 2.1).

We used two colonies for each subspecies and equal number of individuals from these two colonies of each subspecies used for each experiment. Every year, subspecies were routinely confirmed with genetic analysis based on microsatellite variation (Bodur *et al*., 2007; Ivgin-Tunca, 2009). These confirmed colonies have been used in various research (Kence *et al.*, 2013; Perez-Claudio *et al.*, in press), in addition to our ESA conditioning study and activity monitoring assay.

Unfortunately, we lost Syrian honey bee colonies in the winter season between 2016 and 2017. We therefore transferred two colonies of Syrian honey bee from Samandağ, Hatay in 2017 spring. Reversal learning assay and starvation study were conducted with these new Syrian honey bee colonies and colonies of Carniolan honey bee and Caucasian honey bee, which were already present. In addition, we did not use genetic analysis to confirm new Syrian honey bee colonies. In that case, we used morphometric analysis as an alternative and cheaper method for confirmation of

subspecies, which was previously applied on honey bee subspecies in Turkey (Kandemir, Kence & Kence, 2005). Thus, we used geometric morphometrics to check if colonies of subspecies could be discriminated.

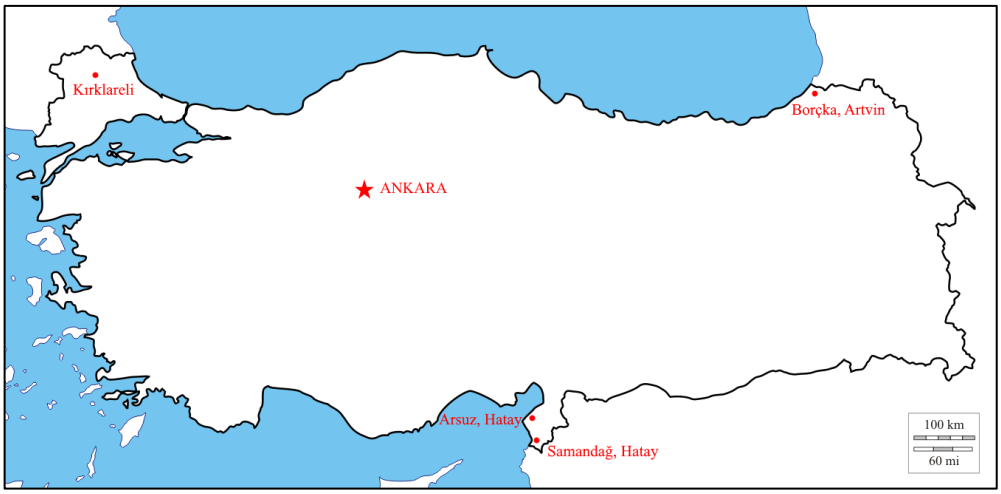

Figure 2.1 Original locations of honey bee subspecies on the map of Turkey.

2.1.1 Morphometric Analysis

We collected the wings of honey bees for geometric morphometrics from two colonies for each subspecies. We only used left wings. Sample sizes were n = 20 for the Caucasian honey bee, n = 20 for the Carniolan honey bee and n = 30 for the new Syrian honey bee. Wings were mounted between two glass slides and photographed with a stereoscopic microscope (LEICA 8AP0) and digital camera (LAS EZ) system. TpsDig version 2.12 software was used to digitize 20 landmarks from each photograph (Figure 2.2) and software produced .tps files which included coordinates of the landmarks. Morpho-J, version 1.06d software was used to process .tps files and realizing the geometric morphometry analysis (Klingenberg, 2011). Differences were clearly observed in the vein pattern of wings of the honey bee subspecies. The Mahalanobis distance between Caucasian and Carniolan honey bees was 7.36 (test on T-square statistic; 10,000 permutations; P <0.0001), Caucasian and new Syrian honey bees was

7.35 (P<0.0001), Carniolan and new Syrian honey bee was 6.56 (P<0.0001) (Figure 2.3).

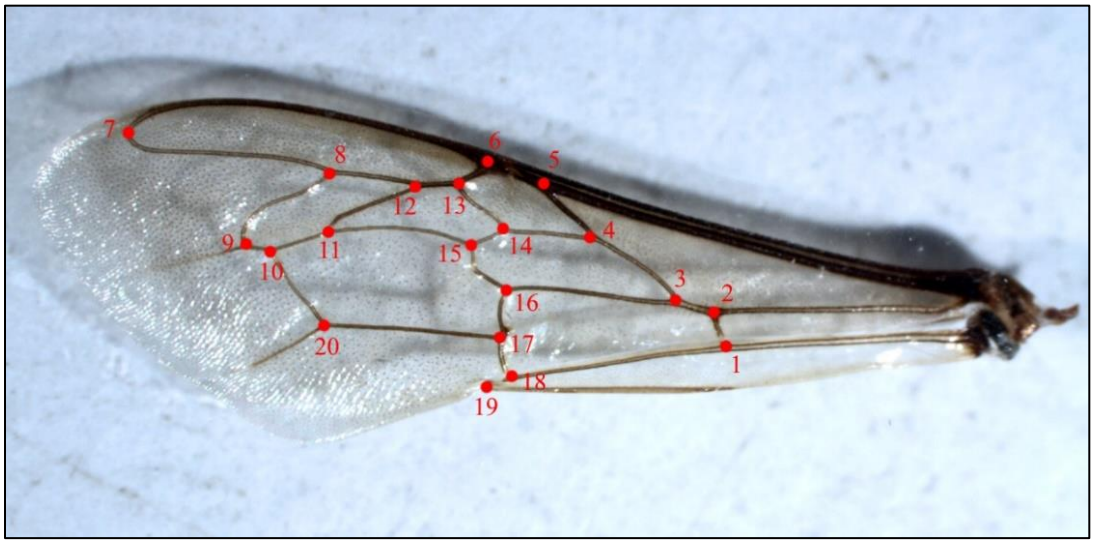

Figure 2.2 Position of the landmarks on the wing of honey bee.

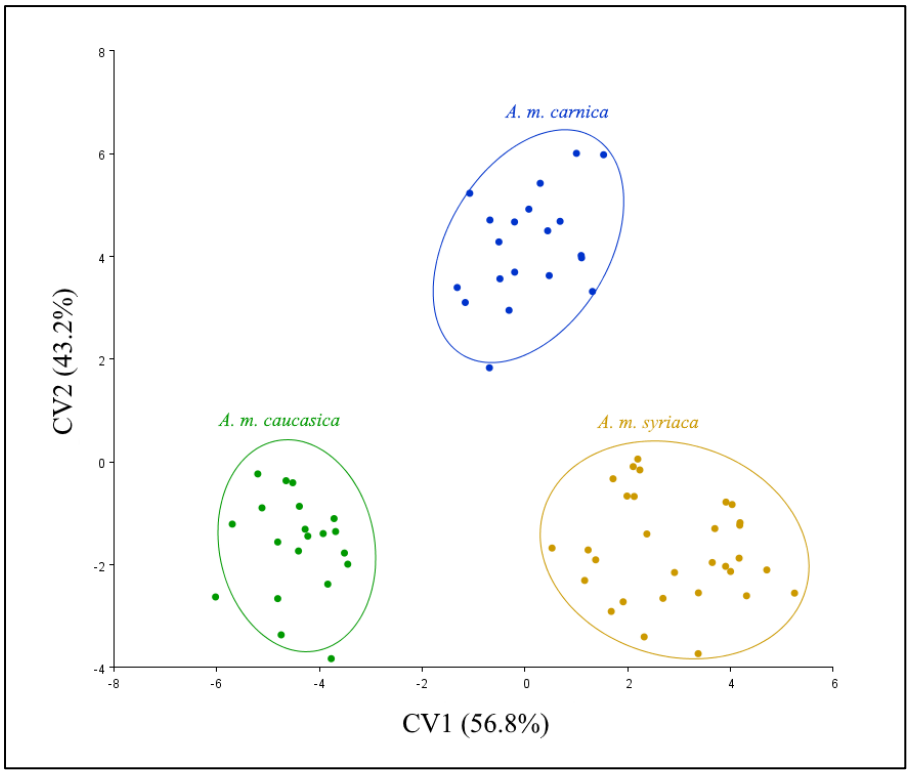

Figure 2.3 Canonical variate analysis (CVA) according to the landmarks on the wing of honey bee. CV1 represents 56.8% of variation and CV2 represents 43.2% of variation.

2.2 Experimental Setups and Protocols

2.2.1 ESA Conditioning Assay

Honey bees were collected from inside the hives and were incubated at 27 °C and 70% humidity in NÜVE TK120 incubator for 3 hours with *ad libitum* feeding in cages before experiment.

We used a shuttle box apparatus measured 15 cm long by 2 cm wide. Upper part of the shuttle box apparatus was made of Plexiglas™ and we applied Vaseline® on the Plexiglas™ which prevented bees from walking on the material and escaping the shock. Under the shuttle box apparatus there was an electric shock grid with wires spaced 0.35 cm apart. Electric shock grid was placed on top of a computer monitor (HP 7550), where two colors: blue (Hex code: # 376092) and yellow (Hex code: # FFFF00) were displayed (Figure 2.4). The shock was presented on the blue colored side of the apparatus, so blue color was determined as CS+ and paired with punishment as electric shock. Shock intensity was 6 V, 50 mA DC from an analog power supply. The yellow colored side of apparatus was not paired with the electric shock, so yellow color was determined as CS-. Similar experimental systems were also used in previous studies (Agarval *et al*., 2011; Dinges *et al*., 2013; Gionnoni-Guzman *et al*., 2014 a; Avalos *et al*., 2017). However, in our control experiments there is no electric shock was applied both blue and yellow sides to investigate any behavioral difference found in naïve honey bees.

When a honey bee was transferred from cage to shuttle box apparatus, we waited for 5 minutes for the recovery of the honey bee, then we started the power supply and began the experiment. An observer recorded the time spent on the shock side versus the non-shock side and counted the number of the crossing border between the sides. Experiments took 5 minutes for each individual and total duration of a set of experiments was 1.5 hours. If a honey bee gave the Sting Extension Response (SER) during the experiment, this honey bee was discarded, because these honey bees show fight response instead of learning, and unfortunately, we did not count the number of

these honey bees. All experiments were conducted between 16:00 and 19:00, because we collected honey bees in afternoon, fed them in cages and prepared experimental setup.

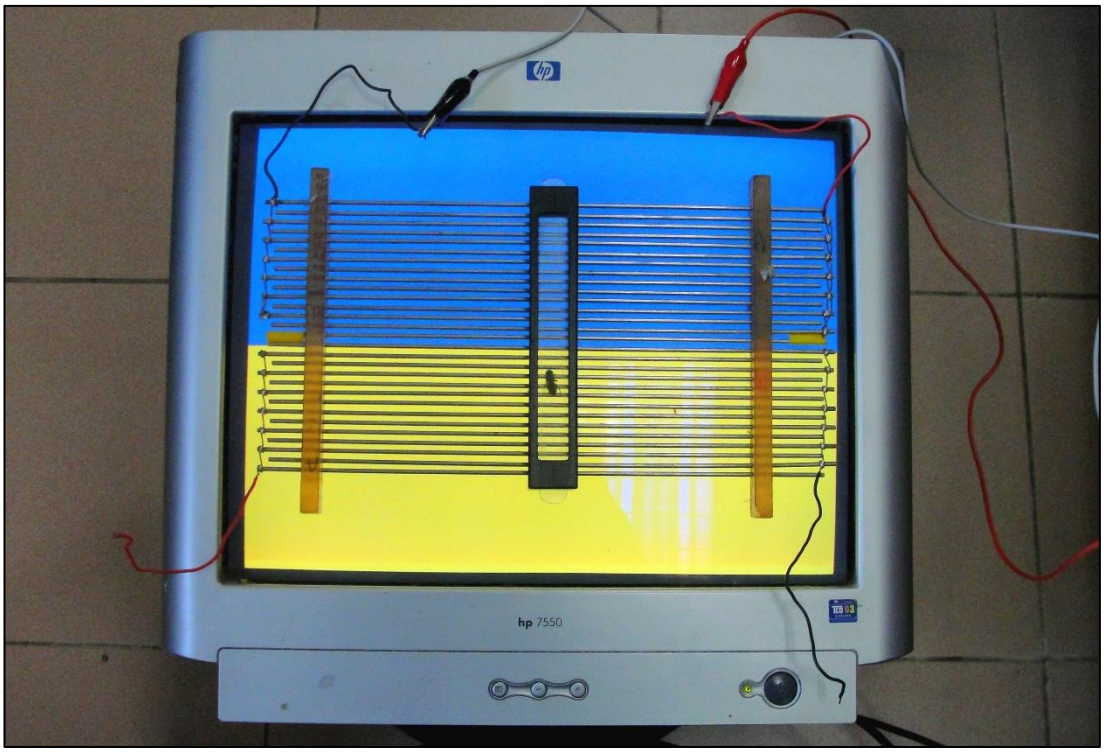

Figure 2.4 Experimental setup of electric shock avoidance assay.

2.2.2 Activity Monitoring Assay

The number of movements in the ESA assay may be related to general differences in activity among subspecies. We therefore studied the baseline activity levels of honey bees between 16:00 and 19:00, which was the hour interval of the ESA conditioning assay, in order to assess our results on the honey bees' movement between the shock side and non-shock in the ESA conditioning assay. We also collected daily activity data to investigate daily activity counts and activity patterns of the honey bee subspecies.

We used a modified *Drosophila* Activity Monitoring (DAM) system called LAM (Large Activity Monitors) of Trikinetics Inc. (Waltham, MA, USA), which are

locomotor activity monitors (Figure 2.5). Each unit has 32 independent activity channels, which measure activity by using three infrared beams and sensors to ensure that recordings are accurate. 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. This system was previously used and described in another research (Giannoni-Guzmán *et al*., 2014 b).

Honey bees were collected from inside the hives and were kept for 1 hour in cages before experiment when we prepared experimental setup. Then, each honey bee was placed into a 15 ml Falcon® centrifuge tube and each tube were placed into a different activity channel of the LAM. The LAM was placed in the incubator (NÜVE TK120) in dark, at 33 °C temperature and 55% humidity conditions with *ad libitum* feeding. Bee candy, a 60:40 mixture of sucrose and honey, was used for feeding. Bee candy was covered with cheese-cloth and put on the top of centrifuge tubes. Activity data was collected from LAM for 24 hours by each minute, and all records begun at 19:00.

Figure 2.5 Large activity monitors.

2.2.3 PER Conditioning Assay

Forager honey bees were collected from the entrances of the hives and were incubated in cages for 1 or 1.5 hours at 27 °C and 70% humidity in NÜVE TK120 incubator. Then, honey bees were anesthetized with ice water bath for harnessing. Honey bees were harnessed in metal bullet casings with a piece of duct tape placed between the head and thorax (Figure 2.6). Metal bullet casings are ideal for our experiment because they do not absorb the odors and are hence reusable. Honey bees were fed with a 10 – 15 µl, 50% (w/v) sucrose solution. Harnessed bees were incubated for 14 – 16 hours at 27 °C and 70% humidity. The average death ratio of honey bees was 14% after incubation.

After the incubation, harnessed bees were placed in a laminar flow bench for reversal learning experiments. Strawberry and lemon odors were used as conditioned stimulus (CS). These odors were used in previous research (Wang *et al*., 2013). Notably, the nearest agricultural area is a field of wheat, 10 kilometers far from apiary of Middle East Technical University, so that the honey bees in our apiary are possibly naïve to these odors. Odors were conveyed to honey bees with air flow from 50 cc plastic syringes for 3 seconds. Syringes were used as "odor cartridge" and contain 1 cm^2 piece of filter paper absorbed essential oil of single fruit. After CS, the cotton swab dipped in sucrose solution was immediately touched on the honey bee's antenna and proboscis for 2 seconds, as the unconditioned stimulus (US) (Figure 2.6). One of the conditional stimuli (CS+) was paired with US and the other is not (CS-). PER of each honey bee in each trial was recorded by an observer.

Before discrimination training, the cotton swab dipped in sucrose solution was touched on the honey bee's antenna and if a honey bee did not give PER, it was excluded from the experiment. The discrimination study contained 12 trails (6 are CS+ and 6 are CS-) with a pseudorandomized sequence of CS+, CS-, CS-, CS+, CS-, CS+, CS+, CS-, CS+, CS-, CS-, CS+ to prevent conditioning to order of CS. Also, each intertrial interval (ITI) took 5 minutes. This discriminant learning experiment protocol is very

similar to method used from Charles I. Abramson (Abramson, Sokolowski & Wells, 2010).

In addition, we continued the experiment with reversal phase, detailed information found in Appendix C.

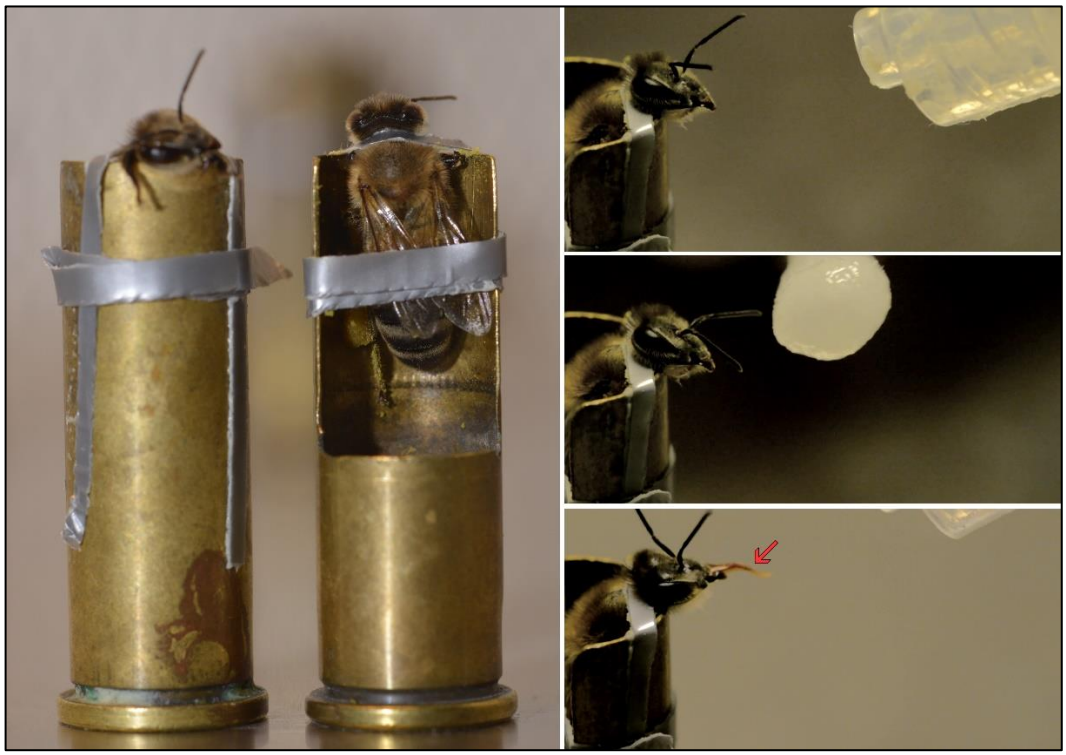

Figure 2.6 Harnessed honey bees in bullet casings (left). As conditioned stimulus, odor is conveyed from syringes (right, upper). As unconditioned stimulus, a cotton swab dipped in sucrose solution is touched on the antenna (right, middle). PER (right, bottom).

2.2.4 Starvation Assay

In this experiment we imitated the conditions of the PER assay to determine the effect of hunger on PER and the durability of subspecies to starvation. Forager honey bees were collected from the entrance of the hives and were incubated in cages for 1.5 hours at 27 °C and 70% humidity. Then, honey bees were anesthetized with ice water bath for harnessing. Honey bees were harnessed in metal bullet casings. They were fed with 50% (w/v) sucrose solution until their hunger was satiated and no PER was observed. Harnessed bees were incubated at 27 °C and 70% humidity in NÜVE TK120

incubator. Honey bees were observed at twelfth, twenty-fourth and thirty-sixth hour after feeding. PER was controlled by touching a honey bee's antenna with a cotton swab dipped in sucrose solution sucked. The number of PER and number of deaths were recorded.

2.3 Statistical Analysis

Statistical analysis of ESA conditioning assay was conducted with R. We used both a multiple linear regression model and a repeated measures ANOVA to compare subspecies. We determined minutes as integer, subspecies groups and individual honey bees as factor, and individuals were determined as error factor within subspecies groups for both multiple linear regression model and repeated measures ANOVA test. We also performed permutation test to verify the results of multiple linear regression model and repeated measures two-way ANOVA tests. R codes of multiple linear regression model and repeated measures ANOVA (Table A.1) and permutation test (Table A.2) are found in Appendix A.

Data of activity monitoring assay were evaluated with IBM SPSS Statistics 24, and one-way ANOVA test was conducted for comparison of subspecies.

Because of assumptions of parametric tests were not met, a Kruskal-Wallis test and chi-squared test were conducted for statistical analysis of PER conditioning assay with IBM SPSS Statistics 24.

We used the chi-squared test to investigate effect of starvation on mortality and PER across subspecies. IBM SPSS Statistics 24 was used for conducting this test.

All graphs were drawn with Microsoft Excel 2016.

CHAPTER 3

RESULTS

3.1 ESA Conditioning Assay

We firstly compared the honey bee subspecies by time spent on the shock side to determine their learning performances. Sample size of the subspecies groups, Caucasian, Carniolan, and Syrian honey bees were 32, 39, and 39, respectively. We had 5 data points for each minute. Means, standard deviations and standard errors of the results in ESA conditioning assay are found in Appendix B (Table A.7).

We used both a multiple linear regression model and a repeated measures ANOVA test for comparison. Both multiple linear regression model and a repeated measures ANOVA test gave same values for F and p. According to their result, significant time effect (F (1, 437) = 99.911, $p < 0.001$), significant group effect (F (2, 107) = 7.006, $p < 0.01$), but no significant time and group interaction effect (F (2, 437) = 2.297, $p > 0.5$) were found (ANOVA table found in Appendix B, Table A.3). Post hoc Tukey's test of model on groups showed that Caucasian honey bee was significantly different from both Carniolan honey bee at $p < 0.001$ and Syrian honey bee at $p < 0.001$, but Carniolan honey bee and Syrian honey bee were not different at $p > 0.5$. Thus, Caucasian honey bee had better learning performance than Carniolan and Syrian honey bees (Figure 3.1).

In addition, the validity of multiple linear regression model and repeated measures ANOVA result on group effect were checked with a permutation test, since assumptions of parametric tests were not met in terms of normality of distribution (a Shapiro-Wilk test on the model: $W = 0.951$, $p < 0.001$, and a Kolmogorov-Smirnov

test results were as follows: *A. m. caucasica*, D (160) = 0.232, $p < 0.001$, *A. m. carnica*, D (195) = 0.177, $p < 0.001$, and *A. m. syriaca*, D (195) = 0.170, $p < 0.001$) and homogeneity of variance (a Levene's test results: F (2, 547) = 26.980, $p < 0.001$). In the permutation tests, we determine the number of permutations as 1000. So, permutation test on group effect of multiple linear regression model with approximation (B = 1000) the difference was significant at $p < 0.001$, in agreement with the multiple linear regression model. Also, permutation test on group effect of repeated measures ANOVA indicated that the repeated measures ANOVA result was indeed significant (B = 1000, $p < 0.05$) and not a fluke of the repeated measures ANOVA test.

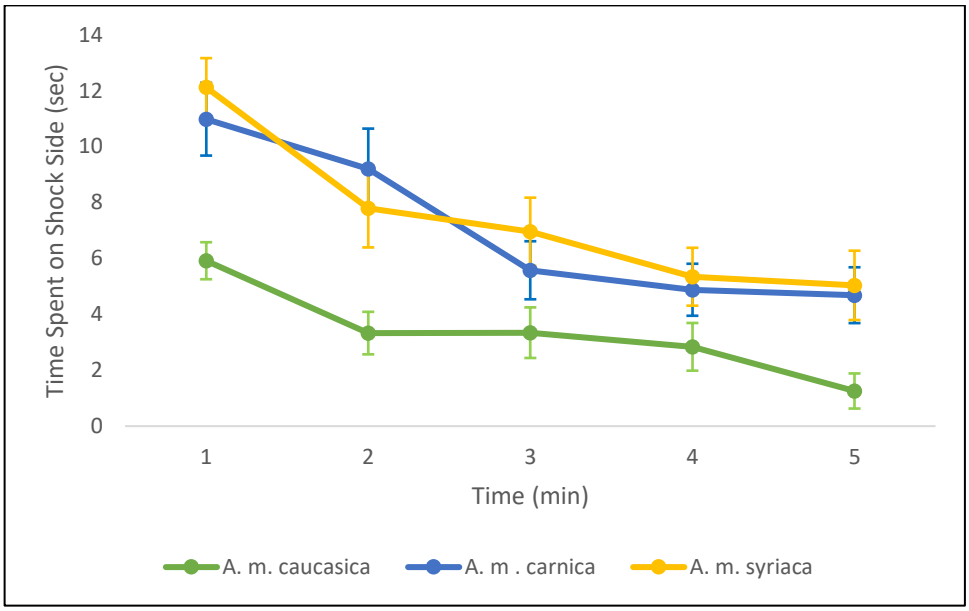

Figure 3.1 Comparison of spatial-avoidance learning rate across honey bee subspecies during ESA assay. Each data point shows mean (± standard error) of the time honey bees spent on shock side during the trial.

Secondly, subspecies were compared in terms of the number of times individuals crossed the border between the shock side and non-shock side. Data were collected from the same experiment described above (used for the comparison of the honey bee subspecies by time spent on the shock side).

Again, we used both a multiple linear regression model and a repeated measures ANOVA test for comparison. Both multiple linear regression model and repeated

measures ANOVA test gave same values for F and p. According to their result, significant time effect (F (1, 437) = 254.838, $p < 0.001$), significant group effect (F (2, 107) = 11.896, $p < 0.001$), and significant time and group interaction effect (F (2, 437) = 5.585, $p < 0.001$ were found (ANOVA table found in Appendix B, Table A.4). Post hoc Tukey's test of model on groups showed that Caucasian honey bee was significantly different from both Carniolan honey bee at $p < 0.01$ and Syrian honey bee at $p < 0.001$, and Carniolan and Syrian honey bees were also significantly different from each other at $p < 0.01$. Thus, this result also indicated that, Caucasian honey bee had better learning performance than Carniolan and Syrian honey bee (Figure 3.2).

The parametric test assumptions were again not met in terms of normality of distribution (a Shapiro-Wilk test on the model: $W = 0.985$, $p < 0.001$, and a Kolmogorov-Smirnov test results were as follows: *A. m. caucasica*, D (160) = 0.229, $p < 0.001$, *A. m. carnica*, D (195) = 0.184, $p < 0.001$, and *A. m. syriaca*, D (195) = 0.163, $p < 0.001$) and homogeneity of variance (a Levene's test results: F (2, 547) = 20.113, $p < 0.001$). We check the validity of multiple linear regression model and repeated measures ANOVA result on group effect with permutation test. Permutation test with approximation ($B = 1000$) the difference was significant at $p < 0.001$, in agreement with the multiple linear regression model. Also, permutation test indicated that the repeated measures ANOVA result was significant ($B = 1000$, $p < 0.01$).

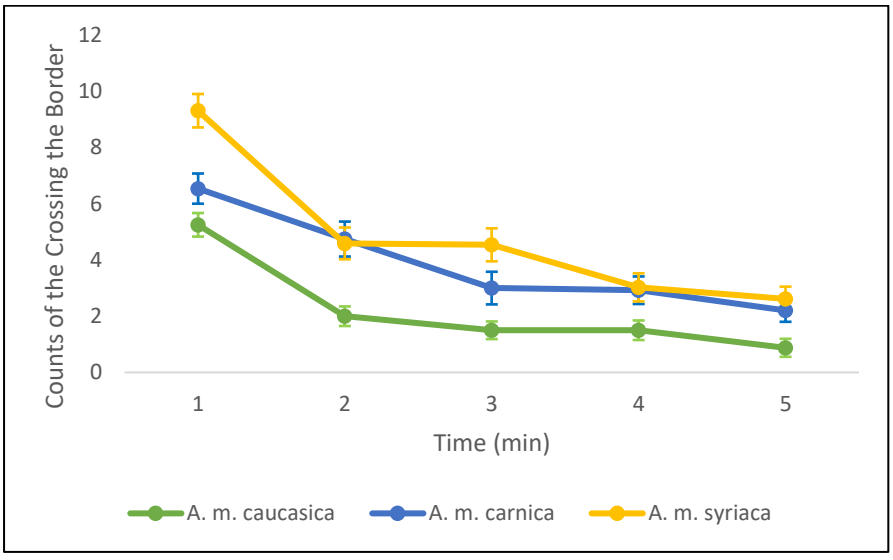

Figure 3.2 Comparison of honey bee subspecies in terms of number of the crossing border between the shock side and safe side. Data are represented as mean ± standard error.

We also analyzed the control groups in terms of both time spent on the blue side and counts of the crossing border between the sides. There were 32 individuals for Caucasian honey bee, 38 individuals for Carniolan honey bee and 26 individuals for Syrian honey bee found in the groups at 5 data point for each minute.

According to both multiple linear regression model and repeated measures ANOVA result, time effect (F (1, 381) = 0.015, $p > 0.5$), group effect (F (2, 93) = 1.812, $p > 0.5$), and time and group interaction effect (F (2, 381) = 0.901, $p > 0.5$) were not significant for comparison of the honey bee subspecies by time spent on the blue side (ANOVA table found in Appendix B, Table A.5). So, there was no color choice differences found across subspecies (Figure 3.3)

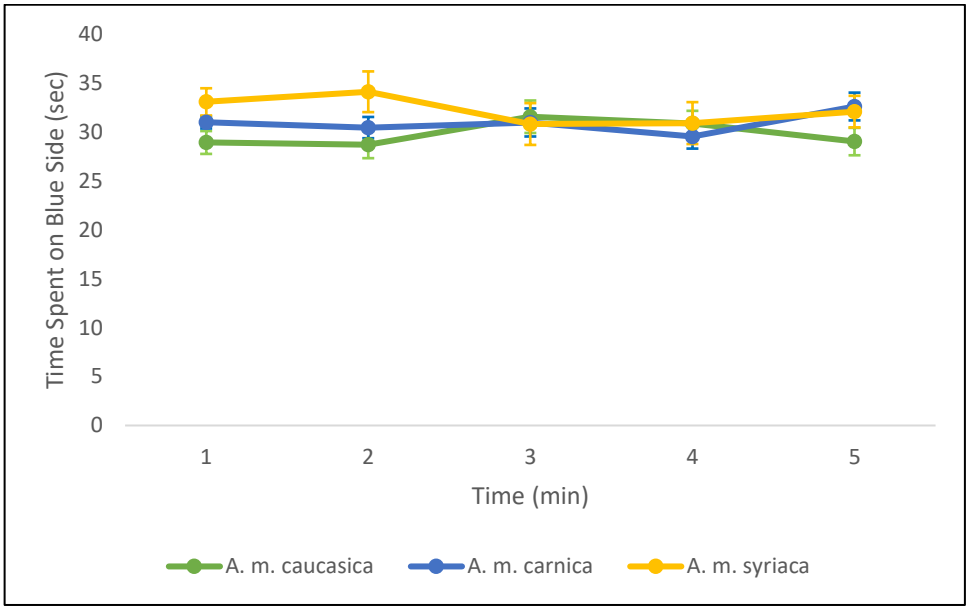

Figure 3.3 Comparison of the honey bee subspecies by time spent on the blue side. Data are represented as mean ± standard error.

Only significant result found in time effect (F (1, 381) = 34.65, $p < 0.001$) for comparison of the subspecies in terms of number of the crossing border between the blue side and yellow side, according to both multiple linear regression model and repeated measures ANOVA result. Group effect (F (2, 93) = 1.288, $p > 0.5$), and time and group interaction effect (F (2, 381) = 0.07, $p > 0.5$) were not significant (ANOVA table found in Appendix B, Table A.6). Significant result for time effect possibly

indicated habituation, in other words, honey bees reduced their movements in time. Meanwhile, there was no difference found across subspecies in terms of their movement behavior (Figure 3.4).

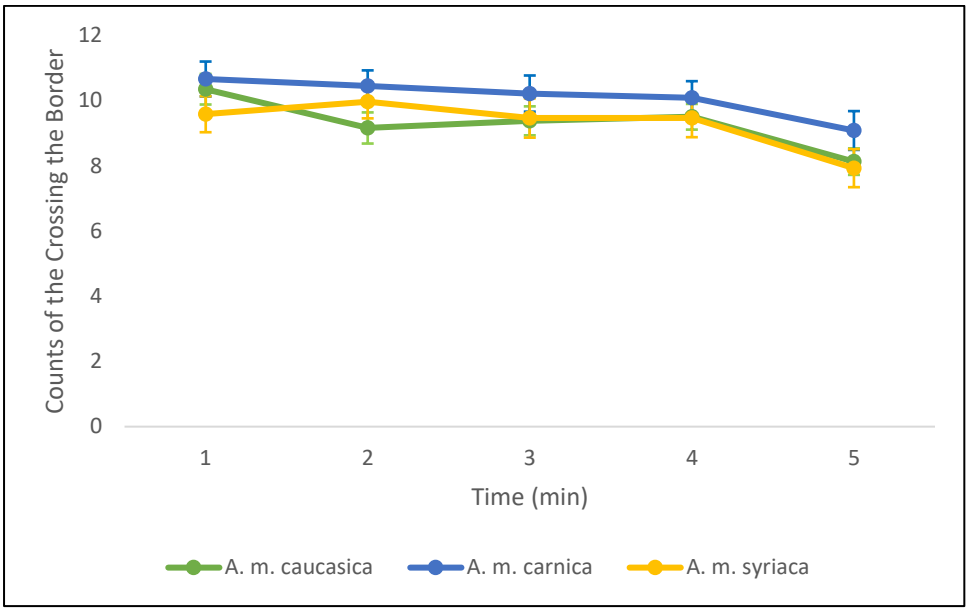

Figure 3.4 Comparison of honey bee subspecies in terms of number of the crossing border between the blue side and yellow side. Data are represented as mean ± standard error.

3.2 Activity Monitoring Assay

Here we compared in activity levels of honey bees between 16:00 and 19:00, because ESA experiments were conducted at this time and we decided to check the background activity of honey bees.

The subspecies sample sizes were n = 34 individuals for the Caucasian honey bee, n = 34 for the Carniolan honey bee, and n = 24 for the Syrian honey bee.

Because of assumptions of parametric tests were not met in terms of normality of distribution, we used square root transformation on locomotor activity counts. After transformation, distributions became normal as affirmed by a Kolmogorov-Smirnov test for normality. The test results were as follows: *A. m. caucasica*, D (34) = 0.103, p > 0.05, *A. m. carnica*, D (34) = 0.095, p > 0.05, and *A. m. syriaca*, D (24) = 0.138, p

> 0.05. Also, a Levene's test results suggested that the homogeneity of variance, F (2, 89) = 0.222, $p > 0.05$.

We next applied one-way ANOVA to compare subspecies. We found a significant one-way ANOVA result, F (2, 89) = 9.538, $p < 0.001$ (ANOVA table found in Appendix B, Table A.8). Post hoc Tukey's test on groups showed that Caucasian honey bee was significantly different from both Carniolan honey bee at $p < 0.01$ and Syrian honey bee at $p < 0.01$, but Carniolan honey bee and Syrian honey bee were not different. Thus, with square root transformation, *A. m. caucasica* (μ = 30.1, *sd* = 10.7) hashigher activity than *A. m. carnica* (μ = 20, *sd* = 10.2) and *A. m. syriaca* (μ = 20.2, *sd* = 11.1) between 16:00 and 19:00, which was the hour interval of ESA conditioning assay (Figure 3.5). According to comparison without square root transformation, *A. m. caucasica* (μ = 1016.53, *sd* = 629.06) has nearly two times higher activity than *A. m. carnica* (μ = 501.74, *sd* = 424.33) and *A. m. syriaca* (μ = 525.08, *sd* = 562.23)

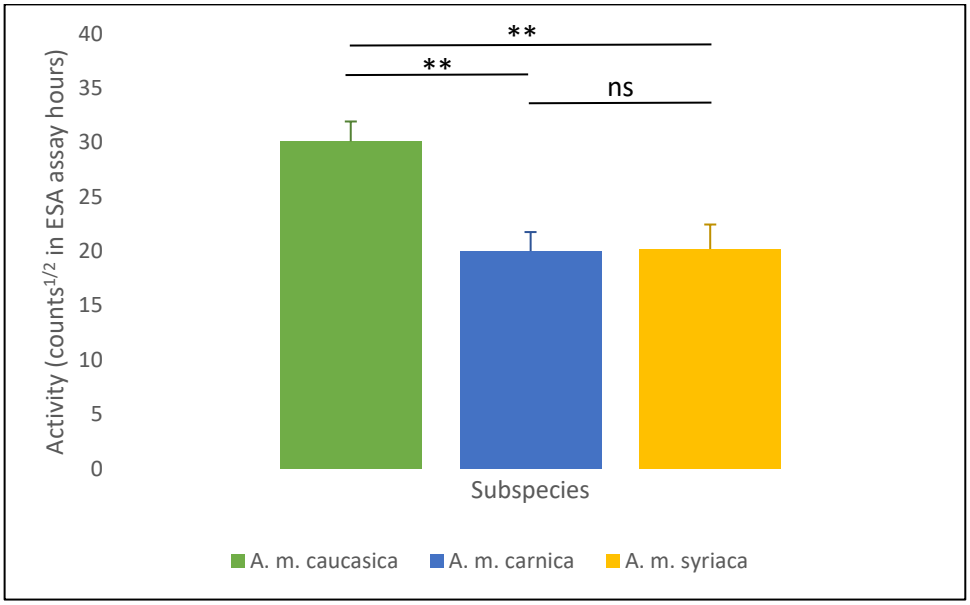

Figure 3.5 Squared root of activity counts between 16:00 and 19:00. Data are represented as mean ± standard error. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

After we found differences in activity across subspecies between 16:00 and 19:00, we suspected that there may be differences in daily activity across subspecies across 24 hours. We evaluated the data of same experiment. We next checked the assumptions of parametric test for daily activity. A Kolmogorov-Smirnov test was used to test for

normality: *A. m. caucasica*, D (34) = 0.106, $p > 0.05$, *A. m. carnica*, D (34) = 0.140, $p > 0.05$, and *A. m. syriaca*, D (24) = 0.166, $p > 0.05$. Also, a Levene's test results suggested that the homogeneity of variance, F (2, 89) = 1.025, $p > 0.05$. Thus, assumptions of parametric tests were met.

We found a significant one-way ANOVA result, F (2, 89) = 5.716, $p < 0.01$ (ANOVA table found in Appendix B, Table A.9). Post hoc Tukey's test on groups showed that the Caucasian honey bee was significantly different from the Carniolan honey bee at $p < 0.01$, while there was no other significant result found in other pairwise comparisons. Thus, *A. m. caucasica* (μ = 3096.1, *sd* = 1431.9) have higher activity than *A. m. carnica* (μ = 1938.4, *sd* = 1287.3), but not significantly higher than *A. m. syriaca* (μ = 2376.5, *sd* = 1584.7) (Figure 3.6).

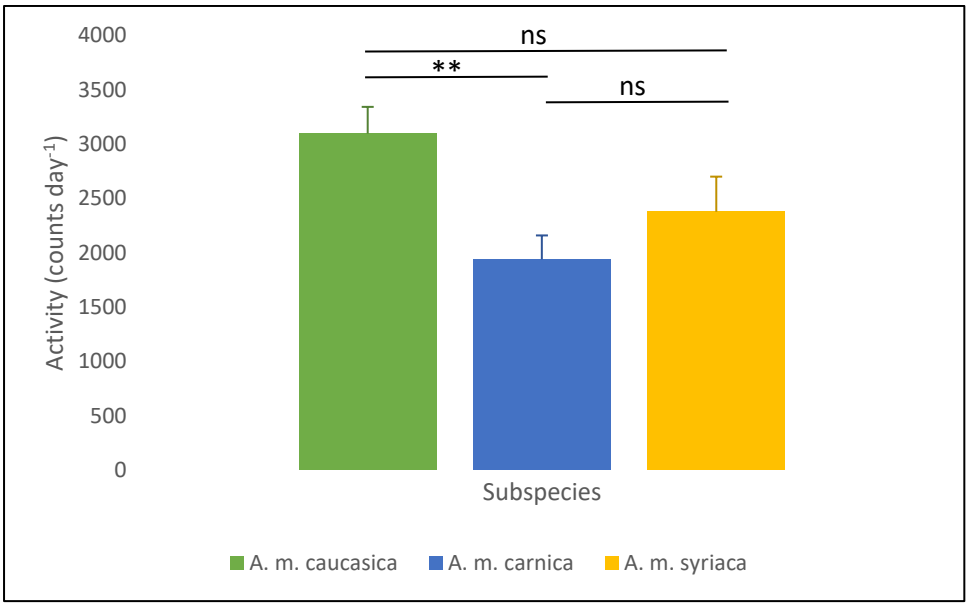

Figure 3.6 Total activity counts in 24 hours. Data are represented as mean ± standard error. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

We also investigated daily activity patterns and how it differed among subspecies. According to 24 hours activity monitoring graph, the activity of Caucasian honey bee was higher than others between 16:00 – 19:00. However, between 10:00 – 14:00, activity of Syrian honey bee was higher than other subspecies. Also, most active hours differentiated between the subspecies, to illustrate Syrian honey bee reached higher activity between 14:00 – 15:00, Carniolan honey bee reached maximum activity

between 16:00 – 17:00 and Caucasian honey bee reached at 17:00 – 18:00 (Figure 3.7).

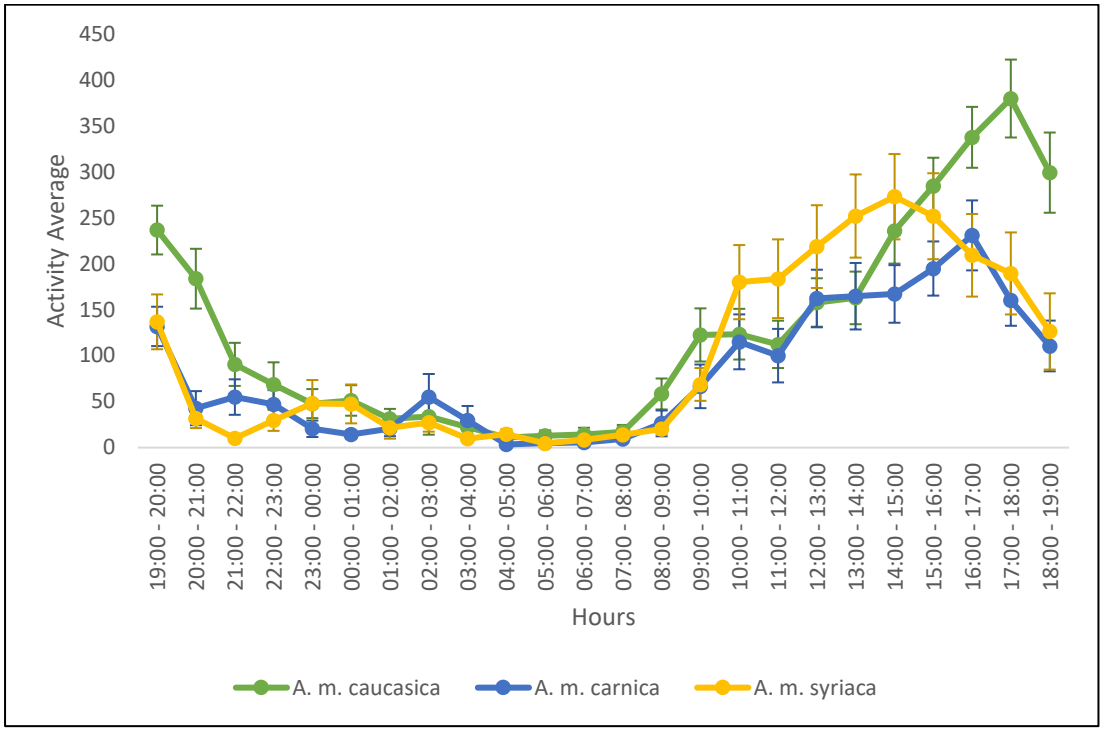

Figure 3.7 Activity patterns of honey bee subspecies for 24 hours. Data are represented as mean ± standard error.

3.3 PER Conditioning Assay

We studied olfactory learning and how it may differ among subspecies using the PER conditioning assay. Sample sizes were n = 29, 37, and 28 for Caucasian, Carniolan and Syrian honey bees, respectively. We used the PER ratio score of each individual to compare subspecies, mathematical formula is as follows:

$$A = \sum_{n=1}^{6} \frac{i_n}{6} \quad \begin{Bmatrix} i = 1 \; if \; + \\ i = 0 \; if \; - \end{Bmatrix}$$

A is PER score of an individual, n is trial, i is PER, + means that PER was observed, and – means that no PER was observed. So, if a honey bee gave PER to CS we counted as 1 and if did not gave PER which was 0. Thus, sum of PER to CS+ divided by total CS+ trial number which is 6, and the same calculation was applied for CS-. Because

of distributions were not normal according to a Kolmogorov-Smirnov test (for CS+, *A. m. caucasica*, D (29) = 0.270, $p < 0.001$, *A. m. carnica*, D (37) = 0.229, $p < 0.001$, and *A. m. syriaca*, D (28) = 0.299, $p < 0.001$, and for CS-, *A. m. caucasica*, D (29) = 0.324, $p < 0.001$, *A. m. carnica*, D (37) = 0.286, $p < 0.001$, and *A. m. syriaca*, D (28) = 0.187, $p < 0.05$), and both square root and log transformation did not maintain the normality, then we used Kruskal-Wallis test for comparison. There was no significant result found for the CS+ ($p > 0.05$), however result was significant ($p < 0.05$) for CS- (Figure 3.8, values of box-and-whisker plots are found in Appendix B, Figure A.10).

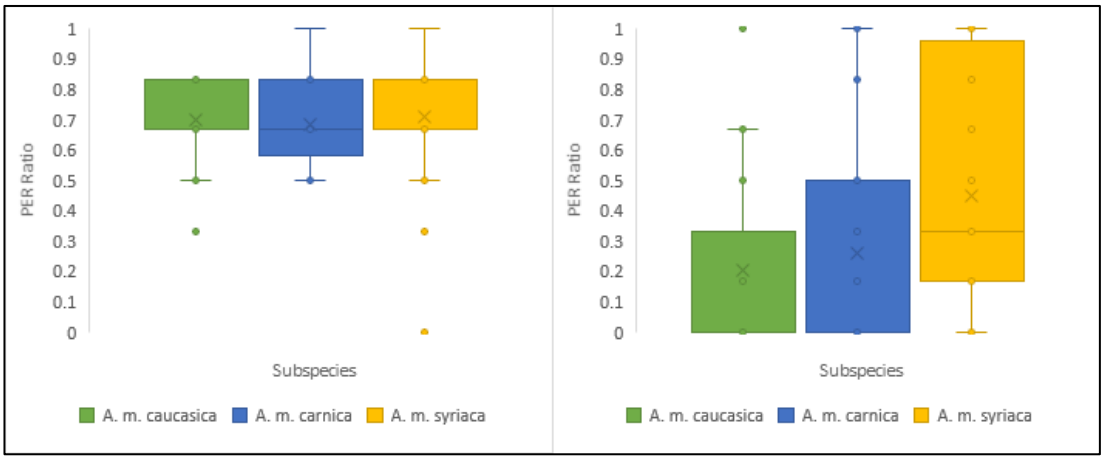

Figure 3.8 Box-whisker plot for comparison of subspecies` PER ratios to CS+ (left), and CS- (right).

All subspecies' PER ratio rapidly increased for CS+, however there was no evident decrement observed for CS- along trials in discriminant learning. This suggests an impairment in discriminant learning, because complete extinction did not occur for any of the subspecies. Also, there was a surprising peak observed on the line of Syrian honey bee (*A. m. syriaca*) (Figure 3.9).

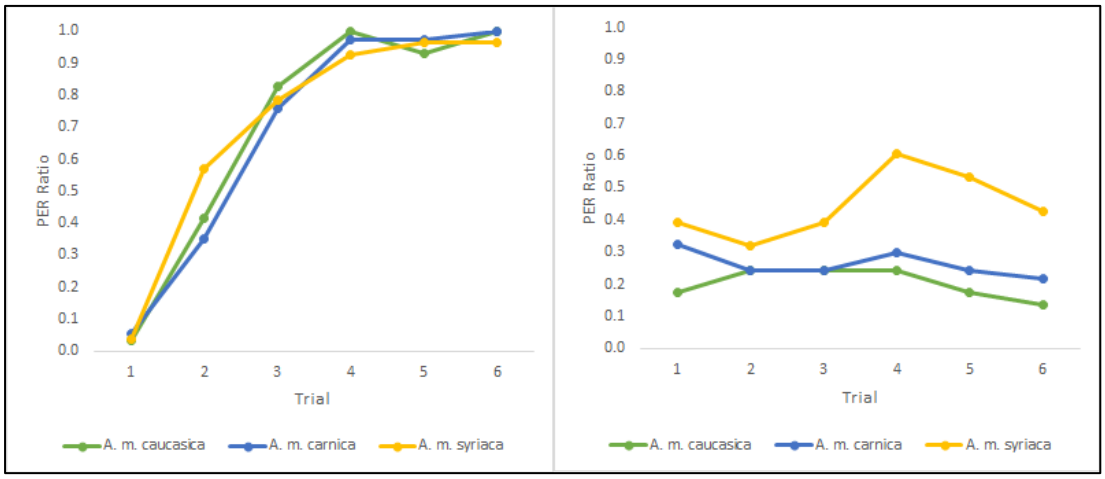

Figure 3.9 Ratio of PER to CS+ (left), and CS- (right) along trials.

Possible explanation of incomplete extinction to CS- is that, some individuals continuously give PER to both CS+ and CS- without any discrimination. Ratio of these individual to sample size are 4/29 for *A. m. caucasica*, 8/37 for *A. m. carnica*, and 9/28 for *A. m. syriaca*. Using with these numbers, we also conducted a chi-square test to examine the relation between subspecies and tendency to give PER unselectively to both CS+ and CS-. The relation between these variables was not significant, χ^2 (2, n = 94) = 4.109, $p > 0.05$.

Then, we excluded these individuals, which still gave continuously PER both CS+ and CS- in last 6 trial, from data and repeated our analysis.

After the data filtering, we observed that the PER ratio rapidly increased for CS+ in all groups similar to the previous result. Differently, PER ratio of Caucasian and Carniolan honey bees slightly decreased for CS- along trials in discriminant learning. However, a sharp peak was still observed, and complete extinction did not occur for Syrian honey bee (Figure 3.10).

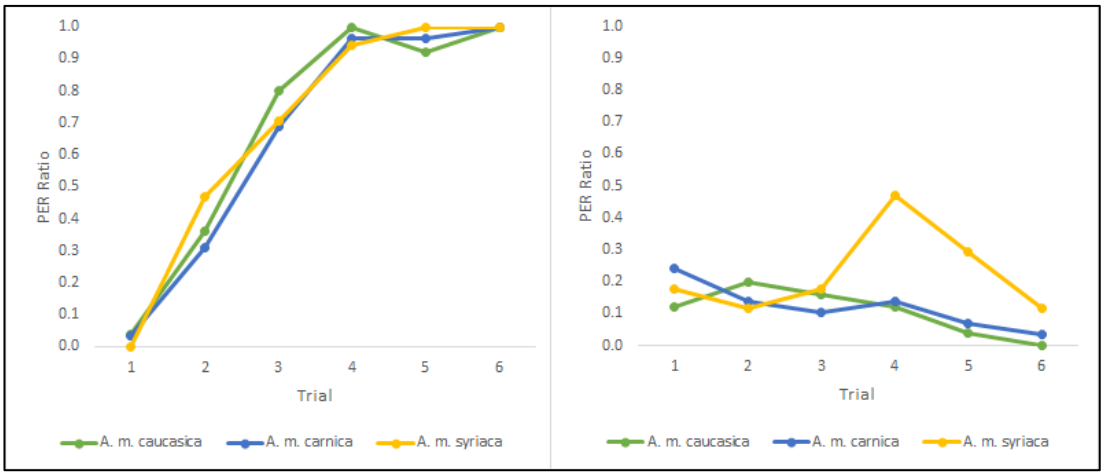

Figure 3.10 After data filtering, ratio of PER to CS+ (left), and CS- (right) along trials.

A Kruskal-Wallis test performed to test differences in terms of PER ratio for comparison across *A. m. caucasica* (n = 25), *A. m. carnica* (n = 29), and *A. m. syriaca* (n = 17). Because of distributions were not normal according to a Kolmogorov-Smirnov test (for CS+, *A. m. caucasica*, D (25) = 0.241, $p < 0.01$, *A. m. carnica*, D (29) = 0.242, $p < 0.001$, and *A. m. syriaca*, D (18) = 0.301, $p < 0.001$, and for CS-, *A. m. caucasica*, D (25) = 0.380, $p < 0.001$, *A. m. carnica*, D (29) = 0.374, $p < 0.001$, and *A. m. syriaca*, D (18) = 0.203, $p < 0.05$), and both square root and log transformation did not maintain the normality, again we used Kruskal-Wallis test for comparison. We had similar results with previous comparison. There was no significant result found for the CS+ ($p > 0.05$), however the result was significant ($p < 0.05$) for CS- (Figure 3.11 values of box-and-whisker plots are found in Appendix B, Figure A.10).

These results indicated that, Syrian honey bee had lower performance in olfactory learning than Caucasian and Carniolan honey bees.

In addition, results of reversal phase found in Appendix C.

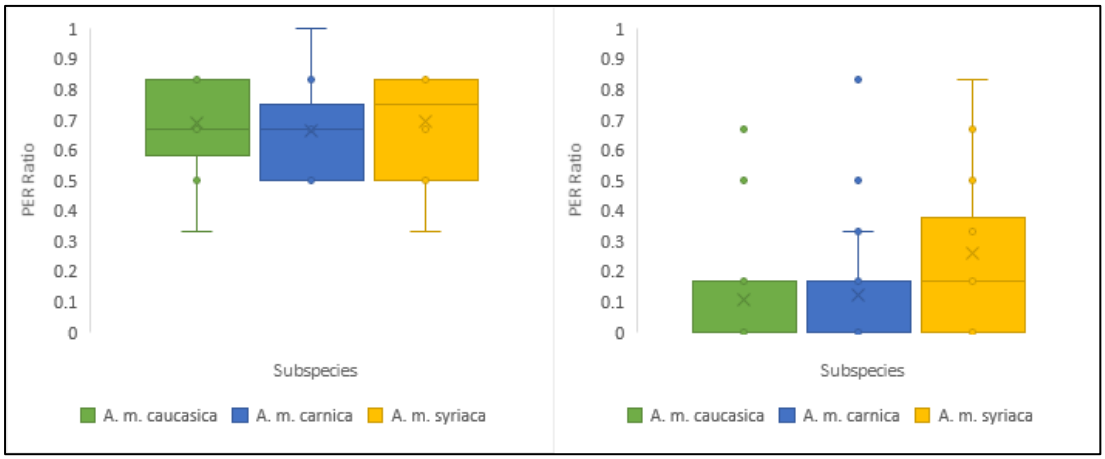

Figure 3.11 Box-whisker plot for comparison of subspecies` PER ratios to CS+ (left), and CS- (right), after data filtering.

3.4 Starvation Assay

We performed chi-squared tests to examine the relation between subspecies and PER under starvation. The subspecies sample sizes were n = 36 individuals for the Caucasian honey bee, n = 39 for the Carniolan honey bee, and n = 38 for the Syrian honey bee. We had three checkpoints in the assay at the 12th, 24th, and 36th hours. The relation between these variables was significant at only the 12th hour checkpoint (Table 3.1).

Table 3.1 Chi-squared test results for PER scores at 12th, 24th, and 36th hours.

Hour	*df*	*n*	χ^2	*p*
12	2	102	23.813	< 0.001
24	2	42	2.014	> 0.05
36	1	16	0.246	> 0.05

According to our results, the Carniolan honey bee gave less PER than Caucasian and Syrian honey bees under starvation (Figure 3.12).

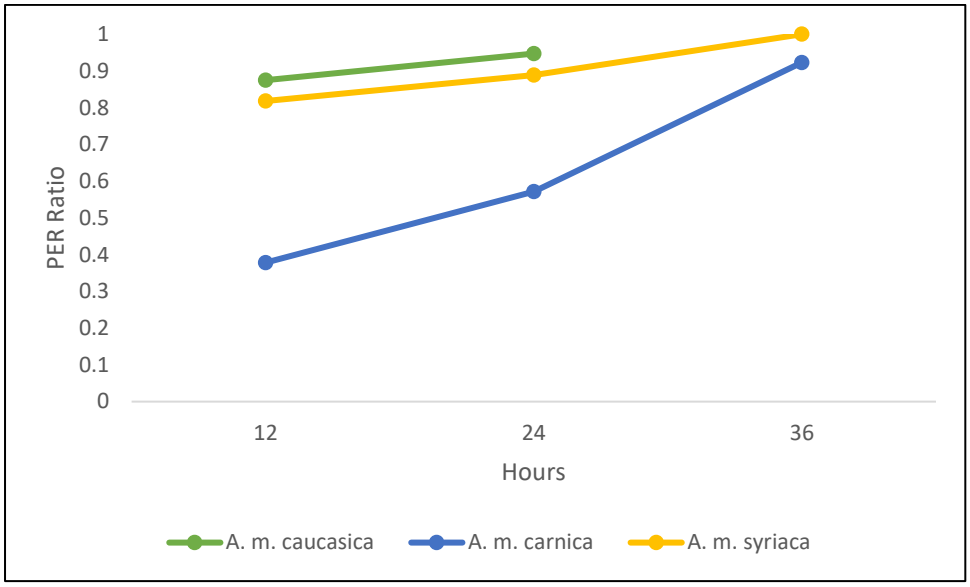

Figure 3.12 PER ratios of subspecies under starvation at 12th, 24th, and 36th hours.

We also conducted chi-squared tests to examine the relation between subspecies and their mortality rates under starvation. The relation between these variables was significant at only 24th, and 36th hour checkpoint (Table 3.2).

Table 3.2 Chi-squared test results on mortality data at 12th, 24th, and 36th hours.

Hour	*df*	*n*	χ^2	*p*
12	2	113	1.526	> 0.05
24	2	113	22.336	< 0.001
36	2	113	17.189	< 0.001

Our results suggest that, Carniolan honey bee was the most resistant subspecies to starvation and Caucasian honey bee was the most susceptible subspecies among three subspecies used in our research (Figure 3.13).

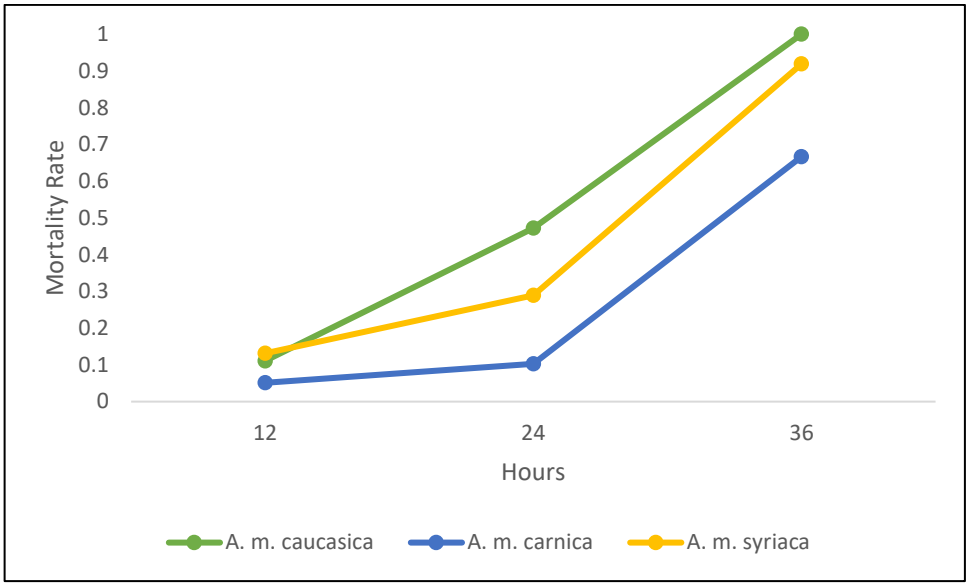

Figure 3.13 Mortality rate of subspecies under starvation at 12th, 24th, and 36th hours.

CHAPTER 4

DISCUSSION

In this study, we showed learning performance differences across three subspecies in Turkey using two main assays which are ESA conditioning assay and PER conditioning assay.

Firstly, in ESA conditioning assay we evaluate two variables. One is time spent on the shock side and the other is number of the crossing border between the shock side and non-shock side. Previous studies only used time spent on the shock side or safe side for evaluation (Agarval *et al*., 2011; Dinges *et al*., 2013; Gionnoni-Guzman *et al*., 2014 a; Avalos *et al*., 2017). However, we decided that this approach may not be enough for a clear comparison. Because, when a honey bee enters to the shock side, it can immediately return to the safe side in a very small time by reflex. If this is true, the honey bee may not learn to avoid the shock side and may still try to the enter shock side, but our statistic (time spent on shock side) would not indicate this lack of learning. In other words, only studying the total duration may not be meaningful for statistical analysis. Because of that reason, we also analyzed the data of number of the crossing border between the shock side and non-shock side, obtained in the same experiment. Meanwhile, we should note that the number of border crossings by itself would also not suffice as a statistic to compare learning performance. Because honey bees decrease their activity along minutes due to habituation, and this habituation effect can be mistaken as learning. Thus, both the time data and border crossing counts should be evaluated together and results of the two analyses should be consistent with each other (Figure 3.1 and Figure 3.2).

In addition, activity differences of honey bees may affect the border crossing counts. To investigate that, we set a control experiment without electric shock and activity

monitoring assay. We could not find any differences between subspecies in the control experiment without electric shock (Figure 3.4). Also, according to activity monitoring assay result, *A. m. caucasica* has higher activity than *A. m. carnica* and *A. m. syriaca* at the hour interval used for the ESA conditioning assay (Figure 3.5). Even though the Caucasian honey bee has higher activity at the hour interval of ESA conditioning assay, they have the lowest means of number of the border crossing between the shock side and non-shock side. This indicates that the Caucasian honey bee may have higher learning performance independent of its activity level. Also, researchers should take account that daily locomotor activities vary in different time of the day among subspecies.

The color preference of honey bees may be another factor affecting the results of the ESA conditioning assay. As mentioned in the Introduction, Giurfa *et al*. illustrated that specific colors under UV wavelengths were being innately chosen by flower-naive forager honey bees (1995). If color preference of honey bee subspecies differs from each other, our results may be drastically affected. Our control experiment without electric shock illustrated that there is no difference across subspecies in color preference, as all groups spent the same amount of time in the blue area (Figure 3.3).

Another speculation is that, animals exhibit flight or fight response against aversive stimulus, and Syrian honey bee is known with its aggressive behavior (Çakmak, Wells & Fıratlı, 1998; Ruttner,1988). If the latter exhibits a fight response rather than flight response, that may be mistakenly evaluated as an impairment in discriminant learning. This speculation does not apply to our situation, because a fight response in honey bees should be observable from the Sting Extension Response (SER), and we discarded the honey bees which gave SER during the experiment (Unfortunately, we did not record how many bees were discarded from each group). Moreover, contrary to the Syrian honey bee, the Carniolan honey bee is known with its gentle disposition (Winston, 1987), and these two subspecies represented similar low learning performance in our ESA conditioning assay. This suggests that fight response differences are not responsible for the observed learning difference.

Overall, these results suggest that the Caucasian honey bee may have higher learning performance than Syrian and Carniolan honey bees in ESA conditioning assay.

Secondly, after we determined that the Caucasian honey bee has higher activity than the Syrian honey bee and Carniolan honey bee at the hour interval of ESA, we investigated daily activity difference across honey bee subspecies. We found that, Caucasian honey bee daily average activity was significantly different from that of Carniolan honey bee (Figure 3.6). Further, we found differences in daily activity patterns among the subspecies (Figure 3.7). The reason of the differences in daily activity could be various, such as geographic and floral differences or only resulted from neutral evolution. Further studies are necessary to determine the reasons for this pattern.

Thirdly, in the PER conditioning assay, we found that the increase in PER to CS+ throughout trials was similar for all subspecies. However, there was no evident decrement observed for CS- along trials in discriminant learning and complete extinction did not occur for any of the subspecies (Figure 3.9). Even so, the Syrian honey bee had a significantly higher PER ratio to CS- (Figure 3.8), which indicates that Syrian honey bee may have more impairment in discriminant learning. The reason for the lack of noticeable decrement observed for CS- throughout the trials for all subspecies is that some individuals continuously gave PER to both CS+ and CS- without any discrimination (Figure 3.9 vs. Figure 3.10). We therefore compared subspecies for investigating if any of subspecies is prone to give PER unselectively, but we did not find a significant difference.

We next performed a data filtering and excluded individuals that unselectively have PER to both CS+ and CS-. After this filtering, the PER tendency found for the Caucasian honey bee and Carniolan honey bee slightly decreased for CS- along trials, as we expected (Figure 3.10). However, for the Syrian honey bee, a sharp peak was observed in the fourth trial of CS- and complete extinction did not occur (Figure 3.10), and this group still had significantly higher PER ratio to CS- than the other subspecies (Figure 3.11).

We then hypothesized that these particular behaviors of *A. m. syriaca* may be caused by a sensitization and it may be more susceptible to hunger. To investigate that, we conducted a starvation assay with imitating the conditions of PER conditioning assay on harnessed honey bees and checked the PER ratios and mortality in different levels of hunger. We found that the Carniolan honey bee has lower PER ratio while PER ratios of Caucasian and Syrian honey bees are similar under starvation (Figure 3.12). Also, *A. m. caucasica* appeared to be the most susceptible to starvation among three subspecies (Figure 3.13). Thus, hunger sensitivity is cannot explain the distinct behaviour of *A. m. syriaca* in discriminant learning.

Another possibility would be that an experimental error might have occured at the fourth trial of CS- while testing the Syrian honey bee. For example, we used lemon odor for CS+ and strawberry odor for CS-, and at fourth trial of CS- we may have mistakenly used lemon odor. We used the sequence of CS+, CS-, CS-, CS+, CS-, CS+, CS+, CS-, CS+, CS-, CS-, CS+ in PER conditioning study and if we had lemon odor instead of strawberry odor at the fourth trial of CS-, we would have expected higher PER ratio at the fourth trial of CS- from fourth trial of CS+. However, PER ratio of fourth trial of CS- is nearly half of PER ratio of fourth trial of CS+ (Figure A.3 in the Appendix D), which argues against this possibility. Another possibility is that we mixed odors only during one part of experiment. However, the individuals which give PER at the fourth trial of CS- are not clumped in the same part of experiment but distributed randomly (Figure A.4 at Appendix D). Thus, it is likely that a simple experimental mix-up cannot explain the unexpected PER peak in the Syrian honey bee.

It is tempting to speculate that the cause of lower success of Syrian honey bee in PER conditioning assay could be related to the particularities of foraging activity of this honey bee subspecies. The Syrian honey bee inhabits an arid habitat with mild winters, so it has longer seasonal foraging periods but lower diversity in terms of nectar source (Kandemir, Kence & Kence, 2000; Kandemir *et al.*, 2006). On the contrary, the foraging period is restricted to short summers and cold winters for the Carniolan honey bee and the Caucasian honey bee, as well as the more sequential blooming of the flowering species. As a result, Carniolan honey bee and Caucasian honey bee have to

maximize the amount of the collected nectar in a limited time period from casual nectar sources (Çakmak *et al*., 2010; Perez-Claudio *et al.*, in press). Thus, making discrimination between flowers is very crucial for Carniolan and Caucasian honey bees. Possibly, we may be observing the reflection of this foraging behavior on our appetitive learning study. This may explain why the Syrian honey bee has lower learning performance than the Carniolan and the Caucasian honey bees in the PER conditioning assay.

In addition, our PER conditioning assay result different from previous studies (Abramson *et al*., 2008: Perez-Claudio *et al.*, in press). Abramson *et al*. could not find any difference across *A. m. caucasica, A. m. carnica* and *A. m. syriaca* in their discrimination comparison with PER conditioning assay (2008), also Perez-Claudio *et al*. could not observe any difference between PER of *A. m. caucasica* and *A. m. syriaca* in acquisition phase of their reversal learning test. First possible reason of the different results of these researches is that some slight differences found in the experimental protocols. For example, Abramson *et al*. used wintergreen and cinnamon oil, Perez-Claudio *et al*. used lavender and cinnamon oil, and we used strawberry and lemon oil as CS. Second possible reason is that we used new colonies of *A. m. syriaca* in our PER conditioning assay. Thus, both genetic or experimental procedure differences may cause the inconsistency of the results among the researches.

CHAPTER 5

CONCLUSION

To conclude, we found differences in learning performance and related behaviors across three honey bee subspecies from Turkey.

First, we conducted an aversive learning study, the ESA conditioning assay. According to the results of this assay, the Caucasian honey bee has a higher discriminant learning performance than the Syrian and the Carniolan honey bees.

Second, we used an appetitive learning study, the PER conditioning assay, to compare discriminant learning performance of the Caucasian honey bee, the Syrian honey bee, and the Carniolan honey bee. According to the results of this assay, the Syrian honey bee has impairment in discrimination. Meanwhile, the Caucasian and Carniolan honey bees have similar success.

Third, we also determined differences in daily activity across subspecies. Thus, subspecies differ from each other with their daily activity patterns, such that the Caucasian honey bee has significantly higher daily activity than the Carniolan honey bee.

Fourth, the Carniolan honey bee appears to be the most resistant and the Caucasian honey bee appears to be the most susceptible subspecies to starvation across the three subspecies.

The results of our study thus provide insight into how learning and behavior-related diversity may have evolved in the history of honey bee subspecies and set a landmark for further studies on this issue.

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APPENDICES

Appendix A: R Codes

Table A.1 R code of multiple linear regression model, repeated measure ANOVA test, Tukey test, and Shapiro-Wilk's test for normality.

```
## time data were determined as integer ##

myData <- within(ESA, {
individuals <- factor(indv)
groups <- factor(GrNo)
time <- as.integer(min)})

## ordering the data ##

myData <- myData[order(myData$individuals), ]


## multiple linear regression model ##

dur.model <- lme(dur~time*groups, data=myData,
random = ~ 1|individuals/(groups))
anova(dur.model)

## repeated measured ANOVA test also gave the same F and p
values of multiple linear regression model ##

dur.aov <- with(myData,
aov(dur~time*groups+Error(individuals/(groups))))
summary(dur.aov)

## Post hoc Tukey test ##

summary(glht(dur.model, linfct = mcp(groups = "Tukey")), test =
adjusted("holm"))

#Shapiro-Wilk's test for normality ##

shapiro.test(residuals(dur.model))
```

Table A.2 R code for permutation test to check the validity of multiple linear regression model and repeated measures ANOVA test.

```
## time data were determined as integer ##

myData <- within(ESA, {
individuals <- factor(indv)
groups <- factor(GrNo)
time <- as.integer(min)})

## package used for permutation test on multiple linear
regression model ##

install.packages("pgirmess")
library(pgirmess)

## multiple linear regression model ##

dur.model <- lme(dur~time*groups, data=myData,
random = ~ 1|individuals/(groups))

## permutation test on multiple linear regression model ##

PermTest(dur.model,B=1000)

## package used for permutation test on repeated measured ANOVA
##

install.packages("permuco")
library(permuco)

## permutation test on repeated measured ANOVA ##

aovperm(dur~time*groups+Error(individuals/groups), data =
myData, np = 1000, method = NULL)
```

We used the same R codes of repeated measures ANOVA test and permutation test by modifying for other comparison in ESA conditioning study.

Appendix B: Statistical Tables of Results Chapter

Table A.3 Repeated measures ANOVA table of comparison of the honey bee subspecies by time spent on the shock side.

```
Error: individuals
Df Sum Sq Mean Sq F value  Pr(>F)
groups      2   1758   878.8   7.006 0.00138 **
Residuals 107  13421   125.4
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Error: Within
Df Sum Sq Mean Sq F value Pr(>F)
time          1   2390  2389.7  99.911 <2e-16 ***
time:groups   2    110    54.9   2.297  0.102
Residuals   437  10452    23.9
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Table A.4 Repeated measures ANOVA table of comparison of the honey bee subspecies in terms of number of the crossing border between the shock side and safe side.

```
Error: individuals
           Df Sum Sq Mean Sq F value   Pr(>F)
groups      2  596.6  298.32    11.9 2.16e-05 ***
Residuals 107 2683.1   25.08
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Error: Within
             Df Sum Sq Mean Sq F value  Pr(>F)
time          1 1508.1  1508.1 254.838 < 2e-16 ***
time:groups   2   66.1    33.1   5.585 0.00403 **
Residuals   437 2586.2     5.9
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Table A.5 Repeated measures ANOVA table of comparison of the honey bee subspecies by time spent on the blue side.

```
Error: individuals
          Df Sum Sq Mean Sq F value Pr(>F)
groups     2    406   203.2   1.812  0.169
Residuals 93  10432   112.2

Error: Within
             Df Sum Sq Mean Sq F value Pr(>F)
time          1      1    0.90   0.015  0.902
time:groups   2    108   53.87   0.901  0.407
Residuals   381  22768   59.76
```

Table A.6 Repeated measures ANOVA table of comparison of the honey bee subspecies in terms of number of the crossing border between the shock side and safe side.

```
Error: individuals
          Df Sum Sq Mean Sq F value Pr(>F)
groups     2   74.4   37.22   1.288  0.281
Residuals 93 2687.9   28.90

Error: Within
             Df Sum Sq Mean Sq F value   Pr(>F)
time          1  138.0  138.02   34.65 8.65e-09 ***
time:groups   2    0.6    0.28    0.07    0.932
Residuals   381 1517.4    3.98
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Table A.7 Means, standard deviations and standard errors of the results in ESA conditioning assay.

Treatment																		
	A. m. caucasica						*A. m. carnica*						*A. m. syriaca*					
	Counts of the Crossing the Border			Shock duration (sec)			Counts of the Crossing the Border			Shock duration (sec)			Counts of the Crossing the Border			Shock duration (sec)		
Minute	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err
1	5.25	2.37	0.42	5.92	3.75	0.66	6.54	3.35	0.54	10.98	8.12	1.30	9.31	3.71	0.59	12.12	6.52	1.04
2	2.00	1.97	0.35	3.33	4.30	0.76	4.74	3.88	0.62	9.20	9.02	1.44	4.59	3.53	0.57	7.79	8.76	1.40
3	1.50	1.78	0.31	3.34	5.12	0.90	3.00	3.63	0.58	5.58	6.48	1.04	4.54	3.66	0.59	6.95	7.60	1.22
4	1.50	1.97	0.35	2.84	4.82	0.85	2.92	3.06	0.49	4.88	5.81	0.93	3.03	3.12	0.50	5.35	6.46	1.04
5	0.88	1.81	0.32	1.26	3.55	0.63	2.21	2.53	0.40	4.68	6.24	1.00	2.62	2.72	0.44	5.04	7.74	1.24
Control																		
	A. m. caucasica						*A. m. carnica*						*A. m. syriaca*					
	Counts of the Crossing the Border			Duration in blue area (sec)			Counts of the Crossing the Border			Duration in blue area (sec)			Counts of the Crossing the Border			Duration in blue area (sec)		
Minute	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err	Mean	St.Dev	St.Err
1	10.34	2.65	0.47	28.92	6.61	1.17	10.66	3.30	0.54	30.97	3.81	0.62	9.58	2.97	0.55	33.07	7.49	1.39
2	9.16	2.70	0.48	28.70	7.80	1.38	10.45	2.93	0.48	30.44	6.69	1.09	9.96	2.75	0.51	34.11	11.21	2.08
3	9.38	2.51	0.44	31.55	9.39	1.66	10.21	3.42	0.55	30.97	8.83	1.43	9.46	3.24	0.60	30.81	11.51	2.14
4	9.50	2.23	0.39	30.87	7.27	1.28	10.08	3.16	0.51	29.53	7.63	1.24	9.46	3.17	0.59	30.90	11.54	2.14
5	8.13	2.30	0.41	29.02	7.93	1.40	9.08	3.66	0.59	32.60	8.74	1.42	7.92	3.14	0.58	32.08	8.69	1.61

Table A.8 One-way ANOVA table of comparison of the honey bee subspecies using with square root of activity counts between 16:00 and 19:00.

ANOVA

SRActESA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2150.050	2	1075.025	9.538	.000
Within Groups	10031.077	89	112.709		
Total	12181.127	91			

Table A.9 One-way ANOVA table of comparison of the honey bee subspecies using with daily locomotor activity counts.

ANOVA

Activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23136305.060	2	11568152.530	5.716	.005
Within Groups	180106917.800	89	2023673.233		
Total	203243222.800	91			

Table A.10 Values of box-and-whisker plots in the PER conditioning assay results.

			Mean	St. Dev.	Min. Value	1st Quartile	Median	3rd Quartile	Max. Value
Before Data Filtering	CS+	*A. m. caucasica*	0.70	0.14	0.5	0.67	0.67	0.83	0.83
		A. m. carnica	0.68	0.13	0.5	0.58	0.67	0.83	1
		A. m. syriaca	0.71	0.21	0.5	0.67	0.83	0.83	1
	CS-	*A. m. caucasica*	0.2	0	0	0	0	0.33	0.67
		A. m. carnica	0.26	0.35	0	0	0	0.5	1
		A. m. syriaca	0.45	0.39	0	0.16	0.33	0.96	1
After Data Filtering	CS+	*A. m. caucasica*	0.69	0.15	0.33	0.58	0.67	0.83	0.83
		A. m. carnica	0.67	0.14	0.5	0.5	0.67	0.75	1
		A. m. syriaca	0.75	0.16	0.33	0.5	0.75	0.83	0.83
	CS-	*A. m. caucasica*	0.11	0.2	0	0	0	0.17	0.17
		A. m. carnica	0.12	0.21	0	0	0	0.17	0.33
		A. m. syriaca	0.26	0.24	0	0	0.17	0.38	0.83

Appendix C: Reversal Phase of PER Conditioning Assay

In acquisition phase, initial discrimination training is realized as defined in thesis. After 5 minutes from acquisition phase, we immediately began to reversal phase using with same individuals of honey bees. In reversal phase, the odor used as CS- shifted to CS+ and *vice versa* for odor used as CS+ in acquisition phase. The same pseudorandomized trial sequence used in acquisition phase also used in reversal phase, which was CS+, CS-, CS-, CS+, CS-, CS+, CS+, CS-, CS+, CS-, CS-, CS+.

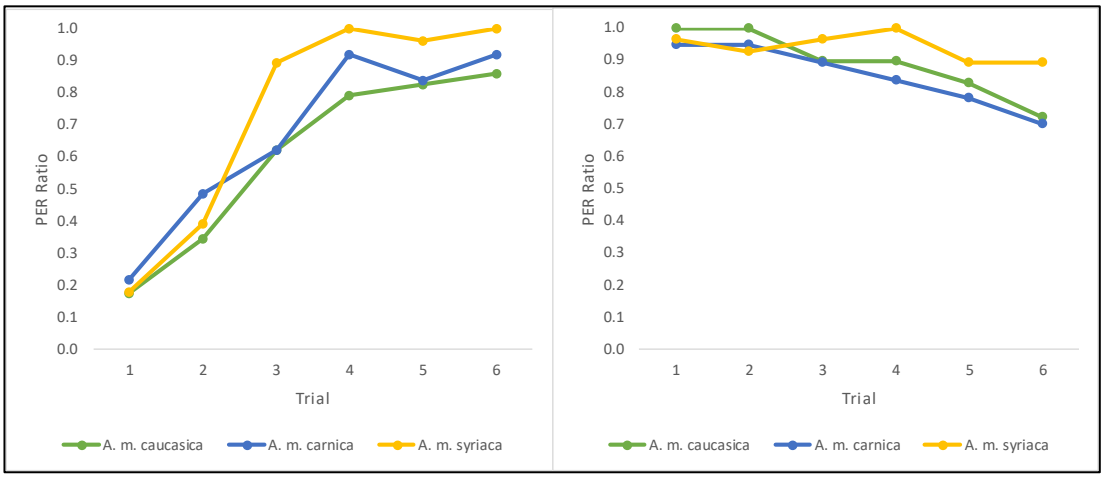

Figure A.1 Ratio of PER to CS+ (left), and CS- (right) along trials in reversal phase.

Sample sizes were n = 29, 37, and 28 for Caucasian, Carniolan and Syrian honey bees, respectively. We used the PER ratio score of each individual to compare subspecies as same as acquisition phase. Because of distributions were not normal according to a Kolmogorov-Smirnov test (for CS+, *A. m. caucasica*, D (29) = 0.216, $p < 0.01$, *A. m. carnica*, D (37) = 0.191, $p < 0.01$, and *A. m. syriaca*, D (28) = 0.242, $p < 0.001$, and for CS-, *A. m. caucasica*, D (29) = 0.383, $p < 0.001$, *A. m. carnica*, D (37) = 0.392, $p < 0.001$, and *A. m. syriaca*, D (28) = 0.481, $p < 0.001$), we used Kruskal-Wallis test for comparison. There was no significant result found for both the CS+ ($p > 0.05$), and for CS- ($p > 0.05$).

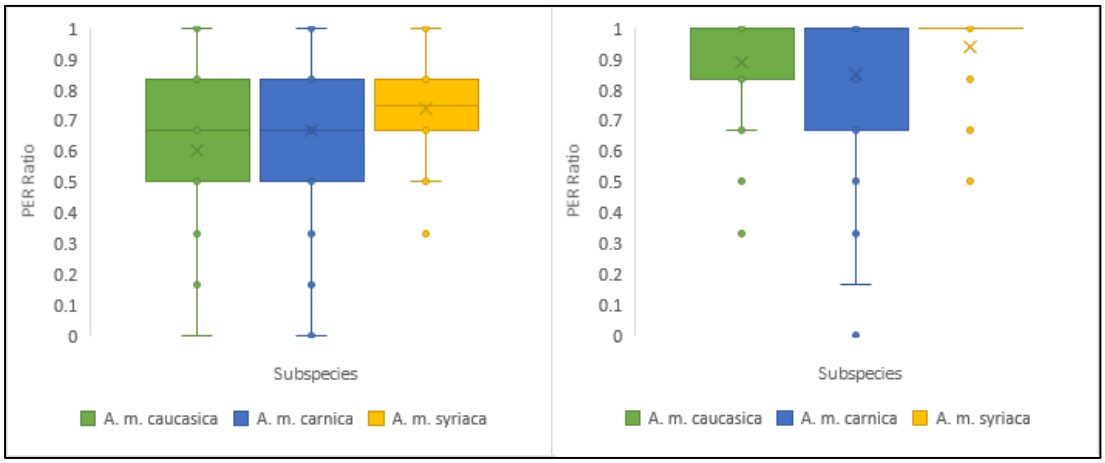

Figure A.2 Box-whisker plot for comparison of subspecies` PER ratios to CS+ (left), and CS- (right) in reversal phase.

Table A.11 Values of box-and-whisker plots in the reversal phase of the PER conditioning assay results.

			Mean	Min. Value	1st Quartile	Median	3rd Quartile	Max. Value
Reversal Phase	CS+	*A. m. caucasica*	0.60	0	0.5	0.67	0.83	1
		A. m. carnica	0.67	0	0.5	0.87	0.83	1
		A. m. syriaca	0.74	0.5	0.67	0.75	0.83	1
	CS-	*A. m. caucasica*	0.89	0.67	0.83	1	1	1
		A. m. carnica	0.85	0.17	0.67	1	1	1
		A. m. syriaca	0.94	1	1	1	1	1

Appendix D: Figures of Conclusion Chapter

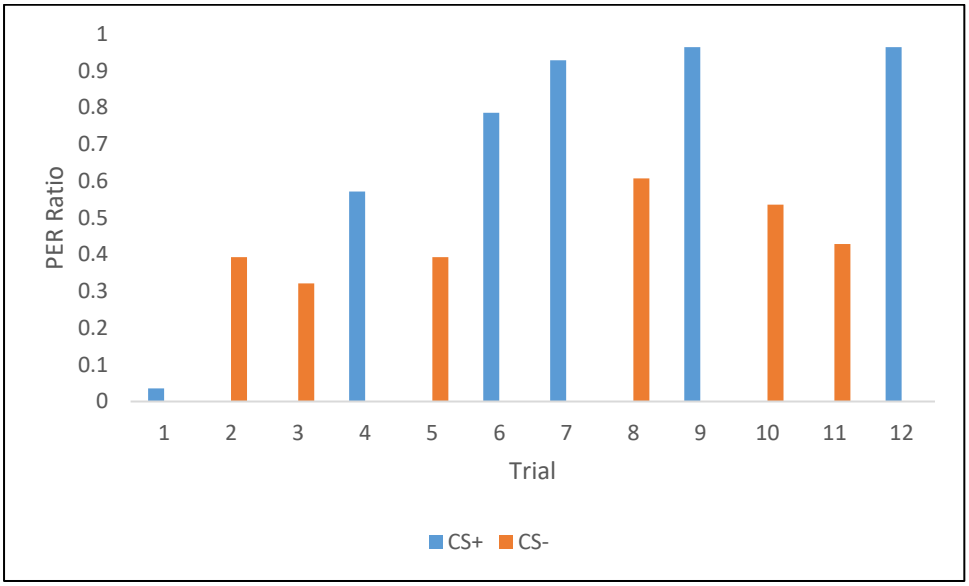

Figure A.3 PER ratio of *A. m. syriaca* in discriminant learning.

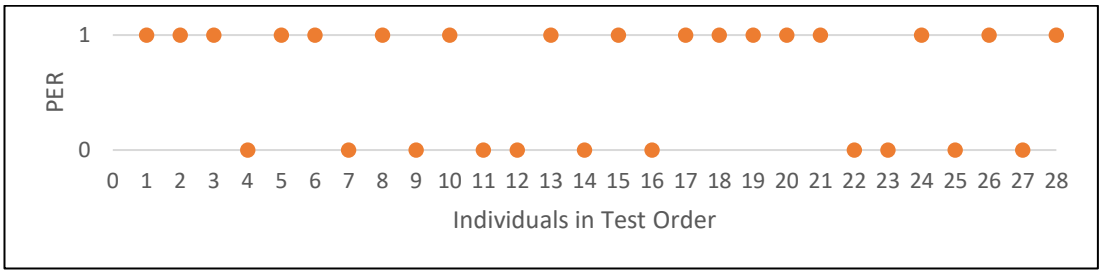

Figure A.4 PER of *A. m. syriaca* individuals at fourth trial of CS-. Individuals are aligned in test order.