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Original article

The effects of instrumental insemination on selected and unselected breeding characteristics in honeybee (*Apis mellifera* L.)

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Abstract – In this study, the effect of instrumental insemination and natural mating on selected and unselected characters in a breeding population was investigated. The experimental colonies were from a population that has been selected for 3 generations in terms of hygienic behavior. Honey yield, brood production, and adult bee population characters were not taken into consideration as a selection criterion. Mother queens and drone fathers were selected from the breeding population. While a significant difference was found between naturally mated queen (NMQC) and instrumentally inseminated queen colony (IIQC) groups in terms of hygiene behavior, there was no significant difference between the groups in terms of performance phenotypes. The average dead pupa removal was 84.44 ± 0.87% in the NMQC; this average increased to 87.70 ± 1.09% larvae/colony by the control of the father in IIQC usage. This result demonstrates that instrumental insemination can be used to produce colonies of equivalent phenotypes compared to open-mated queens.

Honeybee / *Apis mellifera* / Mating / Paternal / Control / Breeding

1. INTRODUCTION

Queens of honey bees mate in the air approximately with 15–20 drones coming from different apiaries and different bee colonies in mating areas within a 13–15-km radius (Koeniger et al. 2014; Cobey et al. 2011). Depending on the season and rearing methods, 10–80 thousand drones can be found in a mating area (Ruttner 1988; Dodologlu and Genc 1997). Thus, it is not possible to know the drones from which apiaries and from which colonies the queen bees mate in the mating area with such large drone populations (Woyke 1962; Page et al. 1985; Cobey 2007; Buchler et al. 2008). Thus, the paternal side of the progeny generation is not known completely, and its control is not possible by natural mating. On the other hand, it is not possible to know the advice genetic effects (additive gene) originating from the father on the character. By using the instrumental insemination (IIQ) method, which was first applied by Watson (1927) in honey bees, that control of the paternal side and thus the genetic structure was completely possible (Cobey 2007; Cobey et al. 2011; Plate et al. 2019). As a matter of fact, it has developed very important genotypes that are productive and resistant to diseases and pests (Buchler et al. 2008; Rinderer et al. 2010; Cobey et al. 2011).

In the past, many studies (Laidlaw 1944; Mackensen 1947; Kaftanoglu and Peng 1980a)

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have been performed to facilitate the application of the IIQ method, such as anesthesia (CO_2), pre-oviposition period, semen dose (amount), time of administration, age of the queen, and collecting large amounts of semen (Washing Technique). Recently, Cobey has made important contributions to the practice and effectiveness of the IIQ method (Cobey 1983, 1998, 2004, 2007, 2015, 2016). The method has become very popular in the last century, and it is widely used in honey bee breeding (Kaftanoglu and Peng 1980b; Rinderer 1986; Laidlaw 1987; Ruttner 1988; Cobey et al. 2011). The IIQ not only provides control of genetic structure and protection of subspecies but also enables hybridization and line development, which is important in today's beekeeping and can not be achieved in natural mating (Cornuet 1986; Cobey 2007). In addition, it will be possible to improve new genetic stocks to overcome possible global warming, diseases, and similar difficulties in the future, with the support of IIQ. Although there are doubts and reluctance to apply the technique (Harbo and Szabo 1984), Cobey's (2007) comprehensive compilation study revealed that there is no significant difference in performance between instrumental IIQ and NMQ colony groups. In fact, many researchers have reported that the performance of IIQ colonies is higher than the NMQ colonies (Wilde 1987; Cermak 2004). The idea that IIQ negatively affects colony performance actually stems from the inexperience of IIQ practitioners and many inappropriate beekeeping practices (Cobey 2007; Rousseau et al. 2015).

In our opinion, the most important issue regarding the application of IIQ is to determine its effects on genetic structure and breeding populations. For example, it is to determine to what extent some characters selected and unselected as selection criteria for several generations have changed in the population or whether they provide improvement. Many researchers (Pritsch and Bienefeld 2002) support this hypothesis. However, although it is possible to control the paternal side with IIQ, we do not have enough data and information about the positive contribution of this control level to the population and each generation. Since the father is cytologically haploid, he transfers all of his genotypic structure to his offspring more confidently (Ruttner 1972; Page et al.1985; Collins 1986; Cornuet 1986). So, it is important to determine the effects of each of the parents on improving characteristics during the application of breeding. On the other hand, queen bee producers generally transfer the larvae from the breeding colonies and produce queens, but most of the producers do not care about the rearing of the fathers (sires of workers). Progeny cannot show the expected performance if the sires of the queens and workers are not used in the queen rearing (Guler 2010). Thus, it is not possible for the beekeeper to obtain the desired yields when using these types of queen bees. By way of instrumental insemination, it is possible to determine the contribution of paternal's control to progeny generation. This contribution also refers to the additive gene effect which is provided by the drone producer queens. Therefore, this hypothesis needs to be questioned.

This study aimed to compare the effects of naturally mated (NMQC) and instrumentally inseminated queen colony (IIQC) groups in terms of performance (honey yield, bee, and brood frame amounts) and hygienic behavior selection in a population that underwent three generations.

2. MATERIAL AND METHODS

The study was conducted at the Bee Research and Application Unit of the Agricultural Faculty at Ondokuzmayis University, Samsun-Turkiye.

2.1. Honey bee material

Caucasian (*A. m. caucasica* G.) colonies which were selected in terms of hygiene characteristics of three generations were used as material. Colonies having one-year-old queen bees were used. Honey yield, brood production, and adult bee population characters were not taken into consideration, or these characters were not used in the selection.

Among those which colonies that removed 95% or more dead pupae in each generation (Spivak 2006; Ibrahim and Spivak 2006; Guler and Toy 2013), 5% were selected as fathers and 20% as mothers colonies (Laidlaw and Page 1986; Ruttner 1988; Bienefeld and Pirchner 1990; Bienefield et al. 2007). Colonies used in this study were produced by controlled rearing and mating of these parents. Experiments were carried out from 2018 to 2020 years. Each year at the beginning of spring (in April), the colonies equalized in relation to the number of frames with bee and brood, nutrition and nurse, comb foundation, disinfection, control, settlement in apiary, and transportation (Delaplane et al. 2013; Guler et al. 2018). Colonies were numbered, and registration system was formed. Medicine (Perizin; Caumaphous=Asuntol) was applied to the colonies against *Varroa destructors* in early spring. Apart from this, no other chemicals were applied to the colonies. The colonies renewing queen bees, having any diseases symptom, and swarming during the course of the study were excluded from the experiment. Migratory beekeeping was applied for determining the colonies' performance characteristics. In the study, 343 artificially inseminated and 563 naturally mated queen colonies were used.

2.2. Queen bee rearing

Queen bees were reared by the Doolittle method (Laidlaw 1985; Buchler et al. 2013). Colonies with bees on an average of 20 frames were used as starters (Buchler et al. 2013). In the study, the colonies, which were the source of the father of sire and queen bee mother of sire, were previously produced within the scope of the breeding study (Ruttner 1972; Page et al.1985; Bienefeld and Pirchner 1990; Koeniger et al. 2014). Queen cells accepted by the starter were kept in the finisher colonies until they became adults. On day 10, queen cells were harvested and introduced into mating (nucleus) colonies with 3 framed bees. Then the colonies were tested the following year after all the worker bees in the colony were converted into queen bees' progeny. Natural mating was prevented by closing the entrance of the queen bee group hives to be instrumentally inseminated with a queen bee excluder (Page and Laidlaw 1997; Buchleret al. 2013; Cobey 2016a, b). In the natural mating group, the queen bees mated naturally without restriction.

2.3. Drone rearing

Since drones become an adult in 24 days and reach sexual maturity in 12 days, drone breeding started approximately 40 days before the instrumental insemination date (Cobey 1983; Ruttner 1988; Rinderer 1986; Buchler et al. 2013). In terms of hygienic behavior, 3-generation selection was applied to drone bees reared from colonies (Ruttner 1972; Bienefield et al. 2007). Colonies selected as paternal parents were given drone-eyed honeycombs between their brood frames in the early spring (Ruttner 1988; Buchler et al. 2013). The queen bee was allowed to lay an unfertilized egg in these cells. After reaching the larval stage, it was transferred to the feeder colonies. Adult drones were colored and used for instrumental insemination after they reached 12 days of age (Ruttner 1988; Cobey 2007).

2.4. Instrumental insemination

Queens were inseminated on their 6–17th birthday (Cobey 2007). A total of 11 μl of semen fluid was collected from drones under the microscope using the vacuum syringe method. This amount of semen fluid was given to all instrumentally inseminated queen bees by the method suggested by Cobey et al. (2013). Instrumentally insemination was performed by the same person (corresponding author). The first carbon dioxide (CO_2) application was made during insemination, and the second application was made one day after insemination (Mackensen 1947; Cobey 2007). Instrumentally inseminated queens have their wings clipped and their thorax numbered. After the anesthetic effect finished, the queen bees were given back to their nucleus colonies from which they were taken, with a cage containing a cake (Laidlaw 1985; Buchler et al. 2013).

2.5. Determination of colony performances

Instrumentally inseminated and naturally mated queen colony groups were maintained in the same apiary during the experiment.

2.5.1. *Honey yield (kg/colony)*

In the first, frames with honey in each colony were determined, and after leaving the required honey for the colony, the remaining was recorded as honey yield. Before the centrifuge process, frames with honey of each colony were weighted by super, and after the centrifuge, the same frames were placed in their own supers and weighted again and their tare was found. Then, the honey amount produced by each colony (kg/colony) was found by excluding tare from the first measurement (Lensky and Golan 1966; Guler and Kaftanoglu 1999; Delaplane et al. 2013; Guler et al. 2018). In each year, honey was harvested in the 3rd week of August.

2.5.2. *Development of worker bee population (frame number/colony)*

A total number of the frames covered with adult bees (frame number/colony) of each colony was recorded every month between the periods of May to November (Guler and Kaftanoğlu 1999; Delaplane et al. 2013; Hatjina et al. 2014; Guler et al. 2018).

2.5.3. *Brood production (frame number/colony)*

Frames covered with open (egg and larvae) and closed (pupae) brood of each colony were counted (frame number/colony) and recorded in May, June, July, August, and September (Guler and Kaftanoglu 1999; Hatjina et al. 2014).

2.5.4. *Determination of hygiene behavior (%)*

In the study, the liquid nitrogen (− 196 ℃) method was used (Spivak et al. 2003). For each application, one frame with pupae was taken from each colony. Approximately 350 ml of liquid nitrogen was poured into the cylindrical metal template covering 165 pupae cells (Spivak et al. 2003; Guler and Toy, 2013). Liquid nitrogen was applied five times in the year (May, June, July, August, and September). The hour at which the frame was placed in the hive was recorded on the colony card. This frame was taken from the colony at 24 h after liquid nitrogen application. The label was fixed near the area where the liquid nitrogen was applied (165 cells) and later photographed by a digital camera. The frame was placed in the hive from which it was taken. At each period, these pictures were loaded onto the computer; later, the removed cells were counted and recorded on the colony card. Empty cells were counted at the beginning and recorded on the colony cards (Spivak et al. 2003; Ibrahim and Spivak 2006; Guler and Toy 2013). This application was used in each generation in the selection. In selection, colonies were ranged from high to low in terms of the mean number and rate of dead pupa removed after the five applications of liquid nitrogen. Then the colonies that removed 95% or over larvae were selected as parents.

2.6. Statistical analysis

Statistical analyses were carried out according to the randomized block with repeated observations design. Duncan's test was used for multiple comparisons. Versus of normality was determined by the Kolmogorov–Smirnov test, and homogeneity of variances was determined by the Levene test. It was determined that the data for all features were normally distributed ($P > 0.05$), and the variances were homogeneous ($P > 0.05$). SPSS 13.0 (2004) (Customer ID: 361835) was used as a statistical program.

Table I Mean and standard error values of adult worker bee population (frame number/colony) and honey yield (kg/colony) of colonies with queens instrumentally inseminated (IIQC) and naturally mated (NMQC) groups

Colonies with queens	*n*	Worker bee population	*n*	Honey yield
Instrumentally inseminated (IIQC)	313	5.86 ± 0.08	141	13.33 ± 0.97
Naturally mated (NMQC)	554	6.00 ± 0.07	173	12.96 ± 0.74
Significance		0.170		0.758

n number of colonies

3. RESULTS

3.1. Performans phenotypes

Adult bee population, honey yield, brood production, and hygienic behavior of IIQC and NMQC groups are given in Tables I and II. There was no significant difference ($P > 0.05$) between the IIQC and NMQC groups in terms of the adult bee population, honey yield, and brood production.

3.2. Hygiene behavior

Hygiene behavior averages of the IIQC and NMQC colony groups were determined to differ significantly ($P < 0.05$) from each other. With an average of 87.70 ± 1.09%, the highest hygienic behavior was determined in the instrumentally inseminated group (Table II).

4. DISCUSION

Colonies used in this study were produced by controlled rearing and mating of the parents. A large number of instrumentally inseminated (IIQC) and naturally mated (NMQC) queen bee colonies were evaluated. In some previous studies, naturally mated queen bee colonies gave more honey and performed better than the IIQC (Harbo and Szabo 1984), but in some other studies (Cobey 1998, 2004; Pritsch and Bienefeld 2002; Cobey et al. 2013) reported no significant differences between IIQC and NMQC. In our study, no significant difference was found between IIQC and NMQC in terms of brood production, adult bee development, and honey yield phenotypes. This might have resulted from the fact that important measures and all kinds of management and administration have been taken to ensure equal environmental effects. Namely, queens were sister and reared

Table II Mean and standard error values of brood production (frame number/colony) and hygienic behaviors (%) of colonies with queens instrumentally inseminated (IIQC) and naturally mated (NMQC) groups

Colonies with queens	*n*	Brood frame number	*n*	Hygienic behavior
Instrumentally inseminated (IIQC)	302	3.97 ± 0.07	265	87.70 ± 1.09[a]
Naturally mated (NMQC)	474	4.00 ± 0.07	559	84.44 ± 0.87[b]
Significance		0.704		0.027

Values within rows with different letters (a and b) differ significantly ($P < 0.05$)

n number of colonies

by transferring larvae from the same age (Woyke 1962, 1971) and genetic source (Laidlaw 1985; Ruttner 1988); the queens were reared in the same apiary, feeding, medicine, beekeeper, foundation comb, and transportation were kept equal. In addition to those preventive measures, many other factors affecting instrumental insemination like rearing queens and drones in the appropriate season (Moritz and Kühnert 1984; Guler and Alpay 2005; Cobey 2007), rearing quality drones (Currie 1987), using quality sperm (Collins 2000; Cobey 2007; Rousseau et al. 2015), using an equal amount of semen, inseminating the queen at the appropriate age (8–12 days old), using adequate CO_2, and performing insemination by an experienced person (Cobey 2007) were taken into consideration. So, the reason for no significant difference between IIQC and NMQC in terms of brood production, adult bee development, and honey yield might be resulted from ensuring and considering all those environmental effects.

In terms of hygienic behavior, breeding material was tested in all colonies in the same apiary and migratory beekeeping conditions. The population showed significant variation in terms of dead pupae removed rate, which is evaluated as hygienic behavior (Spivak et al. 2003). A significant difference was found between IIQC and NMQC in terms of hygiene behavior character. This might have resulted from the fact that it was studied in a herd that was selected in terms of hygienic characteristics of 3 generations and it was due to the controlled mating of the parents in this process. This finding is also supported by some other studies (Harbo 1986; Pritsch and Bienefeld 2002; Cermak 2004). As a matter of fact, Cobey (2007) emphasized that the high performance of the instrumental insemination group was due to the advantage of selection and the effect of the amount of semen used. This difference is essentially the contribution or improvement made by the father to the offspring generation. It was estimated that the variation was due to the difference in the genetic control of the colonies representing the sires of workers by IIQ. This genetic improvement in paternal hygiene was not seen in other phenotypes. Because no selection was applied in terms of paternal lineage in the population. The results of the present study also support this, and this behavior was evaluated as an ability provided by the control of genotypic differences from parents (Moritz and Kühnert 1984; Harbo and Harris 1999; Boecking et al. 2000; Ibrahim and Spivak 2006). As a matter of fact, many researchers (Oldroyd 1996; Spivak and Reuter 1998; De Guzman et al. 2001; Spivak et al. 2003) determined that the level of dead pupae removal behavior may vary depending on the honey bee subspecies. But in this study, thanks to instrumental insemination, this improvement caused by the additive gene effect can be maintained with the controlled use of the paternal side (Bienefield et al. 1989). In our study, while the average dead pupa removal was 84.44 in the NMQC, it increased to 87.70 larvae with the control of the father by instrumental insemination usage. In other words, the progeny population representing the IIQC cleared 3.26% more larvae than the natural mating colony group. This number of clearing more larvae is the contribution of the father. Therefore, the results of our study showed that the queens that are to be given to the beekeepers have to be raised from the breeding mother (dam of the queens) colonies and those queens have to be mated with drones (sires of workers) that are produced from drones produces queens. Of course, the contribution of the parents is possible only with mating control.

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AUTHOR CONTRIBUTION

AG and HO designed the experiment, data analysis by (HO), queen and drone rearing (GK and SB), keeping of colonies (EU), data collection (HT and AA), and instrumental insemination, and the manuscript wrote and participated in the revisions of it (AG). In addition, EU, AA, and GK stayed in the plateau for 4 months in the migratory beekeeping condition to determine the performance of the colonies. All authors read and approved the final manuscript.

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DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are not publicly available due to the study is related to bee breeding and breeding material will be produced. The university does not accept the sharing of data on this breeding material because the breeder certificate will be taken but are available from the corresponding author upon reasonable request.

DECLARATIONS

Ethics approval The Turkiye Central and Ondokuzmayis University Ethic Committees has confirmed that no ethical approval is required for honey bee studies. That is not applicable.

Consent to participate That is not applicable.

Consent for publication That is not applicable.

Conflict of interest The authors declare no competing interests.

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