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Original article (Orijinal araştırma)

Quantitation of neuroxin-1, ataxin-3 and atlastin genes related to grooming behavior in five races of honey bee, *Apis mellifera* L., 1758 (Hymenoptera: Apidae), in Turkey[1]

Türkiye'deki beş bal arısı, *Apis mellifera* L., 1758 (Hymenoptera: Apidae) ırkında tımar davranışı ile ilgili neuroxin-1, ataxin-3 ve atlastin genlerinin kantitasyonu

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Abstract

Although many methods have been used to control *Varroa destructor* Anderson & Trueman, 2000 (Acari: Varroidae), the satisfactory results have not yet been achieved. However, research has shown that some colonies of honey bee, *Apis mellifera* L., 1758 (Hymenoptera: Apidae), exhibit higher resistance or sensitivity to Varroa mites than others. One of the resistance mechanisms based on genetics is grooming behavior and it has been promising for beekeeping. The fact that there are many unanswered questions about grooming behavior led to the idea of this study. Worker bees from five honey bee races in Turkey were individually tested for their grooming behavior in response to *V. destructor* mite infestation. The quantitation of the expression levels of three candidate genes (neurexin-1, ataxin-3 and atlastin) in each honey bee race with and without grooming behavior was evaluated by quantitative polymerase chain reaction. Although expression levels of neurexin-1, ataxin-3 and atlastin genes showed significant differences among individuals, grooming levels of individuals were not related to the expression levels of these genes except in Syrian honeybees. Also, phenotypically no statistical differences were found among the honey bee races in terms of grooming behavior. The results show that grooming behavior may not be associated with neural gene expression alone. However, it is seen that more molecular studies related to grooming behavior are needed.

Keywords: *Apis mellifera,* candidate gene, grooming behavior, quantitation, *Varroa destructor*

Öz

Varroa destructor Anderson & Trueman, 2000 (Acari: Varroidae) kontrolünde birçok yöntem kullanılmış olmasına rağmen, tatmin edici sonuçlar henüz elde edilememiştir. Araştırmalar, bazı bal arısı, *Apis mellifera* L., 1758 (Hymenoptera: Apidae) kolonilerinin Varroa akarlarına diğerlerinden daha yüksek direnç veya duyarlılık sergilediğini göstermiştir. Genetik temele dayanan direnç mekanizmalarından birisi de tımar davranışıdır ve arıcılık için umut vericidir. Tımar davranışı ile ilgili cevaplanmamış birçok sorunun olması bu çalışmanın ortaya çıkmasına neden olmuştur. Türkiye'deki beş bal arısı ırkından işçi arıların *V. destructor* istilasına tepki olarak tımar davranışları test edilmiştir. Tımar davranışı gösteren ve göstermeyen her bir bal arısı ırkında üç aday genin (neuroxin-1, ataxin-3 ve atlastin) ekspresyon seviyelerinin kantitasyonu kantitatif polimeraz zincir reaksiyonu yöntemi ile değerlendirilmiştir. Neuroxin-1, ataxin-3 ve atlastin genlerinin ekspresyon seviyeleri önemli farklılıklar gösterse de Suriye ırkı haricinde tımar davranışı bu genlerin ekspresyon seviyeleri ile ilişkili bulunmamıştır. Ayrıca fenotipik olarak bal arısı ırkları arasında tımar davranışı açısından istatistiksel farklılık bulunmamıştır. Elde edilen sonuçlar, tımar davranışının, tek başına nöral gen ifadeleriyle ilişkili olmayabileceğini göstermektedir. Bununla birlikte tımar davranışı ile ilgili daha fazla moleküler çalışmaya ihtiyaç duyulduğu görülmektedir.

Anahtar sözcükler: *Apis mellifera*, aday gen, tımar davranışı, kantitasyon, *Varroa destructor*

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Introduction

The European honey bee, *Apis mellifera* L., 1758 (Hymenoptera: Apidae) has an important role in honey production and pollination of plants. Close to 84% of agricultural crop species depend on pollinators, especially bees (Gallai et al., 2009). However, colony losses in honey bees have been a major problem since the beginning of the 1800s, when modern beekeeping began. Although a number of factors have been shown to cause colony losses, research indicates that the main cause of the colony losses is *Varroa destructor* Anderson & Trueman, 2000 (Acari: Varroidae) (Eliash et al., 2017). *Varroa destructor* is an obligate ectoparasite primarily feeding on honey bee fat body tissue but also the hemolymph (Oldroyd, 1999; Ramsey et al., 2019). If effective control is not used, a Varroa-infested colony collapses within 2-3 years (Boecking & Genersch, 2008; Rosenkranz et al., 2010).

Honey bee colonies have natural resistance mechanisms for various diseases and parasites, and some colonies may show higher resistance or sensitivity than others. Resistance mechanisms work through physical, behavioral and immune systems due to genetic diversity of honey bee races. Some European honey bee populations have been reported to exhibit one or more behaviors for counteracting diseases and parasites through some physiological properties they have gained through natural selection, and survive without any problem (Fries et al., 2006; Le Conte et al., 2007; Seeley, 2007). For these reasons, in Varroa mite control focused on the determination of resistant races and genotypes by seeking alternative methods such as bee breeding in terms of behavioral resistance (Rosenkranz et al., 2010). One of the most important defensive behaviors against ectoparasites in honey bees is grooming behavior (Aumeier, 2001). This behavior in bees has been developed to protect the health of individual workers and the entire colony (De Figueiró Santos et al., 2016). Grooming behavior in adult bees involves detecting and eliminating mites from their own bodies (autogrooming) or from other bees (allogrooming) (De Figueiró Santos et al., 2016). Removal of mites from other bees is also known as social grooming behavior (Peng, 1988). When bees self-clean quickly with their legs, their body waggles and bends. So, this behavior is also called a grooming dance (Milum, 1947). This stimulates social grooming behavior in temporarily specialized groomer bees, and often clean several other bees in a row (Kolmes, 1989).

Various studies have been conducted on several candidates including genes coding for atlastin, ataxin-3, neurexin-1 (AmNrx1), poly U binding factor kd 68, vitellogenin, autophagy linked FYVE protein, blue cheese (BlCh) and immune-related hymenoptaecin, which have potential neurodevelopmental and behavioral effects, and thought to be related to the grooming behavior of honey bees. Generally, expression levels of genes have been investigated in Varroa mite-treated bees. Navajas et al. (2008), investigated the susceptibility of *A. mellifera* to Varroa mite parasitism and whether mite invasion caused changes in gene expression. As a result of such studies, it was found that most of the genes expressed differently between tolerant and sensitive bees are important in the development of the nervous system. Arechavaleta-Velasco et al. (2012) identified a region on chromosome 5 for honey bee grooming behavior using a QTL mapping approach. This region contained 27 genes, including neuroxin-1, ataxin-3 and atlastin which have potential neurodevelopmental and behavioral effects. Hamiduzzaman et al. (2017) investigated associations between grooming behavior and the expressions of immune, neural, detoxification, developmental and health-related genes. AmNrx-1 expression was found to be significantly higher in bees showing intense grooming behavior. As a result, neurexin-1 has been reported to be useful as a biomarker for behavioral characteristics in bees. Morfin et al. (2020), reported that the rate of mites damaged by mite-biter bees, the severity of the mutilation and winter colony survival was higher in selected Indiana mite-biter honey bees colonies than the unselected Italian honey bees colonies. In addition, the expression of the AmNrx-1 gene associated with grooming behavior was significantly higher in Indiana mite-biter bees. Although the mechanism of grooming behavior has not been fully resolved, it is clear that grooming behavior is negatively correlated with the number of mites in the colonies. It is also known that this behavior varies among honey bee species

and races (Delfinado-Baker et al., 1992; Boecking & Ritter, 1993; Büchler, 1993; Arechavaleta-Velasco & Guzman-Novoa, 2001; Mondragon et al., 2005; Andino & Hunt, 2011; Guzman-Novoa et al., 2012).

This study investigated whether changes in the expression of three candidate genes (neuroxin-1, ataxin-3 and atlastin) were associated with grooming behavior according to grooming in five honey bee races (*Apis mellifera anatoliaca* Maa, 1953, *Apis mellifera caucasica* Gorbachev, 1916, *Apis mellifera carnica* Pollmann, 1879, *Apis mellifera ligustica* Spinola, 1806 and *Apis mellifera syriaca* Skorikov, 1929) in Turkey.

Materials and Methods

Collection of honey bee specimens

This study was conducted in the Akdeniz University Animal Biotechnology Laboratory in 2019. A total of 100 similar physiological age worker bees from the five honey bee races including Anatolian Bee (*A. mellifera anatoliaca*) from Muğla Province, Caucasian Bee (*A. mellifera caucasica*) from Artvin Province, Syrian Bee (*A. mellifera syriaca*) from Hatay Province, Carniolan Bee (*A. mellifera carnica*) from Kırklareli Province and Italian Bee (*A. mellifera ligustica*) from Hatay Province used in Turkey were purchased from beekeepers. Commercially obtained bees were transferred on the same day in five feeding boxes to a laboratory at 30°C. In the boxes, sugar syrup containing 10 ml of 50% sucrose was present. Bees that spent one night in this manner were allowed to de-stress until the next day's grooming test. Considering the phenotypic images and beekeeper's advice, care was taken to ensure that the selected bee samples represent their races in the best possible way.

Collection of Varroa mite samples

Varroa mite samples were obtained from honey bee colonies in Antalya Province. The honeycombs taken from these colonies were brought to the laboratory and mites were removed from the capped drone cells with the help of sterile tweezers. Mites were fed with white-eye phase drone pupae in Petri dishes until used in grooming tests. The mites were stored in Petri dishes at 28°C and 75% RH in an incubator (Huang et al., 2017).

Testing and analysis of grooming behavior

Grooming behavior tests were performed in individual 6-cm Petri dishes of 20 bees for each race. Petri dishes were humidified with moist pieces of paper, and the temperature was maintained at 30°C. Given that the honey bees had been removed from their hives, the tests were performed with minimal delay. In order to prevent stress-induced problems in bees, these tests were completed on the same day for all bees. In order to achieve this, two video camera systems were set above the Petri dishes used in the tests and grooming behavior of bees was recorded. Individually grooming behavior test were a modified version of the method described by Aumeier (2000). When testing grooming behavior, one worker bee from each race was placed in the Petri dish at a time. Then one Varroa mite was put onto its thorax using insect brush. The bees with Varroa mite were monitored for 3 min then each bee immediately stored at -80°C by in sterile 1.5 ml tubes containing RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) solution. A total of 100 worker bees, 20 from each race, were tested for their grooming behavior. After completion of the grooming tests, the video recordings were transferred to the computer and examined to determine whether each bee showed grooming behavior. Also, grooming time for each bee was recorded and individuals who attempted rid themselves of the Varroa mite during the 3 min period were considered as grooming otherwise they were coded as no-grooming.

Total RNA extraction and cDNA synthesis

Thorax of each worker bee tested for grooming behavior was crushed with liquid nitrogen and RNAs were extracted using the Norgen Total RNA Extraction Kit (Norgen Biotek Corp., Thorold, ON, Canada). The quality of the extracted RNAs were determined by Biodrop device. The RNA samples were stored at −80°C. Five µg of extracted total RNAs were converted to cDNA by reverse transcription with EvoScript cDNA Synthesis Kit (Roche, Basel, Switzerland) in Thermal Cycler for use in qPCR.

Primers

A primer sets was used to amplify each candidate genes and β-actin used as a housekeeping gene are given in Table 1. Sequences of the genes required for the primary design were obtained from the National Biotechnology Information Center.

Table 1. The primer sets used to amplified candidate genes to grooming behavior in honey bees and β-actin

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	NCBI ID
Neurexin-1	tctgcacataaagcctgttc	actccattttcacccctc	LOC724217
Ataxin-3	tgcacttttacaaggtccg	ccccaaacttttaatgcactac	LOC410162
Atlastin	ggcatacattagatacagcgg	gggacacaaagggaatgaac	LOC550886
β-actin	gacgaagcccaatcaaagag	ggcgacatacatagcaggag	AB023025

qPCR amplifications

qPCR amplifications were performed using the LightCycler 96 Real-Time PCR instrument with SYBR Green fluorescent dye method. Each qPCR reaction in a 96-well plate contained 1.5 µL H_2O (PCR-grade), 5 µL of SYBR Green Master (Roche LightCycler 480 SYBR Green-I Master), 0.5 µL (for forward and reverse total 1 µL) of each gene-specific primer, and 2.5 µL of DNA in a final volume of 10 µL. Gene-specific primers (Table 1) were designed using Primer3Plus and Primer-BLAST programs.

Statistical analysis

The expression level of candidate genes was calculated using $2^{-\Delta\Delta}$ method (Livak & Schmittgen, 2001). A chi-square test was used to determine whether the number of individuals grooming behavior varied depending between races and whether there was a relationship between races in terms of $2^{-\Delta\Delta}$ values. Kolmogorov-Smirnov normal distribution test was performed on all data before calculating gene expression. Log10 transformation was applied for non-normal data. Box plots was used to visually summarize gene expression data. Differences of the gene expression between the races were compared with Kruskal-Wallis test. The differences of expression levels between individuals with and without grooming behavior were analyzed with the Mann-Whitney U test.

Results

The division of the honey bees into grooming and no-grooming behavior is given in Table 2. The number of individuals grooming was the highest in the Italian race and the least in the Caucasian race. The total number of individuals grooming was slightly less than those not grooming. The movement of mites placed in the thorax of bees towards other body regions also affected the grooming behavior of the bees. The presence of mite in the head region of the bee triggered the grooming behavior more whereas the grooming behavior in the abdomen was less. Grooming behavior was not observed when mites were moved to the propodium. The chi-square test indicated that the was no significant differences between the races in grooming behavior ($p = 0.07$).

Table 2. Classification of grooming behavior success of five honey bee races from Turkey

	Honey bee Races					
	A. mellifera anatoliaca	*A. mellifera caucasica*	*A. mellifera carnica*	*A. mellifera ligustica*	*A. mellifera syriaca*	Total
NG	9	15	13	7	9	53
G	11	5	7	13	11	47
Total	20	20	20	20	20	100

Expressions of neurexin-1, ataxin-3 and atlastin genes in the honey bee races are shown as box plots in Figure 1. The figure is analyzed it is understood that the expression levels of genes showed almost the same pattern for all honey bee races. Interestingly, Carniolan honey bees had the highest values in terms of expression levels for all genes. However, Caucasian bees had the lowest expression of the neurexin-1 gene, Anatolian and Italian bees had the lowest expression for the ataxin-3 gene, and Anatolian bees had the lowest expression of the atlastin gene.

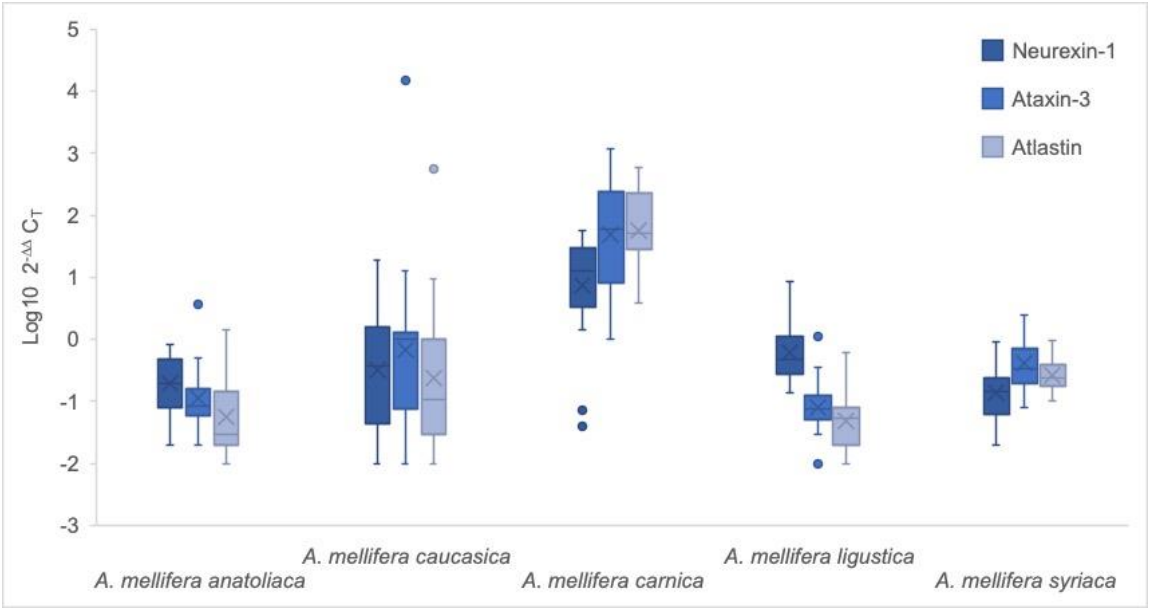

Figure 1. Box plots of Log10 transformed mRNA expression rates of neurexin, ataxin, atlastin in honey bee races.

The expression of neurexin-1, ataxin-3 and atlastin genes had significant differences between bee races ($p < 0.01$). There were also differences in the expression of these genes between individuals within a race. However, based on the Mann-Whitney U test, there were no differences between the individuals with and without grooming behavior in of expression of the genes except for the Syrian race ($p < 0.05$ and $p < 0.01$, respectively).

Discussion

Varroa mites continue to directly and indirectly threaten the health of honey bees. These harmful effects on the health of worker bees and all colony have led to the development of behavioral resistance, including grooming behavior. Although differences in grooming behavior among honey bee genotypes have been demonstrated (Moretto et al., 1993; Guzman-Novoa et al., 2012; Rinderer et al., 2013), the molecular mechanisms underlying grooming behavior are still not fully understood. However, the results obtained in gene expression studies related to neural genes are quite remarkable. On this basis, the aim of this study was to determine whether the expression levels of three candidate genes (neurexin-1, ataxin-3 and

atlastin), which are thought to be related to grooming behavior, change according to five bee races (*A. mellifera anatoliaca, A. mellifera caucasica, A. mellifera carnica, A. mellifera ligustica* and *A. mellifera syriaca*) and grooming behavior.

The individual grooming test described by Aumeier (2001) has been demonstrated to be effective in determining of grooming behavior in different honey bee genotypes. Aumeier (2001) reported that 66% of artificially infested Carniolan bees react to the presence of mites on their bodies within the first 30 s. However, in our study, this was observed in the Carniolan race. Compared to the other bee races, the Carniolan race was the third race in grooming response. In another study, mites that had fallen onto the bottom-board traps from naturally infested colonies were collected, to quantify the active grooming reactions by the Syrian honey bees towards the mite under natural conditions. A total of 22.8% of all dropping mites had body injuries and reported that workers of *A. mellifera syriaca* have an extraordinary potential to actively react to ectoparasitic Varroa mites (Zaitoun et al., 2001). Likewise, our grooming test results showed that the Syrian honey bees were had a 55% grooming rate. The Syrian race, which was noted for its aggressiveness during the tests, is the second most responsive race in terms of grooming behavior and there is little difference between first race, Italian honey bees. Bak & Wilde (2015) reported that *A. mellifera caucasica*, among the five artificially infested honey bee groups (*A. mellifera caucasica* Woźnica line, *A. mellifera mellifera* Augustowska line, *A. mellifera carnica* represented by two lines: Kortówka and Dobra bees, crossbreed of two subspecies: *A. mellifera capensis* × *A. mellifera carnica*) was the second most responsive race in grooming behavior. Also, they reported 86% of Caucasian bees tried to get rid of mites. In contrast to these results, the Caucasian bee known for its calmness was the the least responsive race in our study, and only 25% of the bees showed grooming behavior. In this regard, even in a small area of Turkey genetically diverse honey bee races can be found (Kandemir et al., 2000; Bodur et al., 2007; Solorzano et al., 2009; Kence et al., 2013). Consistently, the high variation in gene expression of Caucasian bees is an indicator of this diversity (Figure 1). Grooming behavior in response to Varroa mites is also associated with injured mites falling from bees in colonies (Arechavaleta-Velasco & Guzman-Novoa, 2001) and the rate of damaged mites per hive varies according to the honey bee race. Van Alphen & Fernhout (2020) reported Italian race had an average of about 6% mite damage. In contrast, Rosenkranz et al. (1997) recorded an average of 45% mite damage in Italian and Carniolan bees, while Africanized *A. mellifera* damaged 39%. In our grooming behavior test, the Italian bees were the most responsive with 65% grooming behavior. When looking at grooming behavior responses, it is not possible to drawn strong conclusions about the races differences. The fact that the results obtained in the tests are variable suggests that this behavior may be affected by the testing method and the parameters of the test conditions. Bak & Wilde (2015) reported that mites were successfully removed when located in the head, legs and distal region of abdomen. They reported none of the bees removed mites when these were on the propodium. Vandame et al. (2002) reported similar results in their studies. Therefore, grooming behavior is also influenced by the location of mites on the bee.

It should be noted that it is difficult to assess the intensity and effectiveness of grooming behavior against Varroa mites (Aumeier, 2001). The preparation of studies in full-size colonies or observation hives (Peng et al., 1987; Moretto et al., 1993, 1997; Bozic & Valentincic, 1995; Fries et al., 1996) is time-consuming, and even then, the continuous recording of the behavior of a particular bee is not guaranteed (Peng et al., 1987; Büchler et al., 1992; Thakur et al., 1997).

Genes expression was the highest in the Carniolan race (Figure 1), however that were relatively low proportion of grooming behavior (Table 2). Caucasian bees failed in terms of both grooming behavior and gene expressions compared to other races. Italian bees, the most responsive race in grooming behavior, did not show the same response in terms of gene expression. The expression profiles of the Syrian and Anatolian bee, which had the same proportion of grooming behavior, has similar expression of the three genes. When the bees with and without grooming behavior were compare in for neurexin-1, ataxin-3 and

atlastin, the expression of these genes was higher in grooming individuals only in the Syrian race. Although there are not many studies, intensive grooming behavior in general has been associated with high neural gene expression. Hamiduzzaman et al. (2017) determined that honey bees that had intense grooming behavior had greater neurexin-1 gene expression. They also reported that intense physical activity during grooming may be related to the nervous system induced byproducts of some neural genes and may cause these genes to suppress some other genes. Morfin et al. (2020) reported in their study that the expression of neurexin-1 was positively correlated the rate of mites injured in bred bees exhibiting of grooming behavior, but did not correlate with the growth of the mite population. Varroa mite-tolerant bees are mainly characterized by differences in the expression of genes regulating neural development, neural sensitivity and olfactory perception, although Navajas et al. (2008) reported that the expression of BlCh (an autophagy-dependent gene) was higher in Varroa mite-tolerated bees, and that the expression of the Dlic2 and Atg18 genes affecting neural reactions decreased. In contrast, the Strn-Mlck neural gene expression decreased in both Varroa mite-tolerant and intolerant bees. According to these studies and our findings, it seems that neural gene expressions may not always be related to grooming behavior. Also, genes selected as candidate genes may not always give consistent results (Navajas et al., 2008).

The present study is one of a few studies using phenotypic methods to address this issue. Also, there appears to have been no earlier studies of the grooming behavior of honey bee races and the comparison of candidate genes that may be responsible for this behavior. However, there is a need for further studies on neurexin-1, ataxin-3 and atlastin genes, which are reported to be candidate genes for grooming behavior in honey bees, and their relationship to grooming behavior. In addition to these candidate genes, other genes that may be involved should be investigated.

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