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Protein supplementation of Africanized honey bee colonies improves drone and semen quality

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ABSTRACT. Honey bee (*Apis mellifera*) drones reared in protein-supplemented colonies may have reproductive advantages over drones reared in non-supplemented colonies. Additionally, climate and genotype can influence drone and semen quality; however, the studies conducted till now have been carried out in temperate climate countries with European honey bee subspecies. Furthermore, it is not known what minerals are associated with sperm quality in honey bees. Therefore, the objective of this study was to evaluate the effect of a protein diet supplement fed to Africanized honey bee colonies on drone and semen quality, as well as on the mineral content and concentration of their semen. The experimental colonies were fed sucrose syrup and a commercial protein supplement, while control colonies were fed only sucrose syrup. Drones reared in these colonies

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were weighed and their thoraxes and abdomens measured. Ejaculated semen collected from the drones was evaluated for volume, sperm concentration, sperm viability, morphological abnormalities, and mineral content. Mineral analyses were also made of drone seminal vesicles. Drones reared in colonies supplemented with the protein diet were significantly heavier and larger than drones from control colonies. Semen volume did not differ significantly between treatments ($P > 0.05$), but the sperm concentration and viability of drones reared in supplemented colonies were significantly higher relative to the control. Furthermore, the rate of abnormalities of sperm cells was significantly lower for drones of supplemented colonies ($P < 0.0001$). There were no differences between treatments for concentration of Mg, P, Cu, Zn, and Se in the ejaculated semen; however, P was significantly more concentrated in seminal vesicles of drones of supplemented colonies. Most of these minerals had not been reported in bee semen before. We conclude that protein diet supplementation provided to Africanized honey bee colonies improves the quality of drones and their semen.

Key words: Africanized bees; Drone; Semen; Nutritional supplement; Minerals; Spermatozoa

INTRODUCTION

The decline of pollinators has negatively impacted natural and managed ecosystems worldwide (Goulson et al., 2015). For honey bees (*Apis mellifera*) in particular, approximately one-third of colonies in North America and parts of Europe have been lost annually since 2007, which is unprecedented (Guzman-Novoa, 2016). These losses have been attributed in part to the so-called Colony Collapse Disorder (CCD), which has multifactorial causes such as pathogens, pesticides, and nutritional stress (VanEngelsdorp et al., 2009). If these factors affect the quality of drones, they could also affect the reproduction and fitness of honey bee populations, since queen bees need to mate with healthy, fit and fertile drones, to produce strong colonies (Rangel and Fisher, 2019). Therefore, the availability of drones and quality of their semen are very important factors to sustain the strength and health of honey bee colonies.

Semen quality may be affected in drone testes during spermatogenesis, which occurs during larval and pupal stages (Yaniz et al., 2020). Three to eight days after drones emerge, the spermatozoa pass from the testes to the seminal vesicles, where they are stored until ejaculation (Hayashi and Satoh, 2019). During the mating process, only 3-5% of the spermatozoa in the semen ejaculated by a drone is stored in the queen's spermatheca to be used in the fertilization of eggs (Lodesani et al., 2004). The volume of the ejaculate, as well as the concentration, viability, and integrity of sperm cells in the semen, can affect the fecundity of the queen, and therefore the fitness of the colony.

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Some important factors that may affect the fertility of drones include age, genotype, body size, season, parasites, and exposure to pesticides (Couvillon et al., 2010; Rousseau et al., 2015; Rangel and Fisher, 2019). Regardless of the above, drones are mainly reared when there are abundant nutritional resources available to the bees, especially proteins. The amount of protein required to raise drones is twice as high as that required to raise workers (Brodschneider and Crailsheim, 2010). Therefore, protein deficiency may affect the quality of drones and semen. Drones reared in colonies with limited access to protein may emerge smaller in size and weight, as well as produce a lower volume of ejaculated semen than drones reared in colonies with unlimited access to protein sources (Czekonska et al., 2015; Szentgyörgyi et al., 2017).

Rousseau and Giovenazzo (2016) reported that the use of protein supplements in European honey bee colonies increased the production of larger drones and improved the quality of their semen, and Gencer and Kahya (2020) showed that the sperm cells of large drones have a higher probability of fertilizing queen eggs compared to those of small drones. Therefore, drones reared in protein-supplemented colonies may have advantages over drones reared in non-supplemented colonies. Additionally, proteins are necessary to produce seminal fluid, which influences sperm viability (Zhao et al., 2021). Another factor to consider regarding the effect of nutrition on the quality of drone semen pertains to the stage of development and age of the individuals. Abdelkader et al. (2014) and Szentgyörgyi et al. (2017) found that restricting protein to adult drones did not affect their fertility, but it did when drone larvae were starved. They concluded that the nutritional investment for drone production occurs during the rearing stage, and that protein supplementation is not important for fertility in the adult stage. This suggests that it is important to use supplemental feeding especially during the rearing stage, which is when colonies must have access to quality protein in sufficient quantity.

Although there is information about the effect of protein supplementation to honey bee colonies on the quality of drone semen, it is not known whether the supplementation of other nutrients such as minerals affects variables such as sperm concentration, viability and morphology. In other animal species it has been found that several minerals play an important role in reproductive aspects. For example, Cu, Se, Mn, Zn, I, Fe, Mo, Cr, Ca, P, and K contribute to sperm viability (Kumar et al., 2011). Additionally, Zn and Se have a high impact on sperm morphological integrity because they are structural components of sperm cells (Behne et al., 1988). Therefore, mineral deficiencies could cause morphological defects in sperm cells.

It is also known that climate and bee genotype may influence drone production and semen quality (Rhodes et al., 2011), but all the studies conducted so far on how nutritional supplementation of honey bee colonies affects the quality of drones and the semen they produce, have been carried out in countries of temperate and cold climates and with honey bees of European subspecies. It is possible that differences in the nutritional quality of pollen sources in temperate and tropical climates might differentially affect the quality of drones produced, and that supplementation might not be needed to rear quality drones in tropical and subtropical environments. Clearly, it is relevant to study whether the effects of colony supplementation are similar for drones

of non-European subspecies reared in tropical and sub-tropical climates. Therefore, this research aimed to evaluate the effect of providing a protein supplement to Africanized honey bee (hybrids of *A. m. scutellata* and European honey bee subspecies) colonies on the size and weight of their drones, as well as on the quality and mineral content of their semen.

MATERIAL AND METHODS

Drone rearing

This study was conducted at the Environmental Centre in Xochimilco, Mexico (19.2º N, 99.1º W; 2200 m altitude), as well as in the Faculty of Veterinary Medicine, both under the jurisdiction of the National University of Mexico, in Mexico DF (19.4º N, 99.1º W). Four Africanized honey bee colonies headed by sister queens were used for this study. The Africanized genotype of the bees was corroborated with morphometric and mitochondrial DNA analyses (Nielsen et al., 1999). The colonies were established in Jumbo size (modified Dadant) hives and were equalized in population, brood, and food stores (Delaplane et al., 2013). Each hive was established with five brood combs covered with bees, along with one comb containing honey, one containing pollen, and two frames with beeswax foundation. The colonies were fed for five weeks during late spring, a period of light nectar flow with several plant species blooming. Two control colonies received a diet of only 50% sucrose syrup (1:1 sucrose: water), and the other two received sucrose syrup and a commercial feed supplement (Ultra Bee, Mann Lake, Hackensack, MN, USA). The supplement contains 50% protein, 6% lipids, and 5% moisture, in addition to vitamins, and minerals (Supplemental Table 1). Patties of 500 g of the supplement were prepared by mixing the formulation powder with sucrose syrup according to the manufacturer's instructions. Sucrose syrup was administered in Doolittle feeders within each hive at a rate of 4 L per week for the two treatments. The patties were placed on the top bars of the brood chambers of supplemented colonies (500 g/week) and were completely consumed by the bees by the end of each week. One week after initiating colony feeding, a frame containing drone cell foundation was introduced into the center of each hive to stimulate drone production. The bees drew comb on these foundation sheets and the queens began to lay eggs in the comb cells within seven days. When the drone cells had been capped, the combs were placed inside confinement cages (46x28x5 cm) that had a 5-square-inch metal mesh to allow the passage of workers but prevented the exit of drones from the cages, so the workers could always attend the drones inside the hive. When emerged drones were first observed, they were left inside the cage for 21 days to reach sexual maturity.

Drone collection

Once the drones reached sexual maturity, the confinement cages were removed from the hives and transported to the laboratory. In the laboratory, each cage was placed

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inside a flight chamber made of acrylic material and screen mesh (40x40x40 cm) where drones were released so they could fly and defecate. Drones were collected individually blindly (i.e., the individual collecting the drones for assessments was blinded to the treatment of the colonies) from the flight chamber and used to evaluate the following parameters: weight, thorax width, abdominal index, ejaculate volume, and semen quality.

Weight and size of drones

Thirty drones per colony were randomly collected and individually weighed on a digital precision scale (Adam PGL2002, Adam Equipment, Milton Keynes, UK). Each drone was introduced into a cylindrical acrylic tube that was kept in a refrigerator (4ºC) for about 1 min to reduce its mobility, and then placed on a scale to register total weight (mg). The weight of each drone was obtained by subtracting the weight of the tube from the total weight. The same 30 drones were used to measure the width of the thorax and length and width of the abdomen (mm). A vernier digital caliper was used for these measurements. In the case of the abdomen, an abdominal index (A.I.) was calculated by multiplying the width by its length.

Semen collection and volume

To collect semen, each of the drones was held with the thumb and index fingers of the left hand, exerting pressure on the abdomen to stimulate endophallus eversion and force ejaculation. The semen was collected in a glass tip adapted to a Harbo syringe (Honey Bee Insemination Service, WA, USA). The average volume of ejaculate per drone was obtained by dividing the amount of semen collected by the number of drones used and three replications of 30 drones per colony were processed.

Sperm concentration

To calculate spermatozoa concentration, the semen was serially diluted in a 0.2% glutaraldehyde solution until obtaining a 1:10,000 (v:v) dilution. A micropipette was used to load a hemocytometer (Neubauer Improved 0.100 mm/0.0025 mm^2 counting chamber; Marienfeld, Mexico City, Mex) with 10 µL of the dilution to perform a sperm cell count under an optical microscope (Leica CME, Fisher, Fair Lawn, NJ, USA). The spermatozoa contained in five grids were counted, the four at the corners and the central one of the hemocytometer. Only spermatozoa with heads within the corresponding grid were counted. Eight repetitions of sperm counts were done per colony. The number of spermatozoa/µL was estimated as per Rousseau et al. (2015).

Sperm viability

Sperm viability was determined using dual eosin/nigrosine staining (EN, Sigma-Aldrich, St. Louis MO, USA), which assesses the integrity of the sperm cell membrane to differentiate living from dead cells (Brito et al., 2003; WHO, 2010). First, a 30%

dilution of semen in Kiev solution was prepared in a 0.5 ml Eppendorf tube (Taylor et al., 2009), and then a smear was prepared. To do this, 5 µL of the diluted semen and 5 µL of the EN stain were mixed, spreading the mixture on the surface of a slide. The stained slide was heat-fixed on a hot plate. The samples were evaluated under an optical microscope. Viable spermatozoa do not show staining since their membrane excludes the dye, being visualized bright on the background, while spermatozoa with an altered membrane allows the dye to penetrate and are stained (Figure 1). The percent viability of sperm cells was determined by counting a minimum of 200 cells per sample, and four samples of pooled semen from 30 drones were obtained and analyzed per colony.

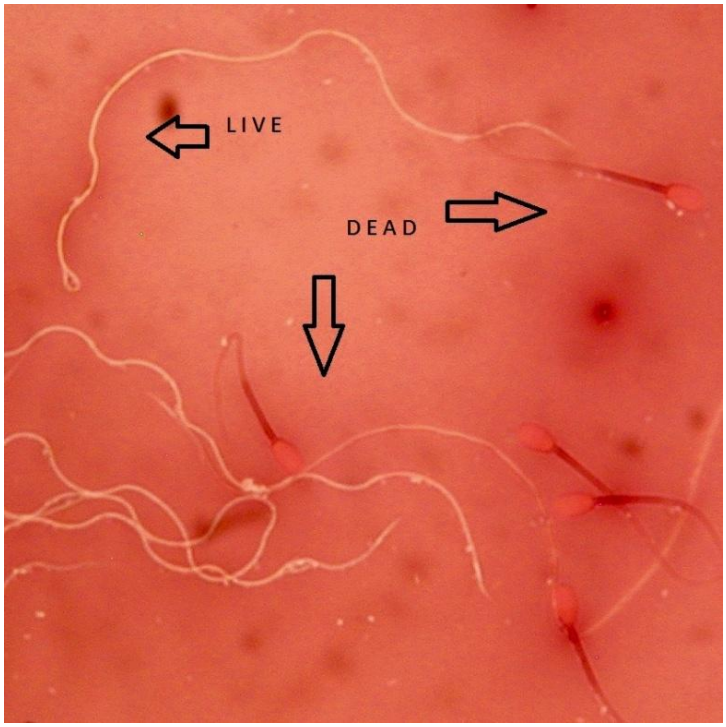

Figure 1. Sperm viability was determined using dual eosin/nigrosine staining. Viable spermatozoa do not show staining because their membrane excludes the dye, while spermatozoa with an altered membrane are stained.

Sperm morphology

To assess sperm morphology, smears of diluted semen samples, like those used to measure viability, were prepared. Two slides were prepared with pooled semen from 30 drones of each colony and 200 spermatozoa per slide were analyzed under the optical microscope (n=400 sperm cells analyzed/colony). The spermatozoa that showed alterations such as those described by Lodesani et al. (2004), such as large heads, double heads, coiled tails, or fragmented tails, were considered abnormal. The percentages of spermatozoa with normal and abnormal morphology were determined, as well as the percentages of the main abnormalities observed.

Minerals in semen and in seminal vesicles

To identify and quantify minerals in ejaculated semen, 30 µL of pooled semen were diluted in 70 µL of deionized water in a 0.5 ml Eppendorf tube, which was the kept frozen until analysis. For mineral analysis in seminal vesicles, these were first obtained as follows. Twenty-five drones per colony were collected and euthanized with ethyl acetate to immediately dissect the seminal vesicles as per Carreck et al. (2013). The dissection of abdomens was performed under a stereoscopic dissecting microscope, removing the exoskeleton and muscles with entomological forceps. Then, the intestine and mucus glands were removed. The seminal vesicles were located and placed in Eppendorf tubes until there were 25 pairs of them per tube. The semen in the vesicles was released by tearing their tissue to fine pieces with fine forceps and then adding 70 µL of deionized water to each tube (Schlüns et al., 2003). The tubes were kept frozen until analysis. Three samples per colony were analyzed for both ejaculated semen and seminal vesicles.

Ejaculated semen and seminal vesicle samples were analyzed according to Khan et al. (2012). Minerals such as Ca, Mg, Cu, Mn, and Zn, were assessed by atomic absorption spectrometry (AAS; García-Alegria et al., 2015), using a Perkin-Elmer 3110 spectrometer (Perkin-Elmer Corporation, Norwalk, CT, USA). For Se, the hydride generation method coupled to AAS was used (Escobar et al., 2011). P was quantified by visible ultraviolet (UV) light spectrophotometry, using the ammonium vanadate technique (Van Gammeren et al., 2008). To calculate the concentration of mineral elements in ejaculated semen and vesicles, the absorbance of the standards of each element read, as well as of each sample of semen and vesicles, was obtained. A linear regression was performed to obtain the coefficient, the intercept, and the slope, as well as the absorbance of the standards, using Microsoft Excel® computer software. The initial concentration or IC (ug/ml) of the mineral elements of each of the samples was obtained with the following formula: $Y = a + b (x)$, where Y is concentration, a is intersection, b is slope, and x is absorbance (Romero-Calderón et al., 2016).

Statistical analyses

Student *t* tests were used to compare control and supplemented treatments and effects of hives within treatments for three drone quality variables (weight, thorax width, abdominal index), two semen quality variables (volume, concentration) and the concentrations of the minerals analyzed in ejaculated semen and vesicles. The distribution and variance homogeneity of the data were examined with the Shapiro-Wilk and Bartlett tests, respectively, before subjecting them to Student *t* tests. Variables such as weight and abdominal index required a power transformation of -0.45, while sperm volume required a power transformation of 6.2 before analysis. The data on quantity of minerals in the ejaculated semen or seminal vesicles did not require transformation. The rates of sperm viability and morphological abnormalities were

analyzed with χ^2 tests. Pearson correlations were also performed between the drone quality variables. The statistical package R 3.3.1 (Foundation for Statistical Computing, Vienna, Austria) was used for statistical analyses.

RESULTS

Drone weight and size

Significant differences between treatments were found for mean drone weight (t=6.14, df=118, P < 0.001). The drones from control colonies weighed on average 202.0±0.01 mg, while the mean weight of the drones from supplemented colonies was 223.1±0.01 mg (Table 1), however, there were effects of hive on drone weight for the supplemented treatment (t=7.19, df=58, P < 0.001). The thorax width of drones from supplemented colonies was significantly greater (5.42±0.01 mm) than that of drones from control colonies (5.28±0.01 mm; t=- 7.61, df=118, P < 0.0001; Table 1) and there were no significant hive effects for this variable (P = 0.15). Conversely, there were no significant differences between both groups of drones for abdominal index (t =-0.82, df=118, P > 0.05; Table 1). Finally, the weight and thorax width of the drones were significantly correlated (r=0.30, P < 0.001), but there were no significant correlations between these two variables and abdominal index (P = 0.25).

Table 1. Weight and size of males and semen quality parameters of drones produced in Africanized honey bee colonies fed or not fed with a commercial diet. Mean individual measurements of weight, thorax width, abdominal index (A. I.), ejaculated semen volume, sperm cell concentration and % sperm cell viability ± SE are shown (n= 120).

Treatment	Weight (mg)	Thorax width (mm)	A. I. (mm)	Semen volume (µL)	Sperm x 10^6/µL	% sperm viability
Supplemented cols.	223.1 ± 0.01*	5.42 ± 0.01*	45.7 ± 0.3	1.5 ± 0.07	6.6 ± 0.3*	87.6 ± 1.8**
Control cols.	202.0 ± 0.01	5.28 ± 0.01	45.4 ± 0.5	1.3 ± 0.09	5.4 ± 0.3	81.1 ± 1.4

* Indicate significant differences (P < 0.01) between treatments based on Student t tests of transformed data.
** Indicate significant differences (P < 0.0001) between treatments based on χ^2 tests. cols. = colonies.

Semen quality

The mean volume of semen ejaculated per drone for males of non-supplemented colonies was 1.3±0.09 µL, while that for drones of supplemented colonies was 1.5±0.07 µL, and there were no significant differences or hive effects for this variable between the two treatments (t=-0.82, df=10, P > 0.05; Table 1). The mean sperm concentration in semen of control drones (5.4±0.3 x 10^6/µL) was significantly lower than that of drones from supplemented colonies (6.6±0.3 x 10^6/µL; t =-2.91, df=30, P < 0.01; Table 1), and there were no effects of colony within treatments (P > 0.05). Sperm concentration in the semen of drones from the supplemented treatment was 22.2% higher compared to the control. The mean sperm viability of control drones was 81.1±1.4%, which was significantly lower than that of drones from the supplemented

colonies, at 87.6±1.8% (χ=25.1, df=1, P < 0.0001; Table 1) and there were no significant effects of colony within treatments (P > 0.05).

The morphological analysis of sperm cells revealed that most of them did not show alterations. However, abnormalities were observed in some of them (Table 2). For the control, spermatozoa with large heads (3.5%), coiled tails (16%) and fragmented tails (6%) were found, while for the supplemented treatment, lower percentages of abnormalities were observed. These were likewise large heads (1.5%), coiled tails (9.5%), and fragmented tails (4%). The percentage of total morphological abnormalities in sperm cells of drones from supplemented colonies (15%) was significantly lower (χ=26.7, df=1, P < 0.0001) than the percentage of morphological abnormalities found in drones from non-supplemented colonies (25.5%).

Table 2. Percent sperm cells that showed abnormalities in the semen of drones produced in Africanized honey bee colonies fed or not fed with a commercial diet. The abnormalities detected included large heads (L. H.), coiled tails (C. T.), and fragmented tails (F. T.) (n= 1600).

Treatment	% L. H.	% C. T.	% F. T.	% Abnormalities*
Supplemented cols.	1.5	9.5	4.0	15.0
Control cols.	3.5	16.0	6.0	25.5

* Indicate significant differences (P < 0.0001) between treatments based on χ^2 tests.

Minerals in ejaculated semen and seminal vesicles

The mean concentration of the minerals analyzed in the samples of ejaculated semen and seminal vesicles of drones from supplemented and non-supplemented colonies are shown in Table 3. No significant differences were found between the treatments for the minerals analyzed in the samples of ejaculated semen (P > 0.05), but for seminal vesicles, P concentration was significantly higher for the supplemented treatment relative to the control (*t*=- 13.45, df=10, P < 0.0001), while that of Cu was significantly lower in the supplemented group (*t*=6.28, df=10, P < 0.0001). There were no significant effects of colony within treatments (P > 0.05).

Table 3. Mean concentration ± SE of five minerals found in the ejaculated (es) and seminal vesicles (svs) semen of drones produced in Africanized honey bee colonies fed or not fed with a commercial protein diet.

Treatment	P (µg/g)	Mg (µg/g)	Cu (µg/g)	Zn (µg/g)	Se (ng/g)
Supplemented (es)	2570	22.6	30.3	23.6	194.0
	± 272	± 1.0	± 1.9	± 4.7	± 20.4
Control (es)	1863	26.3	48.3	19.0	206.7
	± 200	± 3.9	± 8.4	± 2.1	± 18.0
Supplemented (svs)	8354	146188.5	29.3	47.0	460.1
	± 306*	± 13475	± 1.6*	± 9.0	± 29.7
Control (svs)	4138	137400.8	61.5	48.7	391.3
	± 66.8	± 7319.1	± 4.9	± 7.0	± 67.5

* Indicate significant differences (P < 0.01) between svs treatments based on Student *t* tests.

DISCUSSION

This study was carried out to evaluate the effect of providing a nutritional supplement to Africanized honey bee colonies on drone and semen quality, as well as on the mineral content and concentration in the semen. The commercial diet significantly increased the weight and size of the drones produced in the supplemented colonies, although the abdominal index did not vary between treatments and there were effects of colony on the weight of the drones. Previous studies have found that feeding honey bee colonies with protein supplements influences drone development, resulting in increased weight and size of the males that were produced (Rousseau and Giovenazzo, 2016; Rangel and Fisher, 2019), which is consistent with the results of this study, although additional studies with Africanized colonies will be required to confirm these results. It appears that the weight and size of drones is positively related to the quantity and quality of the nutrients that male bees receive during the larval stage (Szentgyorgy et al., 2017), which may determine their body size when they are adults. This is what most likely happened in this study since protein supplementation was provided during the larval stage of the drones.

The size and weight of drones may influence semen quality variables, including sperm concentration and viability (Schlüns et al., 2003; Rousseau and Giovenazzo, 2016; Zhao et al., 2021). Likewise, the size and weight of drones also seem to influence lifespan, sexual maturity, and mating success (Couvillon et al., 2010; Czekońska et al., 2019; Metz and Tarpy, 2019). In Africanized drones, in particular, Duay et al. (2002) found that drones that had a higher concentration of spermatozoa were more efficient fliers, which could contribute to mating success. In this study, the artificial diet fed to the experimental colonies did not result in larger volumes of drone ejaculated semen, but it was associated with increased sperm concentration and viability, as well as with decreased frequency of sperm abnormalities. Drones from supplemented European honey bee colonies that are heavier produce more concentrated semen with higher sperm viability than drones from non-supplemented colonies (Rousseau and Giovenazzo, 2016; Rangel and Fisher, 2019), which was also confirmed in our study. Therefore, most studies, including this one, support the notion that additional supplementation to honey bee colonies is a desirable management practice to produce drones that ejaculate higher quality semen, regardless of their genetic background.

Although the drones of the two treatments did not differ in the volume of ejaculated semen, it was within the range of 0.9 to 1.8 μL of semen/drone that has been reported from other studies (Mazeed, 2011; Rhodes et al., 2011). The fact that differences between treatments were not found for semen volume is not surprising. It has been suggested that semen volume does not necessarily depend on protein availability, but rather on the presence of other specific nutrients, such as sugars, amino acids, vitamins, and lipids, which are present in the seminal fluid (Baer et al., 2009), something that was not analyzed in our study.

Sperm concentration increased with the use of a commercial protein diet in this study. Furthermore, the concentration of sperm cells in drone semen was more than 50% higher than that reported by other authors for bees of European subspecies

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(Rhodes et al., 2011; Rousseau and Giovenazzo, 2016). It is possible that these differences could be due, at least in part, to the genotype of the bees studied. Rinderer et al. (1985) found that Africanized drones produce more concentrated semen than do European drones. This particularity could represent an advantage for Africanized drones, which could result in greater reproductive success because by ejaculating a more concentrated semen, Africanized drones would have a higher probability of fathering more workers of their patriline in a colony, compared with European drones, if the queen mated with the two types of drones (Schneider et al., 2004). An additional factor that might explain the difference in sperm concentration of the drone semen in this study compared to that found in previous studies, is the food source used, something that warrants further investigation.

Another of the parameters evaluated in the semen of Africanized drones was sperm viability. Most studies have reported viability percentages greater than 80% using different staining techniques. The percentage of sperm viability found in both treatments in this study agrees with previous reports (Rosseau and Giovenazzo, 2016; Zhao et al., 2021). It also coincides with the finding that drones from supplemented colonies had a significantly higher sperm viability rate than drones from non-supplemented colonies. Conversely, other studies have concluded that sperm viability is not affected by unlimited or limited access to pollen protein (Czekonska et al., 2015), although pollen restriction resulted in smaller drones. Clearly, additional studies are required to confirm the apparent beneficial effect of supplementing colonies on the viability of sperm from the drones they produce.

As for *A. mellifera* sperm morphology, there are not many antecedents of using abnormalities as an indicator of low fertility. However, the abnormalities detected in our study coincide with those described in other studies that used European drones, mainly in terms of coiled and fragmented tails (Lodesani et al., 2004; Gontartz et al., 2016). These abnormalities could be attributed to the length of the drone sperm cell because the tail or flagellum is very long, measuring up to 270 µm, which could cause it to tangle with the flagella of other sperm cells, or to fragment (Taylor et al., 2009; Yaniz et al., 2020). This would prevent the spermatozoa from reaching the queen's spermatheca. In this study, the frequency of sperm cell abnormalities was significantly lower in the semen of drones from supplemented colonies than in that of drones from non-supplemented colonies. This is a novel result of which there is no history for honey bees. In other animal species, it has been shown that a protein diet and the addition of minerals to the diet influence the structure of the spermatozoa, the maintenance of their membranes, and the motility of the flagella (Lino et al., 2000), which perhaps also occurs in drone sperm cells.

To the best of our knowledge, there are no previous studies that have reported the presence and concentration of more than one mineral in drone semen. Verma (1973) quantified only Mg in semen of European drones but did not correlate it with reproductive variables. Minerals that have been related to reproductive aspects in other species, such as Mg, P, Cu, Zn, and Se, were also found in the semen from drones of both treatments in this study. However, there were no differences in concentration for any of the minerals in the ejaculated semen between drones from supplemented and

non-supplemented colonies. It is possible that the sample size used in this study did not allow observing differences between treatments. Although the concentration of minerals quantified in the ejaculated semen of the two treatments was not different, their presence indicates their potential contribution to the production of antioxidant enzymes such as glutathione peroxidase and super oxide dismutase, present in high quantities in the semen of drones and in the spermatheca of queens (Weirich et al., 2002; Abdelaker et al., 2014). These enzymes need Zn, Se, Mn, and Cu as co-activators.

Our study also analyzed the presence and concentration of minerals in the seminal vesicles of drones. It was found that the concentration of P was higher in the seminal vesicles of drones from the supplemented colonies, possibly because this mineral is included in the supplement. However, unexpectedly, the concentration of Cu in the seminal vesicles of drones from non-supplemented colonies was higher than that in the vesicles of drones from supplemented colonies. This result is difficult to explain, which warrants further investigation. The minerals in the seminal vesicles most likely influence the quality of *A. mellifera* semen because secretions are produced in these organs that help keep sperm cells viable and increase their longevity before being ejaculated (Hayashi and Satoh, 2019). Currently, there are no studies on the type and quantity of minerals necessary in bee diets associated with their benefits for reproduction. Given the little information that exists regarding the role that minerals play in the viability and fertility of honey bee semen, it is necessary to carry out more studies to find out which minerals provide benefits to sperm cells, so that they can be incorporated into supplementary diets in the future.

In conclusion, this study shows that the use of feed supplements in the diet of Africanized honey bee colonies can influence the quality of the drones produced, as well as the quality of their semen, confirming that different honey bee genotypes, including those from sub-tropical climates, can improve reproductive parameters of their males if their colonies receive supplementary feeding. The study also reports for the first time the presence of several minerals in the semen of drones. According to recent studies, the proportion of low-quality drones available for mating with queens is alarmingly increasing (Metz and Tarpy, 2021), highlighting the importance of studying and improving semen quality parameters, since drones contribute significantly to the genotypic and phenotypic characteristics of honey bee colonies. It is critical to carry out single-factorial and multifactorial studies to find out which nutrients and what specific amounts provide benefits to drones and semen, to incorporate them into artificial diets for honey bee colonies.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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