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## Reproductive Dynamics of *Apis mellifera intermissa* Drones in Northern Algeria: Seasonal Patterns in Sperm Production and Mucus Gland Activity

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### Abstract

This study examines the reproductive biology of drones in *Apis mellifera* colonies, focusing on spermatozoa presence and mucus gland activity during breeding and non-breeding seasons. Drones, essential for fertilizing virgin queens, have a reproductive system including testes, seminal vesicles, mucus glands, and an endophallus, which are affected by environmental factors, age, diet, and diseases. The current research was conducted in Azeffoun, Algeria, from March to June and in October 2018. The investigation consisted to analyze the presence of spermatozoa in seminal vesicles and the secretory activity in mucus glands according to breeding and non-breeding seasons.

The results showed the presence of spermatozoa in 62.78% of the seminal vesicles, with higher rates during the breeding season. Drones from hives with two-year-old queens and larger colonies exhibited greater sperm production. Mucus gland activity was observed in 96.51% of drones, with secretion rates between 92.86% and 100% according to the seasons. Sperm production peaked between March and June and declined significantly in October.

Despite reduced sperm production in the non-breeding season, the presence of drones allows beekeepers to initiate queen rearing, ensuring colony survival through winter. This study highlights the critical role of drone reproduction in maintaining honeybee colony health and sustainability.

**Keywords:** Bee Colonies; Drones; Sperm Production; Mucus Glands; *Apis mellifera intermissa*; Histological Analysis.

## Introduction

The social structure of bee colonies includes three castes: the queen, the workers, and the drones. Females populate the hive year-round, but males appear only during the reproductive period, which corresponds to the spring season. During their short lifespan, drones are responsible for fertilizing virgin queens. In fact, the primary role of a honeybee drone is to produce and transmit semen to the queen during the mating flight (Czakońska et al., 2015). The reproductive system of drones includes a pair of testes, a pair of vasa deferentia, paired glands (seminal vesicle and mucus gland) associated with the reproductive tract, and a long ejaculatory duct (Sawarkar and Tembhare, 2015a,b). The copulatory apparatus, known as the endophallus, is a large and complex tube located within the abdomen. Additionally, two male accessory glands have been described in the copulatory apparatus of *Apis mellifera* drones: the bulbous gland (Koeniger et al., 1996; Woyke, 2008; Woyke, 2010; Moors, Koeniger, and Billen, 2012) and the cornua glands (Koeniger et al., 1996; Moors et al., 2009). Honeybee semen consists of spermatozoa diluted in seminal fluid. Various studies have revealed the effect of several factors on semen quality, including drone age (Locke and Peng, 1993; Sturup et al., 2013; Rousseau et al., 2015b), food quality (Sturup et al., 2013; Czakońska et al., 2015; Rousseau and Giovenazzo, 2016), genetic lineage (Rinderer et al., 1985; Rousseau et al., 2015b), storage (Locke and Peng, 1993; Alçay et al., 2019), body size (Schlüns et al., 2003; Gençer and Kahya, 2011; Goins and Schneider, 2013), thermal conditions (Bieńkowska et al., 2011; Sturup et al., 2013), and various contaminants such as *Varroa destructor* (Gençer and Kahya, 2011) and *Nosema* (Peng et al., 2015; Ben Abdelkader et al., 2015; Ciereszko et al., 2017).

Research on honeybee drones has primarily focused on spermatozoa and the factors affecting their fertility, while less attention has been given to the accessory sex glands, despite their significant role in reproductive success. These glands produce crucial molecules for sperm viability. Some sex accessory glands also serve as sperm storage sites until mating (Sawarkar and Tembhare, 2015b). Their secretions are essential for reproductive success (Billen and Morgan, 1998; Klein et al., 2021). In particular, mucus gland secretions may contribute to sperm longevity and storage (Sawarkar and Tembhare, 2015a,b), as well as sperm activation and capacitation (Chen, 1984). Furthermore, gland secretions are involved in spermatozoon nutrition and facilitate sperm movement through the reproductive tract (Colonello and Hartfelder, 2003; Gillott, 1996). Additionally, mucus gland secretions may influence the

queen's post-copulatory behavior and support spermatozoa after their transfer into the female reproductive tract (Wolfner, 1997; Gillott, 1996).

The mucus glands are notably large, occupying a significant portion of the drone's abdomen (Moors et al., 2005), underscoring their crucial role in reproduction. The size of the mucus glands gradually increases during the pupal-to-adult development of drones (Sawarkar and Tembhare, 2010). Secretory material starts accumulating in the lumen of the mucus glands shortly after emergence, with a peak activity within the first five days of adult life in Africanized honeybees (Colonello and Hartfelder, 2003) or between 6 and 12 days in *Apis cerana indica* drones (Sawarkar and Tembhare, 2010).

*Apis mellifera intermissa* is a subspecies found in North Africa, particularly in Tunisia, Algeria, and Morocco (Cornuet et al., 1988; Grissa et al., 1990). In addition to its role in enhancing apiculture production, this subspecies is of particular interest for its role in pollinating native vegetation. In Algeria, it is the dominant hive bee in the apiculture industry. This ecotype is highly adaptable to varying climatic conditions (Adjlane and Haddad, 2014; Barour et al., 2011). It is a small bee with dark pigmentation (Shaibi et al., 2009) and is known for its aggressive and nervous temperament. According to Gadbin et al. (1979), this subspecies exhibits a strong tendency to swarm, particularly in the spring when workers construct numerous royal cells (Clément et al., 2002). This characteristic makes *Apis mellifera intermissa* an excellent producer of royal jelly. Le Conte et al. (2007) demonstrated that this subspecies is highly tolerant to *Varroa* infestations. Furthermore, it exhibits superior hygienic behavior and cleaning abilities compared to other subspecies (Adjlane and Haddad, 2014). It has shown remarkable resistance to various infectious agents, including the *Nosema* fungus, *Paenibacillus larvae* (the causative agent of American foulbrood), and the Deformed Wing Virus (DWV). These adaptive traits make *Apis mellifera intermissa* an excellent candidate for selective breeding programs (Haddad et al., 2015), particularly given its ability to produce propolis.

Numerous studies have investigated drone fertility in honeybee species, focusing on whole semen or individual sperm cells to better understand the negative impacts of beekeeping diseases and pesticides on drone fertility. However, the influence of extrinsic factors such as rearing season, colony size, queen age, and the dynamics of mucus gland secretion has not been thoroughly explored.

Given the economic and ecological importance of *Apis mellifera intermissa*, it is essential to prioritize research on drone reproduction. Thus, the aim of the present study is to investigate the percentage of males showing the presence of spermatozoa in the seminal vesicles and the accumulation of secretory material in the mucus glands according to breeding and non-breeding seasons.

## **Material and methods**

### **Preparation and Observation of Histological Slides**

#### **Sampling**

##### **Animals**

The sampling was conducted in two apiaries located in Azeffoun, Tizi-Ouzou province, Algeria, at altitudes of 150 meters and 450 meters above sea level. Two categories (immature and mature drones) of males were collected from twelve beehives between March and June 2018, a period coinciding with the queen's egg-laying phase. It is important to note that samples could not be collected during the remainder of the year, as the queen ceased egg-laying and no drones were present. Samples were also collected in October, known in Algeria as favorable climatic conditions corresponding to the flowering eucalyptus and viscous inula.

The immature males were aged less than two weeks, and they were harvested inside the hive. They presented less developed bodies, purple eyes, white fuzz, low activity levels, and lacked the typical vibration response. The mature drones (more than two weeks old) were collected from the flight area near the congregation sites. These drones were characterized by large, dark, and hairy bodies, black eyes, and high activity levels, with a vigorous response to pinching. A total of 86 samples were selected for histological examination.

#### **1.2. Reproductive system collection**

The sampling technique consisted of removing the entire reproductive system using a pinching method. Each drone was grasped between the thumb and forefinger, with its head drawn toward the palm and the abdomen facing outward. Using blunt tweezers, the last two tergites were grasped and gently pulled, causing a tear between the 5th and 6th abdominal segments. This allowed the reproductive system, attached by the ejaculatory duct to the bulb in the last pinched segments, to be extracted. The samples were then immediately placed into labeled Eppendorf tubes containing 10% formalin for preservation.

## **2. Histological Sections**

The preparation and observation of histological slides were conducted at Bejaia University, Medical Laboratory.

Upon arrival at the laboratory, the samples were individually placed into histocassettes, each labeled with its respective reference. The samples were then subjected to a dehydration process using an automatic tissue processor. This process involved eight ethanol baths of progressively increasing concentrations (70%, 75%, 80%, 85%, 90%, 95%, 96%, and 100%), with each bath lasting 45 minutes. Following dehydration, the samples underwent two xylene baths, each lasting 1 hour and 30 minutes (for clearing), and then two paraffin baths, each lasting 30 minutes (for impregnation).

The samples were subsequently placed in metal molds and embedded in paraffin to create paraffin blocks. These blocks were solidified on a refrigerated plate. Once hardened, they were sectioned using a microtome. Initial rough sections were cut at 10  $\mu\text{m}$ , followed by refined histological sections of 2  $\mu\text{m}$  thickness after removing the histocassettes from the mold.

The fine histological sections were carefully transferred onto slides pre-soaked in water. They were spread using a water bath, placed on a hot plate at 69°C to dry, and then transferred to an oven set at 75°C overnight for dewaxing.

For each sample, four slides were prepared, with serial sections generated to yield a total of 28 slides from a single specimen.

## **3. Staining**

Before staining, the slides were subjected to a series of preparatory baths: xylene for 30 minutes, 10% ethanol for 10 minutes, followed water bath for 3 minutes. The staining process involved immersing the slides in hematoxylin for 7 minutes, a quick rinse with water, and then dipping the slides in eosin for 3 minutes.

## **4. Slide Assembly**

The slides underwent a series of baths to improve fixation: an ethanol bath, followed by a mixture of ethanol and xylene, then two consecutive baths in pure xylene. Finally, a drop of EUKITT® adhesive was applied to permanently fix the coverslip. The slides were allowed to air-dry for 24 hours.

## 5. Observation of Histological Slides

The histological slides were observed under a microscope to examine the spermatozoa packed in the seminal vesicle or duct, along with the secretory material in the mucus glands. The analysis of histological slides is based on an objective assessment by considering both the presence or total absence of spermatozoa and secretions of the mucus glands.

## 6. Data analysis

The data were entered into Microsoft Excel (2007; Microsoft Corp., Redmond, WA, USA) and imported into SAS software (Statistical Analysis Systems Software, 2001) for statistical analysis. The Chi-square test of independence and Fisher's exact test were used to determine the association between the presence of spermatozoa and secretory mucus glands, and the rearing period, queen age, hive population and drone age. A p-value of 0.05 was used to determine the level of significance.

## Results

### A. Presence of Spermatozoa

In this study, 86 samples were collected from twelve bee hives during the breeding season (March: 29.07%, April: 36.05%, May: 10.47%, June: 8.14%) and in October (16.28%). The age of the queens from the sampled hives ranged from 1 to 5 years. Regarding the hive population, 32.56% were considered heavily populated, 47.67% moderately populated, and 19.77% sparsely populated. Among the studied drones, 41.86% were immature and 58.14% were mature ( $P = 0.13$ ).

The majority of the samples showed the presence of spermatozoa in the seminal vesicles (62.78% vs. 37.22%,  $P = 0.02$ ). During the breeding season (March to June), the percentage of drones with spermatozoa in the seminal vesicles were 60%, 80.65%, 66.67%, and 100%, respectively. In contrast, in the non-reproduction season (October), only 7.14% of the males showed spermatozoa in the seminal vesicles (Figure 1). The presence of spermatozoa was recorded in 65.38%, 85.71%, 68.75%, and 86.67% of drones from hives with queens aged 1, 2, 3, and 5 years, respectively (Figure 2). Hives with queens aged 4 years showed lower spermatozoa presence, particularly outside the breeding season (October).

Hives with a heavily population showed the highest spermatozoa presence rate (89.29%), compared to 70.59% of weak hives and 41.46% of moderate population (Figure 3). Spermatozoa presence was observed in 76% of mature drones, compared to 24% in immature drones ( $P = 0.002$ ) (Figure 4).

### **B. Mucus Gland Activity**

The majority of drone exhibited a secretory activity in the mucus glands (96.51% vs. 3.49%,  $P < 0.0001$ ). The frequency of mucus gland secretions varied from 92.86% to 100% depending on the reproduction season (Figure 5).

The presence of mucus gland secretions was recorded in 96.15%, 85.71%, 100%, 92.89%, and 100% in drones from hives with queens aged 1, 2, 3, 4, and 5 years, respectively (Figure 6). Similarly, the age of drones appears to affect the secretion activity of the mucus glands, with 91.67% and 100% in immature and mature drones. Additionally, mucus secretion was observed in 96.43%, 95.12%, and 100% of drones from strong, moderate, and weak hives, respectively.

## **Discussion**

### **Evaluation of bee male reproduction through a histological approach**

In this study, the reproduction activity of male honey bees was investigation through the presence of spermatozoa in seminal vesicles and the secretory material in mucus glands during the breeding and non-breeding seasons.

#### **1. Sperm Production**

The results showed that during the breeding season, the percentage of drones containing spermatozoa in the seminal vesicles increased from the beginning of the reproduction season (March: 60%) to reach 100% in June. During the non-reproduction season (October), this rate dropped significantly ( $P=0.0001$ ), with only 7.14% of drones showing sperm in the seminal vesicles. The abundant production of drones during the breeding season is positively correlated with sperm production. This is probably related to the abundance of pollen (a source of protein) and nectar (a source of energy).

In spring, the study region is teeming with nectar-producing plants such as oxalis, heather, rockrose, thistle, borage, thyme, lavender, clover, alfalfa, as well as various fruit trees (cherry,

plum, apple, pear, apricot and citrus). The abundance of nectar and pollen is known to stimulate queens laying rate, which leads to the exponential colony growth, particularly thanks to nurse worker bees responsible for raising drones. This reproduction period marked by the abundance of resources, lasts from February to late June, corresponding to the onset of summer heat. However, the months of July and August are characterized as warm and dry periods, which reduce significantly bee activity resulting in a temporary cessation of queen egg-laying. Nevertheless, it is observed that by September, a modest flowering reappears with a few rare nectar-producing plants, such as eucalyptus and inula viscosa. The variable density of these plants and the moderate temperatures allow the queens to resume laying eggs with a sporadic appearance of drones. The current study showed that only 7.14% of drones presented sperm in the seminal vesicles revealing that both the number of drones and their spermatogenesis are strongly affected by the season and, most likely, by the richness of nectar resources and the available food supply.

This has been previously reported where the season appeared as a potent factor with males appearing progressively from spring to early summer. However, according to Boes (2010), bee colonies may adjust drone production based on a combination of environmental factors, such as food availability, worker population, external temperatures, day length, and carbon dioxide concentration in the hive. Rhodes et al. (2011) also reported that the quality of drones varies throughout the bee keeping season, and the rearing of drones depends heavily on the available food resources. Access to pollen during larval development directly impacts the quality of drone reproduction. In fact, Czekońska, Chuda-Mickiewicz, and Samborski (2015) demonstrated that protein deprivation, due to the permanent installation of pollen traps during larval development, leads to the emergence of small-sized drones with low ejaculation capacity compared to drones from hives without pollen traps. However, sperm quality in both groups remains similar in terms of concentration and sperm viability.

Rousseau and Giovenazzo (2016) found that supplementation with pollen (15% protein *ad libitum*) and feeding syrup (1.2 liters of sucrose per week) affected positively the size, weight, sperm production, and sperm viability. On the other hand, Sturup et al. (2013) observed that pollen deprivation after drone emergence does not affect their fertility, and sperm viability remains preserved.

The development of honeybee colonies during the beekeeping season, including drone rearing, is affected by various other factors such as colony size, queen age, availability of stores, nectar

and pollen abundance, and climatic conditions. Particularly, the rising of external temperatures trigger the activity of the colony after its winter dormancy. The plentiful nectar resources, combined with the gradual warming of spring temperatures, boost bee activity and prompt queens to increase their egg-laying.

Sturup et al. (2013) when testing the fertility of drone bees exposed experimentally to extreme temperatures (39°C) for 24 hours, reported a significantly reduced sperm viability. Higher than 40°C, the viability of the males is compromised.

In the semi-arid conditions of the Mediterranean Basin, the emergence of drone bees extends from February to July, with a peak occurring in April and May (Zaitoun et al., 2009). In contrast, in the northern hemisphere, in eastern Canada, bee keeping season runs from May to August. In this region Rousseau et al. (2015a) reported that the sperm volume varies through to the breeding season. In Australia, Rhodes et al. (2011) showed that the reproduction season is occurs in spring, summer, and autumn, corresponding to the period from October to April. They showed that the ejaculation capacity was not affected by the season. However, sperm concentration was significantly influenced by the season of drone rearing. Specifically, sperm volume was significantly higher in drones raised in the spring (October and November). Morais et al. (2022) reported in Brazil that sperm count, viability, and motility were significantly higher during the wet season compared to the dry season. Power et al. (2020), using histological analysis, revealed the lack of seminiferous tubule maturation or even their degeneration in spring (from March to June) in the Campania region in Italy.

## **2. Mucus Gland Secretions**

During drones sexual maturation period, spermatozoa gradually migrate from the gonads to the seminal vesicles, where they are stored in anticipation of mating (Sawarkar and Tembhare, 2015a,b). Concerning the accessory glands, their primary role is the exocrine function related to the secretion of the mucus glands, affecting consequently sperm volume. In this study, histological analysis allowed the evaluation of secretory activity both during the reproduction and the non-reproduction seasons. More than 90% of the samples showed mucus glands secretions both in the reproduction and non-reproduction seasons, with a maximum percentage (100%) in May and June, corresponding to the peak of the breeding season in North Africa.

In domestic honey bees, the drone brood corresponds to the peak availability of nectar-producing resources. This food abundance, affects positively drone reproductive function by stimulating their metabolism. Soon after the emergence of drones, the mucus glands produces a viscous liquid containing proteins (Sawarkar and Tembhare, 2010). The intensity of the secretory activity in the mucus glands is directly dependent on the nutritional status during the larval stage. Rousseau, et al. (2015) reported that at the beginning of the beekeeping season in May, when nectar and pollen resources are still limited, the first cohorts of drones exhibit very small sperm volumes. (Czekońska et al. (2015) also showed that protein deprivation during larval development results in the emergence of small drones with low ejaculation capacity and restricted sperm volumes, due to insufficient secretory activity. However, according to Rousseau and Giovenazzo (2016), supplementation with syrup and pollen during the early stages of drone rearing improves their quality and fertility, with substantial sperm volumes.

### **3. Queen Age and Drone Reproduction**

The current study revealed a marked difference in sperm production when comparing the breeding and the non-breeding season. However, the secretory activity of the mucus glands, showed less differences between these two seasons. These findings are even more striking when drones come from queens of different ages. For instance, the drones collected in the non-reproduction season were produced by a four-year-old queen. These drones exhibited very low values, both in terms of sperm presence and mucus secretion, compared to those sampled during the peak of the breeding season.

In contrast, drones collected during the breeding season were produced by queens from one to five years old. Throughout this period, the drones exhibited high sperm presence and mucus gland activity, peaking in June. This suggests that the queens' age does not significantly influence the drones' reproduction.

### **Conclusion**

This study underscores the significant relationship between the breeding season and sperm production in drones in northern Algeria. Sperm production peaked during the bee reproductive season, remained stable throughout late spring, and then declined sharply outside this period.

The temporary presence of males during the non-reproductive season, albeit with reduced sperm production, provides beekeepers with a valuable opportunity to initiate queen rearing

and restore orphaned colonies. This practice is particularly crucial during periods of intense summer heat and resource scarcity, which often jeopardize colony stability. By enabling colony renewal and bolstering queen production, even during suboptimal reproductive periods, could enhance colony survival through the winter and into the following spring.

The presence of drones and the production of queens during the non-reproductive season, despite their reduced fertility, may contribute to greater colony resilience. This adaptation could play a key role in mitigating the effects of seasonal stressors, ensuring a robust population capable of thriving during the active spring season.

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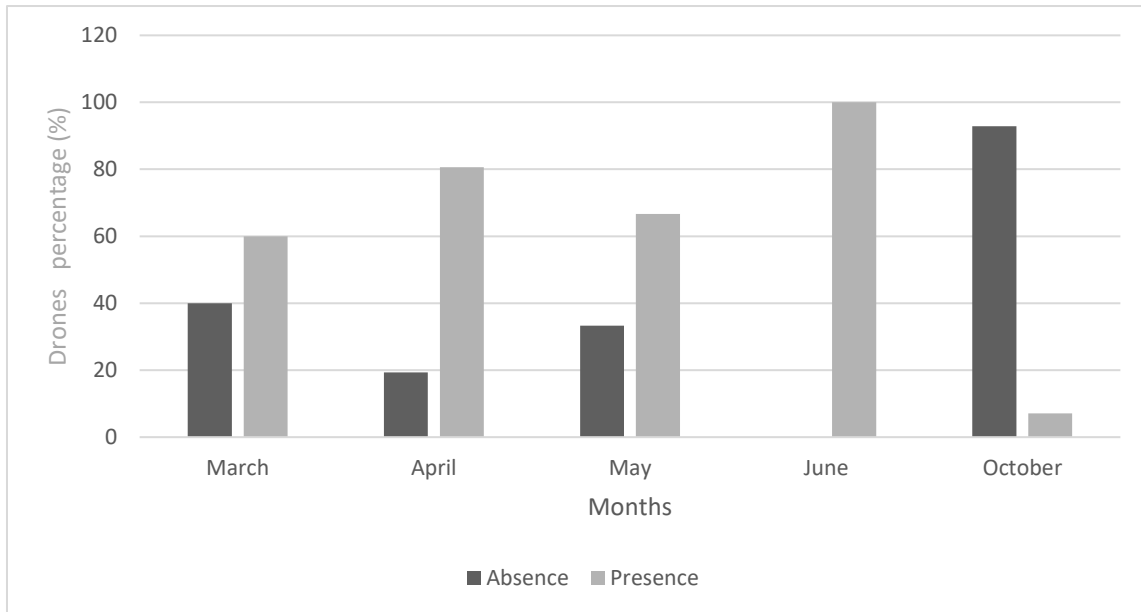
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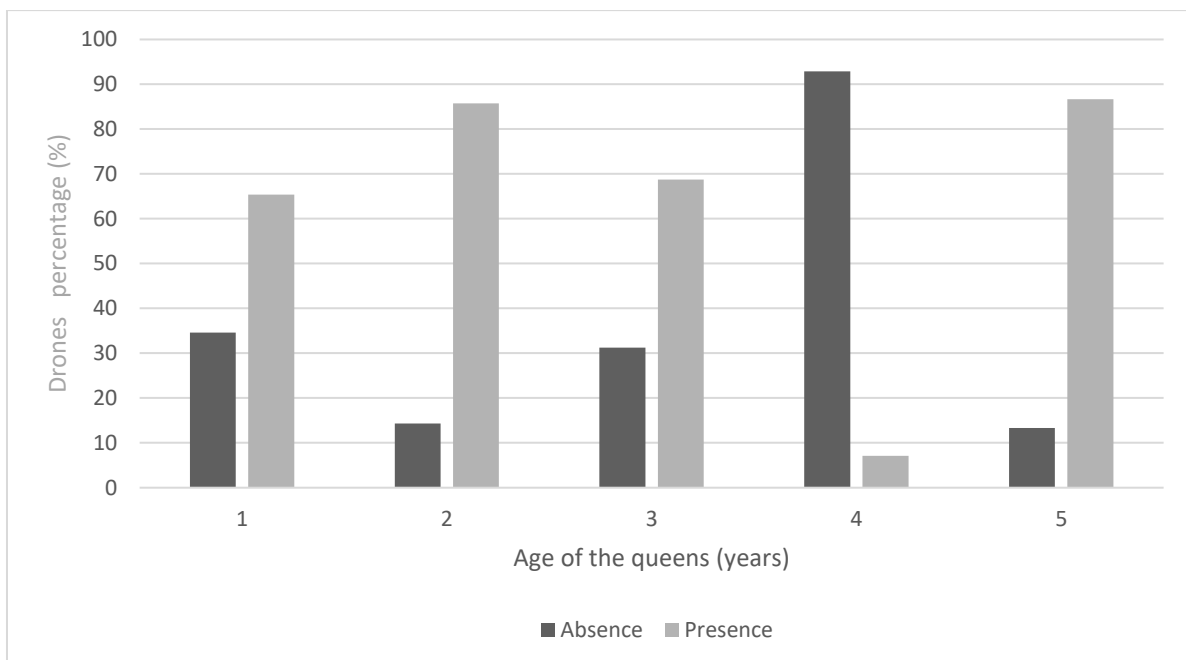
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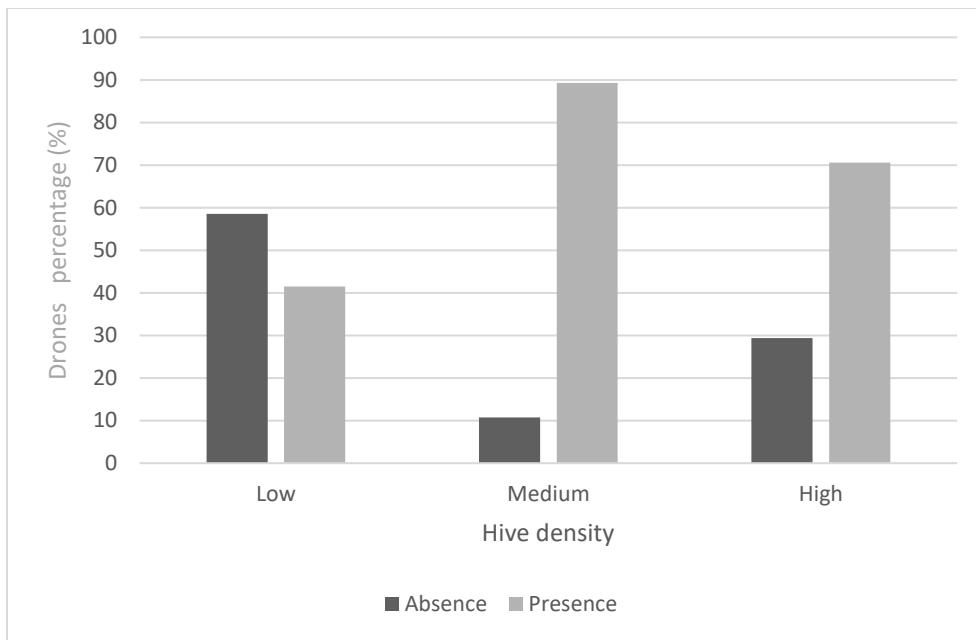
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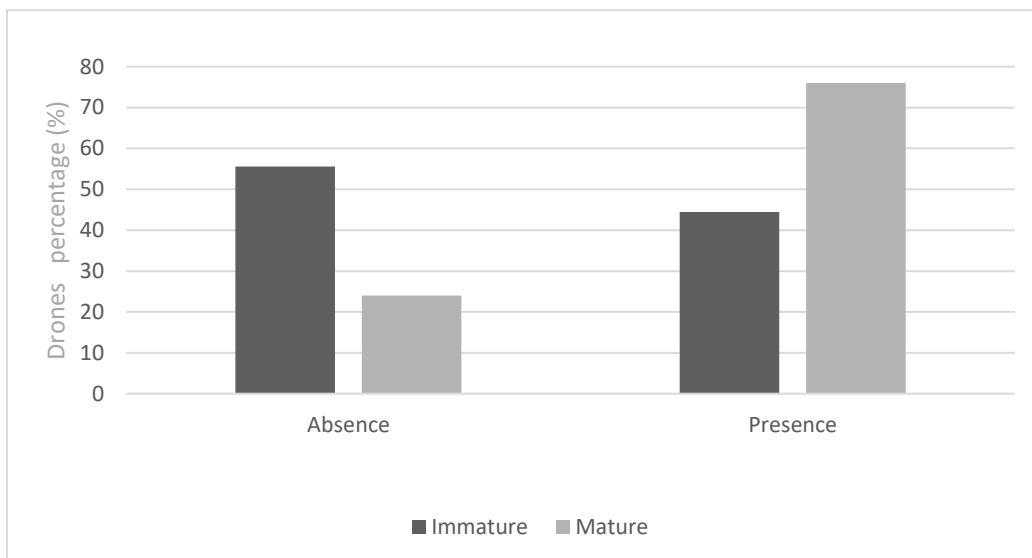
**Figure 1.** Percentage of drones with the presence or the absence of spermatozoa during the breeding (March; April, May, June) and the non-breeding seasons (October).



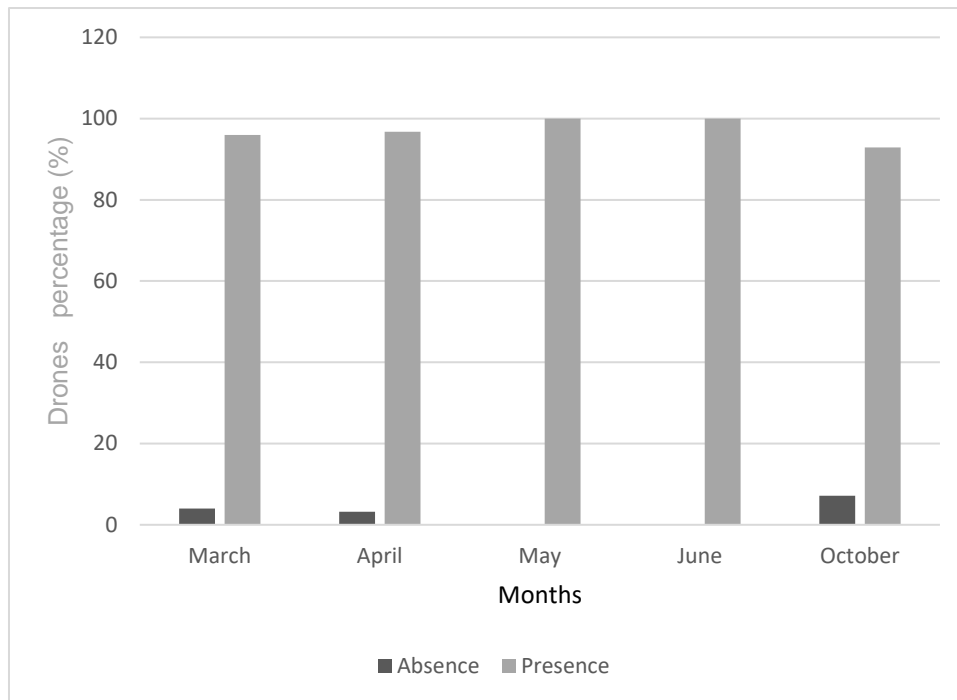
**Figure 2.** Percentage of drones with the presence or the absence of spermatozoa during the study period according to queens age (from 1 to 5 years).



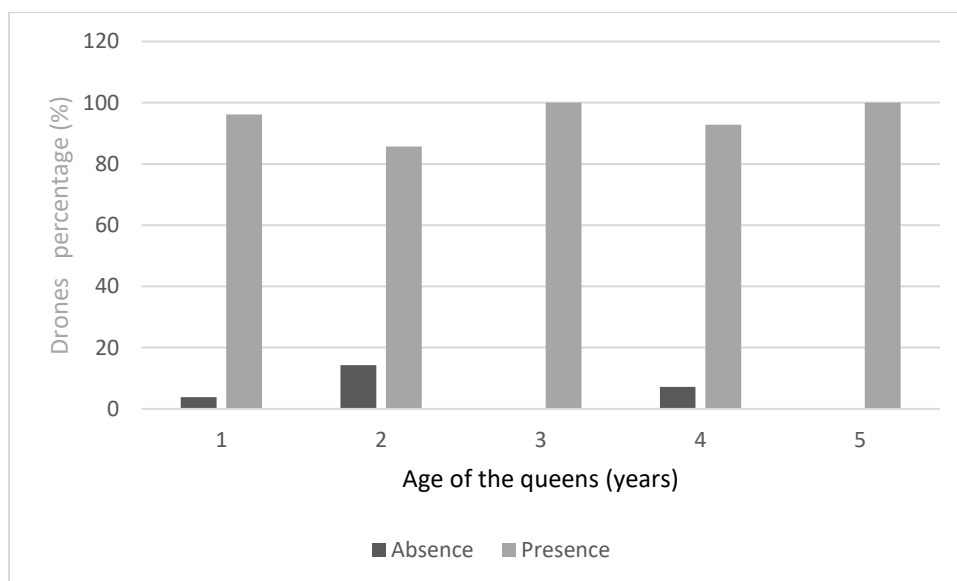
**Figure 3.** Percentage of drones with the presence or the absence of spermatozoa according to hive density (Low, Medium; High) during the study period.



**Figure 4.** Percentage of drones with the presence or the absence of spermatozoa according to drones status (Immature, Mature).



**Figure 5.** Percentage of drones with the presence or the absence of mucous gland secretions during the breeding (March; April, May, June) and the non-breeding seasons (October).



**Fig 6.** Percentage of drones with the presence or the absence of mucous gland secretions according to queens age (from 1 to 5 years).