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Yasin Kahya, H. Vasfi Gençer

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Original article

Reproductive quality in drones bred from Caucasian (*A. m. caucasica)* and Italian (*A. m. ligustica*) honey bee colonies

Yasin KAHYA[1], and H. Vasfi GENÇER[1]

[1] Department of Animal Science, Faculty of Agriculture, Ankara University, 06110 Ankara, Turkey

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Abstract – Many investigations were carried out on how environmental factors affect the reproductive characters of drones. The genetic variation in the reproductive characters of drones has been understudied, however. This research aimed to compare the variation in the reproductive characters of drones bred from two widely used subspecies in apiculture, Caucasian (*A. m. caucasica*) and Italian (*A. m. ligustica*), considering the colony effect. The experimental drones reared under the same spatiotemporal conditions were used to measure semen characters (ejaculate volume and the number, concentration, and viability of spermatozoa) and ejaculation ability characters (ejaculation proportion, ejaculation efficiency, and semen collection efficiency). The results displayed significant differences between subspecies in body weight, ejaculate volume, spermatozoa number in seminal vesicles and ejaculates, ejaculation proportion, ejaculation efficiency, and semen collection efficiency. The differences in the number of spermatozoa in seminal vesicles, ejaculation proportion, and semen collection efficiency were significant between the Italian colonies, whereas no significant difference was found in these characters between the Caucasian colonies. The concentration and the viability of spermatozoa were not significantly different both in subspecies and in colonies. These findings suggest that Caucasian drones appeared to be somewhat fitter than Italian drones.

drone / spermatozoa / ejaculation ability / instrumental insemination / reproductive success

1. INTRODUCTION

A honey bee (*Apis mellifera* L.) colony consists of a single reproductive queen, the mother of all individuals, 5 000 to 60 000 sterile females, workers, and a few thousand seasonally reared reproductive males, drones. The queen plays a central role within the honey bee colony. The colony's productivity, harmony, and stability depend mainly on the queen. Drones do not contribute to colony performance and maintenance, yet they are integral components of the colony as they are the potential mates of virgin queens reared in nearby colonies. The sole apparent function is to produce semen and transmit it to the virgin queen during copulation.

The studies have focused mainly on queen quality. Drone quality is, however, as crucial as queen quality. Poorer drones are expected to contribute fewer spermatozoa to the queen's spermatheca. The queen with few spermatozoa in spermatheca is often superseded earlier by the colony (Laidlaw 1985; Richard et al. 2007). Therefore, plenty of high-quality drones are required in the mating arena to increase the potential reproductive quality of queens. The drone and queen quality is directly linked to reproductive success at the colony level. The reproductive success of a colony depends on the number of drones

Corresponding author: H. V. Gençer,
gencer@agri.ankara.edu.tr
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and queens produced and the individual reproductive success of each queen and drone (Kraus et al. 2003).

Drone production is a period of the annual cycle of the colony. Many drones are produced in the colony during spring and early summer when virgin queens are most abundant under moderate climates (Boes 2010). Drones compose 5–10% of the adult population of the colony (Seeley and Morse 1976). The number of drones produced in the colony is regulated according to the season, the size of the colony, food availability, the amount of drone brood present, and the presence or absence of the queen (Allen 1958; Free and Williams 1975; Rinderer et al. 1985; Lee and Winston 1987; Seeley and Mikheyev 2003; Wharton et al. 2007; Boes 2010).

The key traits responsible for the reproductive competitiveness of drones are the body mass, the number, the concentration, and the viability of spermatozoa and semen volume. Many studies reported environmental factors affecting the reproductive quality of drones during development and after emergence, including the comb cell size (Berg et al. 1997; Schlüns et al. 2003; Gençer and Firatli 2005; Zaitoun et al. 2009; Couvillon et al. 2010; Gençer and Kahya 2011, 2020), food availability (Stürup et al. 2013; Czekońska et al. 2015; Rousseau and Giovenazzo 2016), senescence (Woyke and Jasinski 1978; Mazeed and Mohanny 2010; Czekońska et al. 2013a; Stürup et al. 2013; Yániz et al. 2016; Metz and Tarpy 2019), season (Zaitoun et al. 2009; Rhodes et al. 2011), infestation with varroa mite (Rinderer et al. 1999; Duay et al. 2002), exposure to pesticides (Rinderer et al. 1999; Collins and Pettis 2001; Burley et al. 2008; Kairo et al. 2016; Ciereszko et al. 2017; Fisher and Rangel 2018; Ben et al. 2019), and temperature regimes (Koeniger et al. 2006; Bieńkowska et al. 2011; Stürup et al. 2013; Czekońska et al. 2013b; Czekońska and Tofilski 2020). Nonetheless, much fewer reports on the genetic variation in the reproductive characters of drones exist (Rinderer et al. 1985; Zaitoun et al. 2009; Rhodes et al. 2011; Taha and Alqarni 2013; Rousseau et al. 2015; Halak et al. 2020; Quartuccio et al. 2020).

Several authors reported the genetic differences in body weight and spermatozoa count in the seminal vesicles of drones. Rinderer et al. (1985) compared the body weights, seminal vesicles and mucus glands weights, and the spermatozoa counts of European and Africanized drones. They determined that European drones were heavier in weight (220.2 and 194.6 mg, respectively) and had more spermatozoa (5.7 and 4.6 million in single seminal vesicles, respectively) than Africanized drones. Zaitoun et al. (2009) compared the average body weight of drones from Italian and Syrian subspecies. They reported that Italian drones were heavier in weight (203 and 181 mg, respectively) and had more spermatozoa in seminal vesicles (10.2 and 8.8 million, respectively) than Syrian drones. Likewise, Taha and Alqarni (2013) compared the body weight, testis, seminal vesicle, and mucus gland sizes of newly emerged drones from the Carniolan and Yemeni subspecies. In Taha and Alqarni (2013), Carniolan drones were determined to be heavier at emergence (227.2 and 190.1 mg, respectively). Carniolan drones had more spermatozoa in seminal vesicles (12.7 and 9.3 million, respectively) than Yemeni drones.

Italian (*A. m. ligustica*) and Caucasian (*A. m. caucasica*) honey bees are two of three subspecies that are the most widely used in apiculture (Ruttner 1988). The natural distribution area of the Italian honey bee is the Apennine Peninsula, which is separated from continental Europe by the Southern Alps. The Caucasian honey bee is native to the high valleys of the Central Caucasus, and its range extends to the northeastern part of Turkey. The Caucasian and Italian subspecies that evolved in different natural environments display significant distinctions in various characters. The brood rhythm is the remarkable distinction between Caucasian and Italian honey bees. Unlike the Italian honey bee, brood production of the Caucasian honey bee is restricted at the end of July due to the significant accumulation of stores in the brood nest area. The Italian honey bee tends to rear brood lasting long into autumn, regardless of nectar flow (Ruttner 1988). The reproductive characters of drones of

these subspecies have not previously been compared in a separate study. We aimed to compare the variation in the reproductive characters of drones bred from Caucasian and Italian subspecies, considering the colony effect. We proposed some novel characters to assess the ejaculation ability of drones as measures for colony fitness by interpreting the difference in spermatozoa counts between seminal vesicles and ejaculates.

2. MATERIALS AND METHODS

2.1. Drone rearing

The experiment was conducted in 2012 at Ankara University, Turkey (39°57′47.3″N, 32°52′00.1″E). The experimental drones were reared simultaneously from Italian (*Ita1*, *Ita2*, and *Ita3*) and Caucasian (*Cau1*, *Cau2*, and *Cau3*) colonies. The instrumentally inseminated queens of the Caucasian colonies (*Cau1*, *Cau2*, and *Cau3*) were reared from one of the naturally mated purebred Caucasian queens in the research apiary. The purebred Caucasian queens were obtained from a Caucasian breeder farm in northeast Anatolia. The instrumentally inseminated queens of Italian colonies (*Ita1*, *Ita2*, and *Ita3*) were reared from one of the instrumentally inseminated purebred Italian queens obtained from the apiary at Adnan Menderes University campus, Aydin. The queen of each experimental colony was confined on an empty drone comb by a queen excluder cage ($44.5 \times 25.5 \times 6.0$ cm). After each queen laid unfertilised eggs entirely on both sides of the comb within 48 h, the queen excluder cage was removed. At the expected emergence day, the drone brood comb in each colony was again put into the queen excluder cage. Approximately 5600 drones that emerged in experimental colonies within 24 h were marked with a paint marker (Edding 751) on their thoraces. A different color was used for each maternal colony. The marked drones were transferred to a foster colony arranged by dequeening two days before the emergence of drones. The marked drones were permitted to fly freely outside the hive until dissections for the measurements. The drones were dissected when they were 3 weeks old.

2.2. The outline of reproductive characters measurements

We categorised the characters assessed as semen characters and ejaculation ability characters. Semen characters measured in individual drones were spermatozoa counts in the ejaculate and seminal vesicles, ejaculate volume, and the concentration and viability of spermatozoa. Ejaculation ability characters calculated from individual values of multiple drones were ejaculation proportion, ejaculation efficiency, and semen collection efficiency. Since a single individual cannot be appropriately used for all these characters, the marked drones were randomly allocated to four sets of different characters. The drones in the first, second, and third sets were for semen characters. These drones were individually weighed on the electronic balance (Sartorius BP121S) to the nearest 0.01 mg before the measurements.

The first set of drones was used to determine the ejaculate volume and the number and concentration of spermatozoa in the ejaculate. Measuring the spermatozoa concentration requires determining the ejaculate volume and counting spermatozoa in the ejaculate. However, spermatozoa in the ejaculate do not present the number of spermatozoa produced. We, therefore, determined the number of spermatozoa in intact seminal vesicles in the second set of drones. Besides, we aimed to determine the difference in the number of spermatozoa between intact seminal vesicles and the ejaculate. The drones manipulated for the ejaculate volume measurement could be further tested for the viability of spermatozoa. However, we did not use the ejaculate for the spermatozoa viability test due to the viability loss during the eversion and ejaculation process (Gençer et al. 2014). Instead, we used the third set of drones for the spermatozoa viability test. Finally, the remaining drones as the fourth set were used to determine the ejaculation ability characters.

2.3. Semen characters of drones

Ejaculate volume: Ten drones from each colony were used to determine the ejaculate volume, number, and concentration of spermatozoa. Each drone was manually provoked for the eversion of endophallus and ejaculation, as described by Woyke (2008). The ejaculate on the bulb of the endophallus was carefully drawn into the glass tip of the Harbo syringe connected to the Gilmont micrometer (Gilmont Instruments; Barrington, IL, USA) under × 40 magnification. The Harbo syringe was mounted on Schley instrumental insemination instrument (Peter Schley Equipment, Lich, Germany). The ejaculate volume was determined by reading the beginning and finishing points of the semen column on the scale of the Gilmont micrometer to an accuracy of 0.1 µl. Only one person handled all drones for the eversion of the endophallus and the collection of semen.

The number and the concentration of spermatozoa in the ejaculate: The number and the concentration of spermatozoa in the ejaculate were determined with the spectrophotometer (Harbo 1975; Gençer and Kahya 2011). The ejaculate in the glass tip was discharged into the quartz cuvette (Hellma, 1.4 ml capacity) of the spectrophotometer (Shimadzu UV1800) containing 1000 µl modified Kiev solution (MKS). The absorbance readings of kinetic measurements at 260 nm wavelength in 180 s were converted into spermatozoa numbers by using the calibration curve estimated by the computer-based software (Shimadzu-UV Probe, Version 2.33; Gençer and Kahya 2011). The spermatozoa concentration (million/µl) was calculated by dividing the number of spermatozoa by the volume of ejaculate.

The number of spermatozoa in seminal vesicles: Forty-eight drones (the second set) were dissected under a macroscope (Leica Z16 APO) at × 20 magnification. The intact seminal vesicles of each drone were taken from the abdomen and then placed in a sterile watch glass (diameter = 6.5 cm) containing 150 µl of MKS. The semen content of both seminal vesicles was completely discharged in MKS. The emptied seminal vesicles were removed, and the semen was dispersed. The semen-MKS mixture was transferred from the watch glass into the quartz cuvette containing 850 µl of MKS (total volume 1000 µl). As explained above, absorbance readings of kinetic measurements and the conversions of absorbance values into the spermatozoa counts were done (Gençer and Kahya 2011).

The viability of spermatozoa in seminal vesicles: Another six drones from each colony (36 drones, the third set) were dissected for the spermatozoa viability test. The intact seminal vesicles taken from the abdomen were grasped with forceps from their narrow ends and then washed with a drop of MKS to prevent semen from contamination by hemolymph or any residue. Then the seminal vesicles were placed in a sterile watch glass containing 150 µl MKS. The semen in each vesicle was released by compressing it gently with the forceps in MKS. After the emptied seminal vesicles were removed, the released semen was dispersed by shaking the watch glass gently with a hand (Gençer and Kahya 2011). The damage of spermatozoa due to physical manipulation of semen during mixing and handling (Collins, 2004) may artificially cause a low estimation of spermatozoa viability (Holman 2009). Therefore, the seminal vesicles were meticulously emptied without any contact of semen to the tips of forceps. Moreover, as a previous study suggested, no pipette was used when dispersing semen in MKS to maintain the integrity of spermatozoa (Gençer et al. 2014). Then, 150 µl diluted semen was transferred from the watch glass into a sterile microcentrifuge tube (1.6 ml).

The viability of spermatozoa was measured with a Live/Dead Sperm Viability Kit (L-7011, Molecular Probes) by dual staining method as explained in a previous study (Gençer and Kahya 2011). At least 400 sperm cells from each sample were counted. The viability of spermatozoa was

defined as the percentage of live spermatozoa to the total spermatozoa counted.

2.4. Ejaculation ability characters of drones

Two experimental colonies of each subspecies were used for the ejaculation ability characters of drones since there were no adequate drones left in one of the three colonies from each subspecies after the measurements of semen characters. The ejaculation ability of drones was represented by ejaculation proportion (%), ejaculation efficiency per drone (µl), and semen collection efficiency per drone (µl). The ejaculation efficiency and semen collection efficiency were suggested for the first time in this study. Each drone was manually provoked to ejaculate semen. The drone that fully everted its endophallus and released semen was considered successful, but the drone that did not evert its endophallus or not release semen was assumed unsuccessful. The semen of each drone was drawn into the glass tip of the Harbo syringe. The numbers of successful and unsuccessful drones used to collect a dose of 4 µl semen into the glass tip were registered. The total volume of semen (4 µl) collected from successful drones was measured by the Gilmont micrometer.

The ejaculation proportion (%) was calculated by dividing the number of successful drones by the number of total drones used to collect 4 µl of semen. The ejaculation efficiency per drone (µl) was calculated by dividing the unit volume of semen (4 µl) by the number of successful drones used to collect the unit volume of semen. The semen collection efficiency per drone (µl) was calculated by dividing the unit volume of semen by the number of total drones used to collect the unit volume of semen.

2.5. Statistical analyses

The data of drones from experimental colonies were applied to nested ANOVA (the colonies within the subspecies), followed by the Tukey test for multiple comparisons. Statistical analyses and boxplot graphics of the data were performed using Rstudio (Version 1.3.1073) with the *rstatix*, *dplyr*, *tidyverse*, and *ggplot2* libraries.

3. RESULTS

3.1. Body weight

In total, 144 drones were weighed when they were dissected. The body weight of drones ranged from 170 to 236 mg (Supplemental Table I). The mean body weights of Caucasian and Italian drones were 201 and 197 mg, respectively. Moreover, Caucasian drones were significantly heavier than Italian drones ($P = 0.029$; Fig. 1a; Supplemental Table III). The differences in body weight were not statistically significant between colonies within each subspecies ($P > 0.05$; Fig. 1b).

3.2. Ejaculate volume

The ejaculate volume of 60 drones from 6 colonies ranged from 0.80 to 1.30 µl. The mean ejaculate volumes of Caucasian and Italian drones were 1.01 and 0.95 µl, respectively (Supplemental Table I). Caucasian drones ejaculated more semen than Italian drones. The difference (0.06 µl) was statistically significant ($P = 0.033$; Fig. 2a; Supplemental Table III) between Caucasian and Italian drones. No statistically significant differences existed between colonies within each subspecies ($P > 0.05$; Fig. 2b).

3.3. The number and the concentration of spermatozoa in the ejaculate

The spermatozoa number in the ejaculate ranged from 5.19 to 8.28 million. The mean number of spermatozoa in Caucasian drones was 7.05 million. Italian drones had a mean of 6.57 million spermatozoa (Supplemental Table I). Caucasian drones had 7% more spermatozoa than Italian drones. The difference (0.48 million) was statistically significant between Caucasian

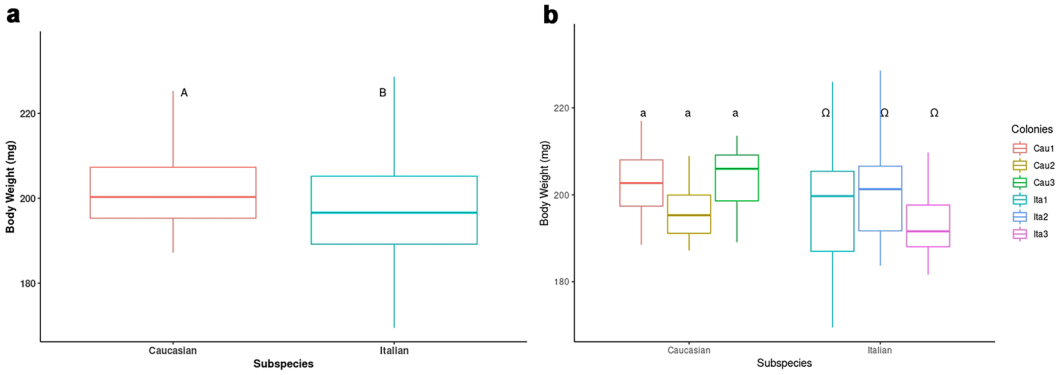

Figure 1. The variation in body weight (mg) of drones from Caucasian (*Cau*) and Italian (*Ita*) subspecies (**a**) and Caucasian (*Cau1*, *Cau2*, and *Cau3*) and Italian (*Ita1*, *Ita2*, and *Ita3*) colonies (**b**). Different capitals (A and B) denote the significant difference between subspecies. Caucasian colonies are not significantly different from each other (small letter: a). Italian colonies are not significantly different from each other (symbol: Ω).

and Italian drones ($P = 0.012$; Fig. 3a; Supplemental Table III). The spermatozoa count in the ejaculate did not differ in colonies within each subspecies ($P > 0.05$; Fig. 3b).

The mean concentration of spermatozoa in the ejaculate was 7.00 million/µl. No significant difference was found between Caucasian (7.03 million/µl) and Italian (6.96 million/µl) drones ($P > 0.05$; Fig. 4a; Supplemental Table III). There was also no significant difference between colonies ($P > 0.05$; Fig. 4b).

3.4. The number of spermatozoa in seminal vesicles

The number of spermatozoa in seminal vesicles ranged from 7.19 to 13.89 million (Supplemental Table I). The mean spermatozoa count in seminal vesicles of Caucasian drones was 10.62 million. But, Italian drones had a mean of 9.55 million spermatozoa. The difference (1.07 million) was statistically significant ($P = 0.019$; Fig. 5a; Supplemental Table III). Caucasian drones produced 10% more spermatozoa than

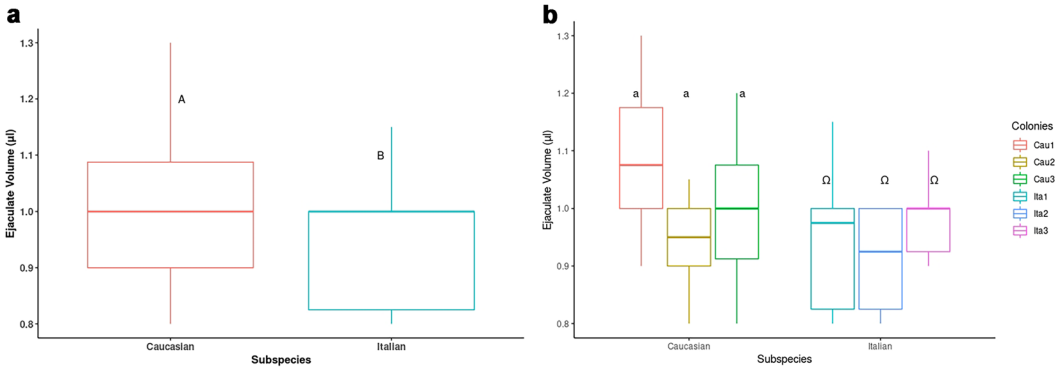

Figure 2. The variation in ejaculate volume (µl) of drones from Caucasian (*Cau*) and Italian (*Ita*) subspecies (**a**) and Caucasian (*Cau1*, *Cau2*, and *Cau3*) and Italian (*Ita1*, *Ita2*, and *Ita3*) colonies (**b**). Different capitals (A and B) denote the significant difference between subspecies. Caucasian colonies are not significantly different from each other (small letter: a). Italian colonies are not significantly different from each other (symbol: Ω).

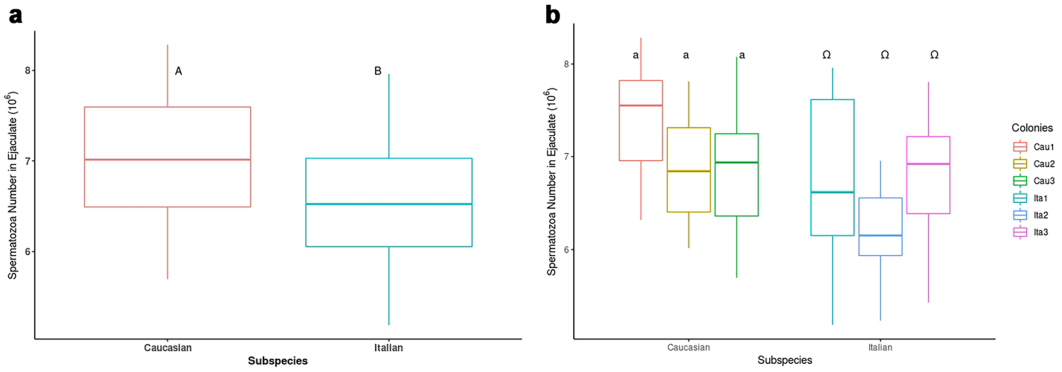

Figure 3. The variation in spermatozoa number in the ejaculates of drones from Caucasian (*Cau*) and Italian (*Ita*) subspecies (**a**) and Caucasian (*Cau1*, *Cau2*, and *Cau3*) and Italian (*Ita1*, *Ita2*, and *Ita3*) colonies (**b**). Different capitals (A and B) denote the significant difference between subspecies. Caucasian colonies are not significantly different from each other (small letter: a). Italian colonies are not significantly different from each other (symbol: Ω).

Italian drones. The number of spermatozoa in seminal vesicles did not differ in Caucasian colonies ($P > 0.05$). Nevertheless, the number of spermatozoa in seminal vesicles differed in Italian colonies. The drones from *Ita2* (8.43 million) had significantly (Fig. 5b) fewer spermatozoa than those from *Ita3* (10.68 million, $P = 0.033$).

3.5. The viability of spermatozoa in seminal vesicles

The mean viability of spermatozoa was 97.9%. There was no significant difference between subspecies (Caucasian: 98.0% and Italian: 97.7%; $P = 0.19$; Fig. 6a; Supplemental

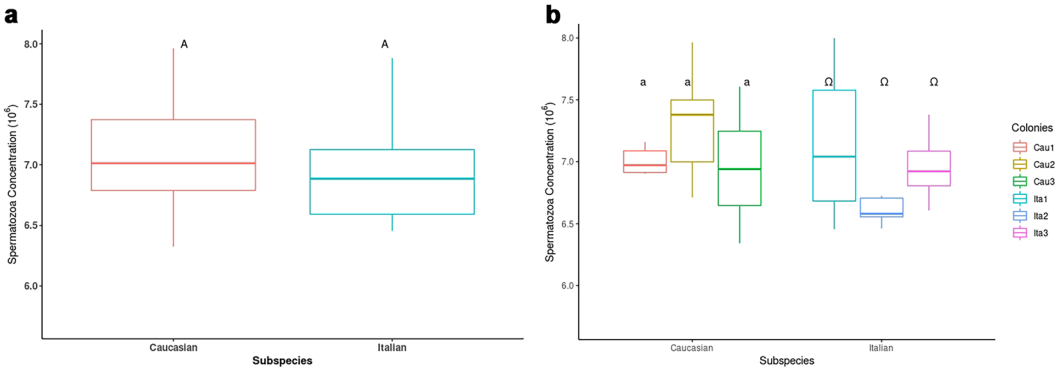

Figure 4. The variation in spermatozoa concentration in ejaculates of drones from Caucasian (*Cau*) and Italian (*Ita*) subspecies (**a**) and Caucasian (*Cau1*, *Cau2*, and *Cau3*) and Italian (*Ita1*, *Ita2*, and *Ita3*) colonies (**b**). Subspecies are not significantly different from each other (capital letter A). Caucasian colonies are not significantly different from each other (small letter: a). Italian colonies are not significantly different from each other (symbol: Ω).

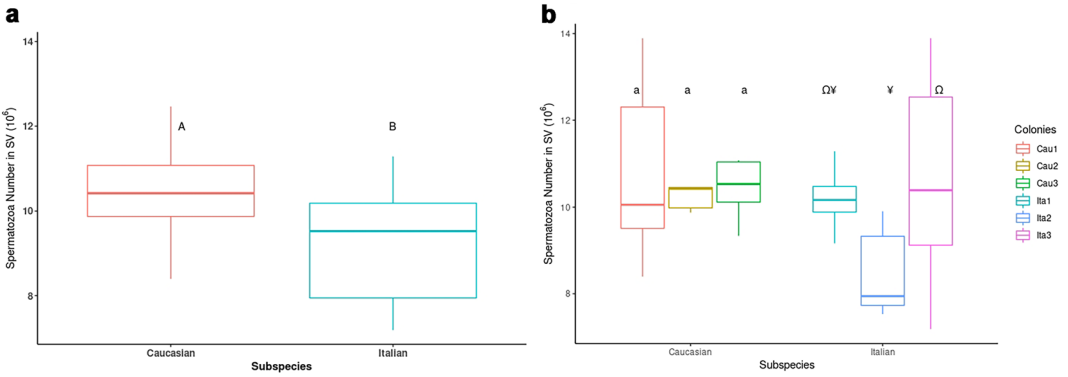

Figure 5. The variation in spermatozoa number in seminal vesicles of drones from Caucasian (*Cau*) and Italian (*Ita*) subspecies (**a**) and Caucasian (*Cau1*, *Cau2*, and *Cau3*) and Italian (*Ita1*, *Ita2*, and *Ita3*) colonies (**b**). Different capitals (A and B) denote the significant difference between subspecies. Caucasian colonies are not significantly different from each other (small letter: a). Different symbols (Ω and ¥) denote the significant difference between Italian colonies.

Table III). Significant differences were also not detected between colonies ($P > 0.05$, Fig. 6b).

3.6. Ejaculation ability of drones

We collected a total of 118 times 4 µl semen (472 µl) into the Harbo syringe from Caucasian (59×4 µl) and Italian drones (59×4 µl) for the ejaculation ability measurements. We used 1113 drones (407 Caucasian and 726 Italian) to collect this amount of semen.

The number of successful drones used to collect 4 µl semen ranged from 4 to 10 (Supplemental Table II). The mean number of successful Caucasian drones (5.8 drones) was significantly ($P = 0.001$; Supplemental Figure 1a; Supplemental Table III) less than the mean number of successful Italian drones (6.9 drones). The difference between *Ita1* (7.1 drones) and *Ita2* (6.7 drones) was insignificant ($P > 0.05$). Similarly,

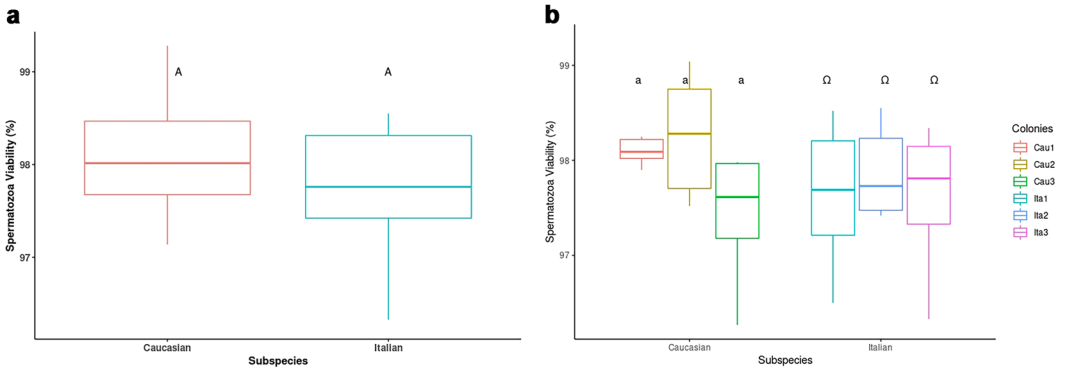

Figure 6. The variation in spermatozoa viability in seminal vesicles of drones from Caucasian (*Cau*) and Italian (*Ita*) subspecies (**a**) and Caucasian (*Cau1*, *Cau2*, and *Cau3*) and Italian (*Ita1*, *Ita2*, and *Ita3*) colonies (**b**). Subspecies are not significantly different from each other (capital letter A). Caucasian colonies are not significantly different from each other (small letter: a). Italian colonies are not significantly different from each other (symbol: Ω).

the difference between *Cau1* (5.8 drones) and *Cau2* (5.7 drones) was not significant ($P > 0.05$).

The number of total drones used to collect 4 µl semen ranged from 4 to 40 (Supplemental Table II). The mean number of total Caucasian drones (6.9) was significantly ($P < 0.001$; Supplemental Figure 2a) less than the mean number of total Italian drones (12.3). There was a significant difference between Italian colonies ($P < 0.001$) but not between Caucasian colonies ($P > 0.05$). The total number of drones used from *Ita1* (21.0 drones) was significantly more than those from *Ita2* (7.5 drones; Supplemental Figure 2b; Supplemental Table III).

A significant difference was determined between Caucasian (86.0%) and Italian drones (73.2%) in the ejaculation proportion ($P < 0.001$; Figure 7a; Supplemental Table III). The ejaculation proportion differed in Italian colonies ($P < 0.001$; Fig. 7b) but did not differ in Caucasian colonies ($P > 0.05$). The ejaculation proportion obtained from *Ita2* (91.1%) was significantly higher than that from *Ita1* (40.9%). The lowest ejaculation proportion was obtained from *Ita1* (40.9%), whereas the highest was from *Ita2* (91.1%) among all experimental colonies.

The mean ejaculation efficiency (µl) in Caucasian drones (0.71 µl/drone) was significantly ($P < 0.001$; Figure 8a; Supplemental Table III) higher than the mean ejaculation efficiency in Italian drones (0.60 µl/drone). The difference was not statistically significant ($P > 0.05$) between *Ita1* (0.59 µl/drone) and *Ita2* (0.61 µl/drone). Similarly, no significant difference ($P > 0.05$) was detected between *Cau1* (0.71 µl/drone) and *Cau2* (0.73 µl/drone).

The mean semen collection efficiency in Caucasian drones (0.62 µl) was significantly ($P < 0.001$; Fig. 9a; Supplemental Table III) higher than the mean semen collection efficiency in Italian drones (0.44 µl). Statistically significant difference ($P < 0.001$) was also detected between *Ita1* (0.24 µl) and *Ita2* (0.55 µl). However, the difference between *Cau1* (0.63 µl) and *Cau2* (0.59 µl) was not statistically significant ($P > 0.05$).

4. DISCUSSION

Our results demonstrated significant differences in body weight, ejaculate volume, spermatozoa counts in seminal vesicles and ejaculate, the number of successful and total drones used to collect per 4 µl of semen, ejaculation proportion, ejaculation efficiency, and semen collection efficiency between the two subspecies. The concentration and the viability of spermatozoa were not significantly different in subspecies. The number of spermatozoa in seminal vesicles, ejaculation proportion, and

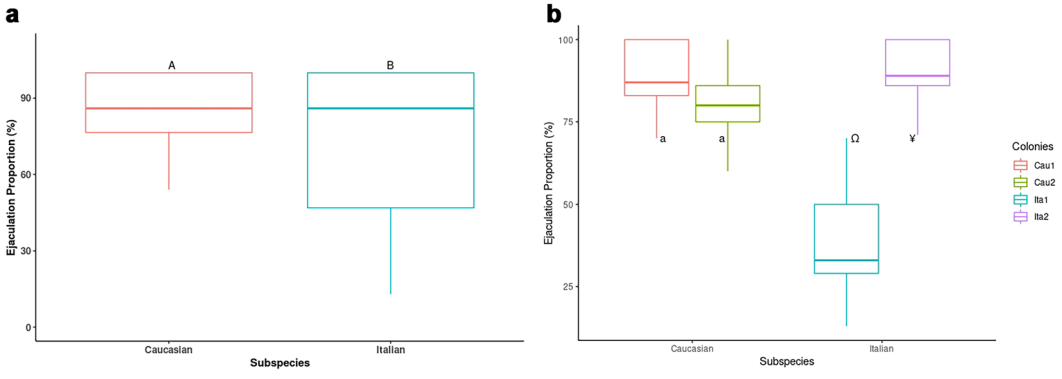

Figure 7. The variation in ejaculation proportion in drones from Caucasian (*Cau*) and Italian (*Ita*) subspecies (**a**) and Caucasian (*Cau1* and *Cau2*) and Italian (*Ita1* and *Ita2*) colonies (**b**). Different capitals (A and B) denote the significant difference between subspecies. Caucasian colonies are not significantly different from each other (small letter: a). Different symbols (Ω and ¥) denote the significant difference between Italian colonies.

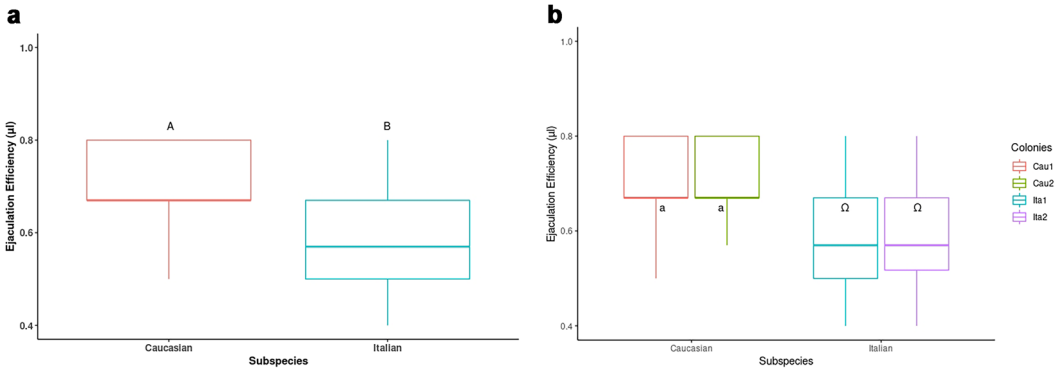

Figure 8. The variation in ejaculation efficiency of drones from Caucasian (*Cau*) and Italian (*Ita*) subspecies (**a**) and Caucasian (*Cau1* and *Cau2*) and Italian (*Ita1* and *Ita2*) colonies (**b**). Different capitals (A and B) denote the significant difference between subspecies. Caucasian colonies are not significantly different from each other (small letter: a). Italian colonies are not significantly different from each other (symbol: Ω).

semen collection efficiency differed significantly between Italian colonies. In contrast, no significant difference was found in these characters between Caucasian colonies. However, body weight, ejaculate volume, spermatozoa counts in the ejaculate, the concentration and viability of spermatozoa, and ejaculation efficiency did not differ between colonies within both subspecies. Caucasian drones were slightly heavier than Italian drones. They had a larger volume of semen, more spermatozoa in both seminal vesicles and ejaculate, and higher ejaculation proportion, ejaculation efficiency, and semen collection efficiency.

The number of spermatozoa is the most studied character as a critical measure in evaluating the reproductive success of individual drones and maternal colonies. We determined the spermatozoa count in intact seminal vesicles and ejaculates by the spectrophotometric method. Many researchers estimated the spermatozoa count either in seminal vesicles (Rinderer et al. 1985; Schlüns et al. 2003; Gençer and Firatli 2005; Mazeed and Mohanny 2010; Taha and Alqarni 2013; Hayashi and Satoh 2019; Metz and Tarpy 2019) or in the ejaculates (Gençer and Kahya 2011; Rhodes et al. 2011; Czekońska et al. 2015;

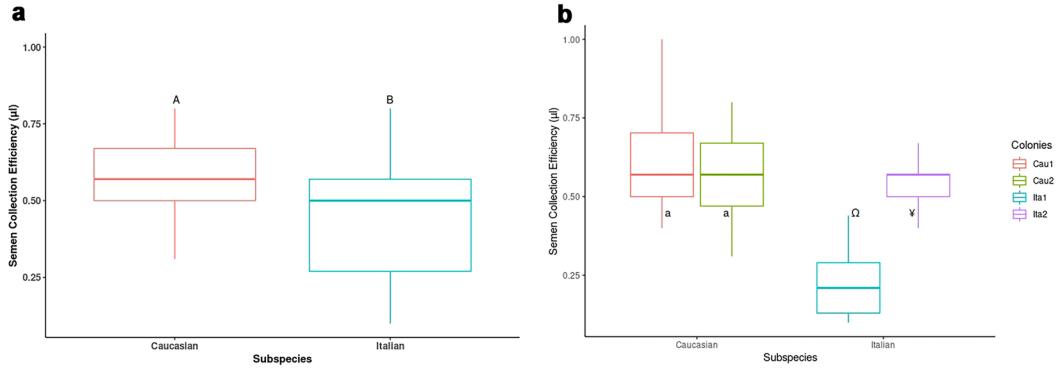

Figure 9. The variation in semen collection efficiency of drones from Caucasian (*Cau*) and Italian (*Ita*) subspecies (**a**) and Caucasian (*Cau1* and *Cau2*) and Italian (*Ita1* and *Ita2*) colonies (**b**). Different capitals (A and B) denote the significant difference between subspecies. Caucasian colonies are not significantly different from each other (small letter: a). Different symbols (Ω and ¥) denote the significant difference between Italian colonies.

Rousseau et al. 2015; Rousseau and Giovenazzo, 2016) by microscopic or spectrophotometric methods. To our knowledge, nobody, however, has reported spermatozoa count in seminal vesicles and the ejaculate together up to now. We did both spermatozoa counting to detect if the ejaculation ability of drones was affected by the number of spermatozoa in seminal vesicles.

Although *Ita2* had the lowest spermatozoa count in seminal vesicles, its ejaculation proportion is the highest among all colonies. In contrast, *Ita1* had the lowest ejaculation proportion among all colonies. However, *Ita1* and *Ita2* drones had similar spermatozoa in their seminal vesicles. These findings demonstrated that the colony rather than the genetic factor affected the ejaculation proportion of drones. These findings also indicated that the number of spermatozoa in seminal vesicles did not affect the ejaculation ability of drones. Therefore, we concluded that the ejaculation ability of drones does not depend on the number of spermatozoa in their seminal vesicles.

No matter how many drones are produced in a colony, drones become worthless unless they ejaculate semen. In addition to semen characters, the ejaculation ability characters should be regarded as indicators of the reproductive success of individual drones and their source colonies. The proportion of drones ejaculating semen (ejaculation proportion) was reported in a few studies. Rhodes et al. (2011) determined the ejaculation proportion in drones reared in different seasons from different genetic lines, ranging from 32.8 to 92.3% (overall: 59.4%). According to Rousseau et al. (2015), the ejaculation proportion in drones reared from Italian and Buckfast colonies ranged from 24.7 to 92.9% (overall: 55.3%). Czekońska et al. (2013b) determined the ejaculation ratio of 69.9 and 88.2% in drones incubated at 32 and 34 °C during capped brood stage. The ejaculation proportion in our study was 86.0% for Caucasian drones and 73.2% for Italian drones. During the measurements of ejaculation ability characters, we observed that Italian drones had more difficulty in everting endophallus than Caucasian drones. More Italian drones were required to collect the same volume of semen. We also observed that collecting ejaculated semen from Italian drones was more laborious and time-consuming than Caucasian drones because the ejaculates of Italian drones were more dispersed on the endophallus than Caucasian drones. The mean ejaculation efficiency (µl) in drones from Italian colonies was 0.11 µl less (about 10% of the average value) than in drones from Caucasian colonies. This situation should be taken into account in the instrumental insemination process.

Ita2 had only 22.3 and 17.3% fewer spermatozoa in seminal vesicles and the ejaculate, respectively, than *Cau1*, which had the highest count in seminal vesicles and in the ejaculate and the highest ejaculation proportion. Nevertheless, *Ita1* had the lowest ejaculation proportion, which was less than half the ejaculation proportion of *Cau1*. *Ita1* and *Ita2* ejaculated nearly equal volumes of semen, and they had similar spermatozoa counts in their ejaculates. However, there was a significant difference in the ejaculation proportion between *Ita1* and *Ita2* (41 and 91%, respectively).

Semen collection efficiency (µl) is a function of ejaculation proportion (%) and ejaculation efficiency (µl). Our findings demonstrated that ejaculation proportion is a noteworthy measure of semen collection efficiency. The difference (0.31 µl) in semen collection efficiency between *Ita1* and *Ita2* resulted not from ejaculation efficiency but from ejaculation proportion. These results also revealed that the ejaculation ability characters are more critical than semen characters (volume, number, concentration, and viability), especially for semen collection in instrumental insemination.

The eversion of the endophallus is a complex anatomical process. Hemolymph plays an essential role in this process as the eversion of endophallus and ejaculation occur under hemolymph pressure inside the abdomen (Woyke and Ruttner 1958; Woyke 2008). The effect of *Varroa destructor* infestation on drone characters was reported in some studies. The drones infested with varroa mites during development produced fewer spermatozoa (Rinderer et al. 1999; Duay et al. 2002), and their flight durations (Duay et al. 2002) and body weights (Duay et al. 2003) decreased with increasing infestation

levels. All colonies in our research apiary were treated against varroa in the early spring to prevent possible differences in drone development due to varroa infestation. However, Italian colonies are inclined to produce more drones than Caucasian colonies, starting earlier and finishing later than Caucasian colonies. This situation may have allowed more varroa reproduction in Italian colonies than in Caucasian colonies. The lower ejaculation proportion in Italian drones might be explained by having less hemolymph pressure, which leads to difficulty in the eversion of the endophallus. It can also be concluded that besides the direct effect, the genetic factor could have indirectly affected the reproductive characters of drones by creating different environments for varroa reproduction.

We did not monitor the cycle of drone brood production in experimental colonies throughout the reproductive season, as it was not the scope of the study. The experimental colonies might vary as to the timing of life history in drone production; some experimental colonies might have started drone production earlier than others and produced more drones at the expense of reducing quality. The reproductive success of a colony increases with the increasing number of drones and daughter queens that were produced and the individual fitness of drones and queens (Kraus et al. 2003). Some colonies produce drones more likely to mate with a queen and have higher paternity shares (Kraus et al. 2003; Couvillon et al. 2010; Gençer and Kahya 2020). The colonies invest considerable incomes in drone production during the reproductive season. The colonies of different subspecies may prefer to produce more drones with lower reproductive quality or fewer drones with higher reproductive quality.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

YK and HVG conceived and designed the experiments, performed the experiments, and analyzed the data; YK prepared the figures and tables; HVG wrote the paper; both authors read and approved the final draft.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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