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Effects of uncontrolled queen importation and migratory beekeeping on the racial purity and spermatological parameters of honey bee (*Apis mellifera anatoliaca*) population in Central Anatolia

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Abstract

Widespread immigrant beekeeping activity, inadequate quality queen bee production and the excess of uncontrolled crossbreeding are great problems for the quality of honey bee races in Türkiye. However, the effects of uncontrolled crossbreeding on the spermatological parameters are not fully known. In this study, samples were analyzed in terms of morphometric parameters such as cubital index, hantel index and discoidal shift. Drones belonging to the same colonies were investigated in terms of spermatological parameters such as total motility, Plasma Membrane Integrity (PMI), Mitochondrial Membrane Potential (MMP) and spermatozoa concentration (SCON). The wing index values of the samples were similar to those of some races. Racial similarities rates were 50% Caucasian (*A. m. caucacia*), 49% Anatolian (*A. m. anatoliaca*), 24% Brown (*A. m. mellifera*), 13% Italian (*A. m. ligustica*) and 8% Carnolian (*A. m. carnica*). These results showed that the expected Anatolian had changed greatly race in the region and there is a danger of crossbreeding of bee races (*Apis mellifera anatoliaca*) in Central Anatolia Region. The averages of spermatological parameters were 85% motility, 82% PMI, 78% MMP and 5.9 $\times 10^9$/ml sperm concentration. In addition, no significant correlative relationship was found between morphometric and spermatological parameters. In this study, the samples of honey bee colonies in the Central Anatolia Region showed random and uncontrolled crossbreeding among different races. The spermatological parameters were found to be sufficient for fertility but no significant relationship was detected statistically between the spermatological and morphometric parameters.

Key words: *Apis mellifera anatoliaca*, Drone, Morphometry, Sperm, Türkiye

Introduction

Honey bees are included in the genus Apis, belonging to the order Hymenoptera and consist of 10 different species. These species are classified as *Apis florea, Apis dorsata, Apis cerana, Apis mellifera, Apis nuluensis, Apis laboriosa, Apis koshevnikovi, Apis nicrocincta, Apis andreniformis and Apis binghami* (Engel,

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1999; Otis, 1996). Ruttner's (2013) study, honey bee samples were taken from different parts of the world, using the morphometric analysis method, and were classified into four main lineages: A (Africa), M (Western Europe), C (Eastern and Southeast Europe) and O (Middle East) major evolutionary lineage.

In his classification of the Anatolian peninsula, Rutter stated that there are Anatolian bees in the Central Anatolia, Aegean, Mediterranean and Black Sea Regions, Iranian bees in the Southeastern Anatolia Region, Caucasian bees in the Northeast Anatolia Region and Carniolan bees in the Thrace Region. Many studies using classical morphometry, geometric morphometry and molecular methods support this grouping (Alattal et al, 2019; Henriques et al, 2020; Modaber et al, 2019). In a study, race differences were observed in some regions and it was concluded that the reason for this was due to commercial queen sales and migratory beekeeping (Kambur and Kekeçoğlu, 2018).

For the morphometric classification of honey bee data, such as body size and shape, wing width and length, leg length, as well as genetic material are used. Honey bees can be classified using only the wing index parameters (cubital index, dumb-bell (hantel) index and discoidal shift values). For example, Romanian honey bees were classified using the wing index values (Cauia et al, 2008). Similarly, the same method was used for the morphometric analysis of bees in the Tomsk region of Russia (Ostroverkhova et al, 2015). In study, samples belonging to the Central Anatolia Region were examined in terms of morphometric parameters and honey bee race similarities were determined.

Spermatological parameters are an important factor for the reproductive ability of the honey bees and survival of the colony. It has been proven that these parameters are affected positively or negatively by many factors such as age of drones (Rhodes et al, 2011), body size (Bratu et al, 2022), genetic factors (Rhodes et al, 2011), temperature (Stoian et al, 2020), nutrition (Zhao et al, 2021), colony management (Ben Abdelkader et al, 2014), seasonal fluctuations (Rhodes et al, 2011), diseases (Collins and Pettis, 2001), insecticides (Ciereszko et al, 2017), miticides (Johnson et al, 2013), semen cryopreservation (Loeza-Concha et al, 2019) and sperm retrieval method (Collins, 2004).

In this study, racial similarities and spermatological parameters of the colonies in the Central Anatolian region were determined. In addition, it was investigated whether these parameters were related to each other.

Material and Methods

Collection of bee samples: All samples were collected from Central Anatolia, Türkiye (39° 50' 23.222" N 33° 30' 31.961" E). The apiaries where the samples were collected consist of centers and districts.

Collection of drones: Three colonies were randomly selected from 15 different apiaries in the Central Anatolia Region. Samples were collected in mid-July, with 50 samples from each hive. Thus, 50 drones were collected from each hive representing a study group. A total of 2250 drones and 45 colonies were used for the study.

Collection of worker bees: In the same way, 50 worker bees were obtained from the same hives from which drones were collected. In total, 2250 worker bee samples were obtained from 45 colonies. Each hive represented a study group.

Morphometric analysis: After the worker bee samples were kept on blotting paper for a while, their right front wings were dissected with the help of forceps and a stereo microscope (Euromex Nexius Zoom/Netherlands). The wings placed on the slide were fixed with the help of tape. Each wing photograph was transferred to the computer environment with the help of a camera adapted to a stereo microscope at the same position and suitable clarity. Forty

wings were used for each colony. Eight landmarks on the wing were marked with the help of the Tps Dig (v. 1.1) program. These values were analyzed as whether or not a relationship with the index values of the Caucasian, Anatolian, Brown, Italian and Carniolan races with the help of the CBeeWing program (American spelling) (Bouga et al, 2011). With the help of the program, the similarity of each colony to the mentioned races was obtained as a percentage (%).

The racial similarity percentages and wing index values (cubital index values, hantel index values and discoidal index values) were also statistically correlated with spermatological parameters (motility, PMB, MMP, SCON) (Table 2, Table 3).

Collection of semen from drones: Artificial insemination device and stereo microscope were used for sperm collection. In the sperm collection process, the semen collection technique suggested by Cobey et al. (Cobey et al, 2013) was used. Averages of 30 µl of semen were obtained from each colony.

Motility: The percentage of sperm motility was determined using a phase-contrast microscope (DM1000, Lecia, Germany) with a heated stage at 33°C. Sperm diluted with Kiev buffer at a ratio of 1:30 (Murray et al, 2022). After**,** 5 µl of diluted semen were determined by examining 40 × objective lens microscope slide and covered with a coverslip. Motile sperm rate was determined as a percentage (Quartuccio et al, 2020).

Assessment of concentration of spermatozoa (SCON): Pure sperm (10 µl) was diluted with Hancock solution in the Eppendorf tube. Three or four times pipetting were applied to homogenize the sperm. Thoma slide and phase-contrast microscope was used for sperm counting (Ben Abdelkader et al, 2021).

Assessment of plasma membrane integrity (PMI): For the assessment of plasma membrane integrity, Hoechst 33342 (Sigma-Aldrich Catalog No: B2261) and PI (Sigma-Aldrich Catalog No: B4170) fluorescent dyes were used. The semen was diluted with Kiev buffer to 100-200 × 10^6 spermatozoon/ml. Firstly, 10 µl Hoechst 33342 (5 µg/ml) was added to 50 µl diluted semen and incubated for ten minutes. After**,** 5 µl PI (10 µg/ml) and Hancock solution (3 µl) were added to the solution before analysis. Afterward, a 5 µl sample was taken from the solution and examined using an inverted microscope with fluorescence attachment (Leica DMI 3000b, Germany) and an "A" filter cube on a 40 × objective lens microscope slide and was then covered with a coverslip. During the evaluation, those with a blue color in the head of the spermatozoa were considered to have intact plasma membrane integrity and those with a red color were considered to have damaged plasma membrane (Figure 1). For each sample, 200 spermatozoa were counted with the help of the Cells Calculator (v. 2.2) program and plasma membrane integrity (%) was determined as a ratio (Morais et al, 2022).

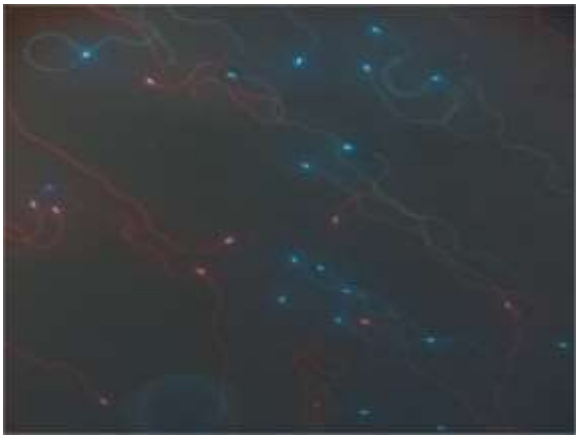

Figure 1. Assessment of plasma membrane integrity (Red: Dead Spermatozoon, Blue: Live Spermatozoon).

Assessment of mitochondrial membrane potential (MMP): For the assessment of mitochondrial membrane potential, the JC-1 fluorescent dye (M34152, Molecular Probes, Eugene, OR, USA) was used. After the semen was diluted with Kiev buffer to be 100-200 × 10^6/ml, 5 µl of JC-1 (2 µM final concentration) was added to 50 µl of

semen and incubated at 33°C for thirty minutes. Hancock solution (3 µl) was added to the solution before the analysis. Then, 5 µl sample was taken from the solution and examined using an inverted microscope with fluorescence attachment and a "I3" filter cube on a 40 × objective lens microscope slide and covered with a coverslip. The orange colored spermatozoa were evaluated as high MMP and the green colored spermatozoa were evaluated as low MMP (Figure 2). In this study, 200 spermatozoa were counted with the Cells Calculator program and determined as the rate (%) regarding high MMP.

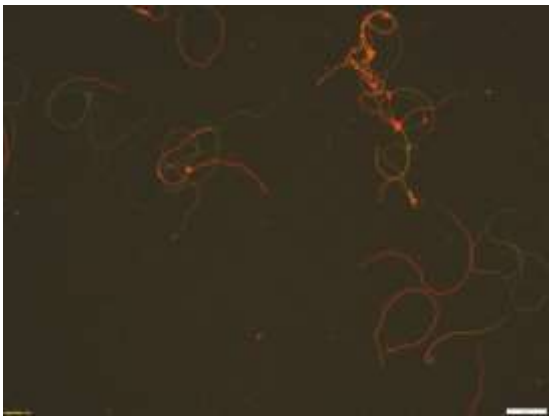

Figure 2. Assessment of mitochondrial membrane potential (Orange: High mitochondrial membrane potential, Green: Low mitochondrial membrane potential).

Statistical analysis: Statistical calculations of the obtained data were made using the SPSS package program (v. 15.6/2007). Differences between spermatological and morphometric parameters were determined by one-way ANOVA. The relationship between the parameters was determined using two-way correlation analysis.

Results

The findings of the study are shown in figures and tables. The minimum, maximum and mean values of spermatological and morphometric value are shown in Figures 3 and 4. According to the morphometric data, both the Anatolian race and the Caucasian race were the most common in the colonies, while the Carniolan honey bee race was the least common race (Figure 4). While the Anatolian race was the most common with a maximum similarity of 85% among the colonies, the Carniolan race was the least similar with 33% similarity. As shown in Figure 5, although the differences between beekeeping apiaries in terms of motility and PMI were significant ($P \leq 0.05$), no difference was found in terms of high MMP and SCON ($P > 0.05$). Regarding morphometry, only the difference in the similarity rate of the Anatolian race was found significant among the apiaries ($P \leq 0.05$) (Figure 6). Although significant differences were not observed regarding the relationships between the spermatological and morphometric parameters obtained in this study, a low-level positive correlation was determined between motility and plasma membrane integrity, as shown in Table 1 (R= + 0.35). It was determined that there was no significant relationship between spermatological and morphometric parameters (Table 2) on the one hand, and that there was no significant relationship between wing index values and spermatological parameters (Table 3) on the other.

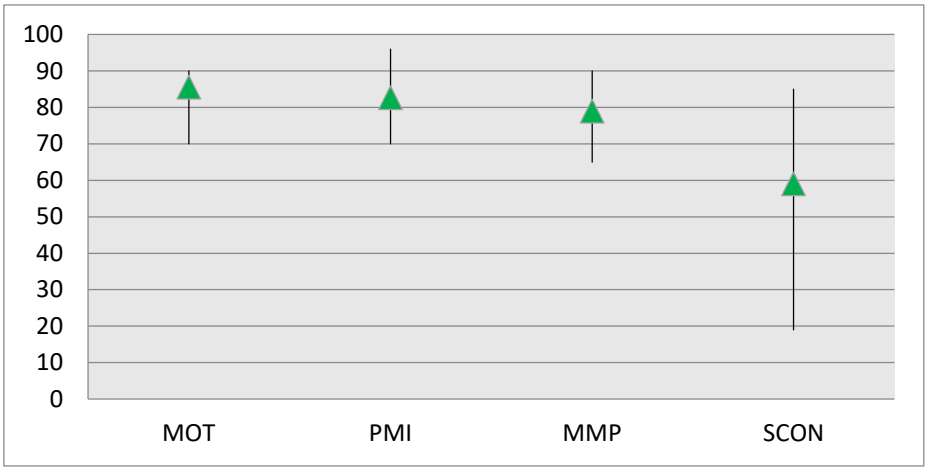

Figure 3. Minimum, maximum and mean percent values of spermatological parameters of colonies (The graph bar shows the minimum and maximum values. The mean value is indicated by the green arrowhead. MOT=% Motility, PMI= % Plasma Membrane Integrity, MMP= % High Mitochondrial Membrane Potential, SCON= Sperm Concentration (×10^8/ml)).

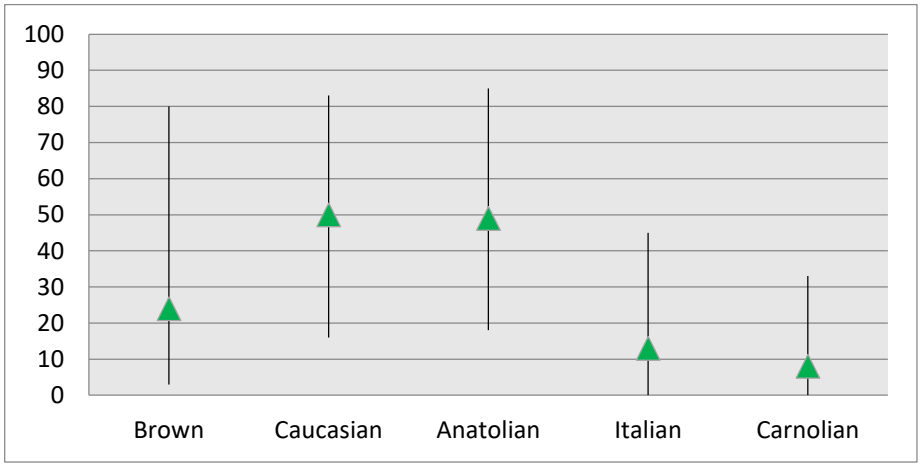

Figure 4. Minimum, maximum and mean percent values of morphometric parameters of colonies (The graph bar shows the minimum and maximum values. The mean value is indicated by the green arrowhead).

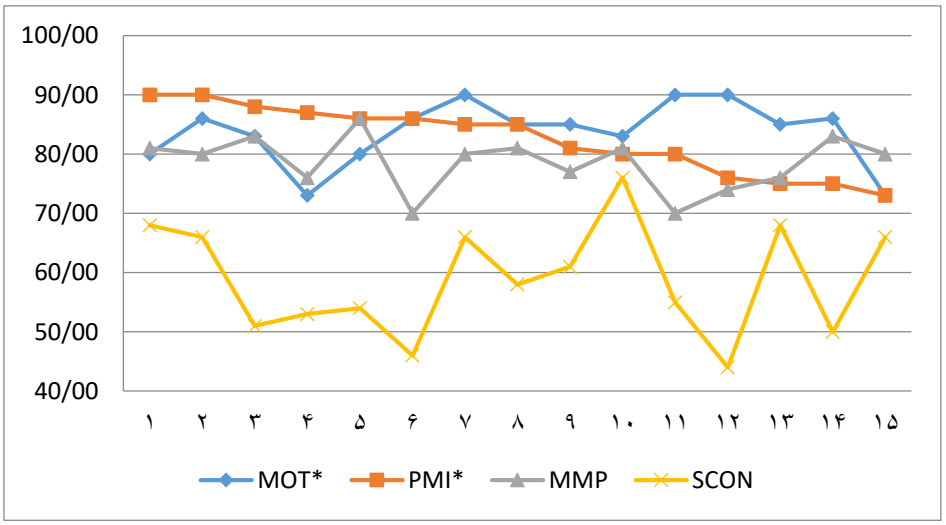

Figure 5. Situation beekeeping apiaries in terms of spermatological parameters (The vertical axis shows the parameter values. The horizontal axis shows the beekeeping apiaries. *:Indicate statistical differences (P ≤ 0.05). MOT=% Motility, PMI= % Plasma Membrane Integrity, MMP= % High Mitochondrial Membrane Potential, SCON= Sperm Concentration (×10^8/ml)).

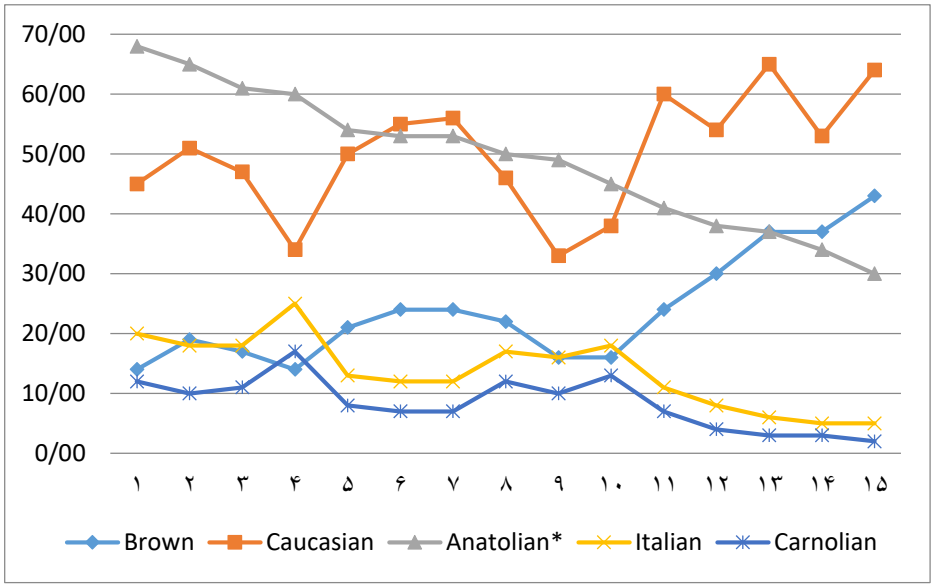

Figure 6. Situation beekeeping apiaries in terms of morphometric parameters (The vertical axis shows the parameter values. The horizontal axis shows the beekeeping apiaries.*: Indicate statistical differences (P ≤ 0.05).)

Table 1. Correlative relationship level of spermatological parameters of colonies (R)

n = 45	Motility	PMI	High MMP
PMI	0.35*		
High MMP	- 0.11	0.15	
SCON	- 0.17	0.17	0.12

*: Indicate statistical differences (P ≤ 0.05).

Table 2. Importance levels of the relationship between spermatological parameters and morphometric parameters (R)

n=45	Brown	Caucasian	Anatolian	Italian	Carnolian
Motility	-0.039	0.010	-0.095	-0.042	-0.038
PMI	-0.147	-0.014	0.064	0.075	0.090
High MMP	0.034	0.217	-0.044	-0.097	-0.088
SCON	0.235	0.268	-0.195	-0.287	-0.297

*: Indicate statistical differences (P ≤ 0.05).

Table 3. Importance levels of the relationship between spermatological parameters and wing index values (R)

n=45	Motility	PMI	High MMP	SCON
Cubital index	-0.166	0.197	0.176	0.162
Dumb-bell index	0.073	0.054	-0.147	-0.259
Discoidal Shift	-0.021	-0.001	-0.172	-0.369

*: Indicate statistical differences (P ≤ 0.05).

Discussion

In this study, samples belonging to 45 different colonies were examined and accordingly mean, 85% motility, 82% PMI, 78% MMP and 5.9 × 10^9/ml SCON were determined. There are many studies examining the effects of spermatological parameters on reproductive performance. For example, in a study investigating the effects of spermatozoa motility on hatching rate, the rate of hatching from the eggs of bees that were artificially inseminated under suitable conditions using sperm with 50% or more motility was over 70% (Almeida and Espencer Egea Soares, 2002). Therefore, in this study, it is understood that the average motility value of 85% **is** appropriate in terms of hatching rate. In other studies on drone semen, plasma membrane integrity values in colonies of various races were obtained on average between 81% and 95% (Yániz et al, 2020). The plasma membrane integrity in the present research is appropriate with the values in the aforementioned studies.

In many studies, the drone spermatozoa concentration was between 3.63 × 10^6 and 11.9 × 10^6/µl spermatozoa. In the present study, the SCON determined as 5.9 × 10^9/ml is parallel with other studies (Yániz et al, 2020).

Mitochondria have an important role in the energy supply necessary for spermatozoa to perform their physiological functions, thus giving important clues about their vital functions. The drone sperm needs a high MMP value to ensure the continuity of its movement during the migration from the spermatheca to the oviduct during the migration of the spermatheca after mating and during the egg-laying (Ciereszko et al, 2017; Slater et al, 2021). To our knowledge, no study was found on detecting high MMP in sperm with JC-1 fluorescent dye, but in two studies, using Rh 123 (Rhodamine 123) fluorescence dye, high MMP values were between 59% and 93% in different control groups (Alçay et al, 2022; Ciereszko et al, 2017). Since the high MMP value detected at the rate of 78% in this study is compatible with these data, it can be said that mitochondrial activity is at an appropriate level.

According to the morphometric analysis data performed on the worker bee samples of the same colonies, the average race rates were 50% Caucasian, 49% Anatolian, 24% Brown, 13% Italian and 8% Carniolan. This shows that the Anatolian bee race, which is expected to be resident in the Central Anatolia Region, has undergone significant crossing, either consciously or unconsciously. The change of bee race expected to be in the region may cause many problems. These can be counted as inability to adapt to seasonal changes, decrease in resistance to diseases, low wintering ability, incompatibility with vegetation and loss of yield. There is a danger of crossbreeding of bee races in Central Anatolia Region. The presence of the Caucasian race is at a similar rate to the Anatolian race in the region. The presence of many Caucasian queen bee producers in the nearby region, which is adjacent to Central Anatolia, may be the reason why the apiaries bought queen bees from these places. In addition, the propaganda about the honey-gathering and calm temperament of the Caucasian race for many years has also caused beekeepers to buy this race. Similarly, the Brown race may have been preferred in the region to reduce winter losses due to its good wintering ability. It is thought that the presence of Italian and Carniolan races in the region is because they were brought from abroad illegally or the hybrids of this race were favored by the beekeepers. Central Anatolia Region is located in the migration route of beekeepers belonging to the coastal regions, which have spent the winter season and early spring in the coastal areas. It is also possible to say that the race differences in the region are caused by widespread uncontrolled

mating through colonies of different races from different regions due to these migratory beekeeping activities.

When the data of the present study were evaluated, no data with a ratio of "r" value above -0.29 was found between spermatological and morphometric parameters. Based on these results, it is observed that the race changes do not have an effect on spermatological parameters. In studies investigating the relationship between race changes and spermatological parameters, the SCON of bees belonging to the Carniolan race was in the range of 6.76-7.08 × 10^9/ml. In a similar study, Duay et al. (Duay et al, 2002) found the SCON of the same race as 7.54 × 10^9/ml. Koeniger et al. (2005) stated that the differences in SCON are due to environmental effects, individual differences or method differences and that the race difference does not correlate with to this. In the present study, it was observed that there were different levels of randomly-uncontrolled crossbreeding and satisfactory spermatological parameters.

Finally, it was determined that there was no relationship between uncontrolled crossbreeding and spermatological parameters. But confirming these results obtained with classical morphometry with some molecular and genetic analysis may provide more accurate results.

Conflict of interest

The authors declare that they have no conflict of interest.

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