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ORIGINAL ARTICLE

Transition in the production of diploid-female to haploid-male eggs in bumblebee colonies: sperm quality or depletion?

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Abstract

Bumblebees establish annual colonies that start with the emergence of workers in spring and end with the production of sexuals, the majority of which are males, in late summer. To date, the causes responsible for the transition in the production of diploid-female offspring to haploid-male offspring during the decline phase of colonies remain elusive. Using flow cytometry, we tested whether such a caste shift is correlated with a decline in sperm number and quality (i.e., sperm viability and sperm DNA fragmentation) in the queen spermatheca over time, from mating to the emergence of the first males. We found that sperm number and viability significantly decreased, while sperm DNA fragmentation increased in the spermatheca over time. These results suggest that the shift towards male production during the decline phase of a bumblebee colony stems at least partly from a combination of a drop in sperm count and sperm quality in queens' spermatheca.

Significance statement

In social Hymenoptera, sex determination is based on the haplodiploidy system in which males develop from unfertilized eggs and are haploid, and females develop from fertilized eggs and are diploid. So far, the proximal mechanisms responsible for the social transition from diploid-female egg production to haploid-male egg production remain unknown. We show that the shift towards male production during the decline phase of bumblebee colonies is associated with a reduction in the quantity and quality of the sperm stored in the queen spermatheca over time. More generally, it suggests that sperm conservation is limited and likely adapted to the relatively short lifespan of bumblebee queens, which produce small and annual colonies.

Keywords Bumblebees · Male production · Sperm · Flow cytometry

Introduction

In sexually reproducing species, fertilization success depends on two key factors: (i) the quality of the germ cells and (ii) their synchronous arrival at the fertilization site (Suarez and Pacey 2006; Baer 2011; Fitzpatrick and Lüpold 2014). In species with internal fertilization, mating does not necessarily coincide with ovulation. The spermatozoa transferred to females during mating are then kept in specific storage organs until ovulation, at which point they are released for fertilization of the eggs. Sperm storage by females was selected when (i) the probability of finding a mate is limited, (ii) the period of gamete production by the two sexes is dissociated, and/or (iii) copulation is made risky by predation (Birkhead and Møller 1993). Sperm storage duration by females varies among species, ranging from a few days in mammalian sperm reservoirs to several months or years in the storage tubules of birds and reptiles, respectively

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(Birkhead and Møller 1993; Suarez 1998; Sasanami et al. 2013).

Females of eusocial hymenopterans (ants, social bees, and wasps) unquestionably hold the record for the longest sperm preservation time in the animal kingdom. Mating proceeds during a single nuptial flight, at the beginning of adult life for both sexes. Males die shortly after mating. On the other hand, reproductive females (queens) store a lifetime's supply of sperm in their spermatheca, which they use to fertilize their eggs for years, sometimes decades depending on the species (Hölldoble and Wilson 1990; Keller and Genoud 1997; reviewed in Degueldre and Aron 2023b). Once inseminated, queens found new colonies by producing non-reproductive workers. Depending on the species, it takes a colony a few months to a few years to reach maturity, at which time the colony starts to produce males and new queens that will repeat the cycle (Michener 1974; Hölldoble and Wilson 1990; Starr 2021). The unique mating event characteristic of social Hymenoptera imposes strong selective pressures on queens to conserve a finite stock of sperm to ensure colony growth (Baer 2011; Chérasse and Aron 2018; Degueldre and Aron 2023b). Several mechanisms have been identified to preserve sperm in the spermatheca, including (i) production of antioxidant proteins, (ii) reduction of the metabolic rate, delaying cell ageing and limiting reactive oxygen species (ROS) production, (iii) production of antimicrobial peptides, (iv) spermiophagy, whereby dead spermatozoa are removed from the spermatheca preventing a potential source of toxic molecules such as ROS, (v) the energy supply of stored sperm from the glandular tissues of the female's reproductive tract, or (vi) promotion of anaerobic metabolism of stored spermatozoa to limit ROS production (Baer 2011; Chérasse and Aron 2018; reviewed in Degueldre and Aron 2023b).

Unlike most social hymenopterans, bumblebee queens have a relatively short lifespan, averaging 11 months, and colonies are annual (Goulson 2003). Mating proceeds in late summer, during which queens typically mate with a single male. Sperm transferred by the male are released into the *bursa copulatrix* of the queen and, within approximately 1 h, migrate through the spermathecal duct into the spermatheca where they are stored until fertilization. The amount of sperm transferred to the female is around 40,000–50,000 spermatozoa (Röseler 1973). After a diapause period (from October/November to March for temperate species; Schmid-Hempel and Schmid-Hempel 2000), the queens found their colony. The first eggs laid after diapause, in spring, are diploid and develop into workers. This ergonomic phase lasts several weeks, with colonies reaching a few hundred workers (± 300–400 workers) (Velthuis and Van Doorn 2006). Early summer corresponds to the beginning of colony decline: production of diploid-female eggs gradually decreases, and haploid-male eggs are laid (males develop from unfertilized eggs in Hymenoptera). This phase marks the "switch point" (SP) in the colony's lifecycle (Duchateau and Velthuis 1988). The emission of the queen pheromone progressively decreases and the last diploid eggs laid are raised into sexual females (virgin queens) (Duvoisin et al. 1999; Aron and Passera 2000). Simultaneously, workers start to lay (haploid) eggs leading to overt queen-worker conflict over male production, a phase known as the "competition phase (CPh)." The CPh is characterized by worker oviposition, egg consumption (oophagy), and the opening or destruction of egg cups (laid or not by the queen). Notably, the timing of the CPh is independent of whether the SP occurs early or late in the colony's life. In some colonies, the CPh can manifest 3 weeks after the SP, indicating that these two events are not causally linked (Duchateau and Velthuis 1988). Workers could adaptively delay egg laying until they obtain information allowing them to maximize their kin-selected interests (Bourke and Ratnieks 2001).

Several ecological, demographic, and population genetic factors were shown to influence the ratio of haploid and diploid eggs laid by queens in social Hymenoptera (reviewed in Aron 2012). These factors encompass fluctuations in sexual production throughout the seasons, the stage in the colony's life cycle, as well as the genetic structure of the population and the intra-colony competition among related offspring. In several temperate ant species, the initiation of haploid egg production by queens and the rearing of diploid eggs into new reproductive queens is triggered by the emergence from hibernation. Queens' ability to control the ratio of haploid and diploid eggs laid over time has also been documented in the honeybee *Apis mellifera*, with the proportion of drone eggs to worker eggs being higher during the swarming season than in other times of the year (Sasaki et al. 1996). This ratio of drone and worker eggs laid is influenced, at least partially, by a negative feedback mechanism in drone egg production (Sasaki et al. 1996; Wharton et al. 2007). Furthermore, Wharton et al. (2007) suggested that, in *Apis mellifera*, the queen's choice to lay a haploid or diploid egg is influenced by the size of the cell.

In contrast to ants and honeybees, bumblebee colonies are annual and limited to a few hundred individuals and limited to a few hundred individuals. So far, the proximal mechanisms responsible for the social transition in the production of diploid-female offspring to haploid-male offspring marking the SP in bumblebee colonies remain unknown. Two non-mutually exclusive hypotheses can be envisaged to account for the increase in the proportion of males reared in early summer: (1) sperm depletion, that is queens no longer have enough sperm in their spermatheca to fertilize all their eggs, and/or (2) a decline in sperm quality, that is, queens retain a sufficient supply of sperm to fertilize their eggs but

the spermatozoa undergo progressive degradation in the spermatheca leading to infertility; hence, the production of unfertilized-male eggs.

Here, we examined these two hypotheses in the buff-tailed bumblebee, *Bombus terrestris*. Queens are typically single-mated (monandrous); they store the sperm transferred in their spermatheca for around 11 months, which corresponds to the time between mating (late summer) and the end of the colony's life (early summer of the following year) (Baer 2003; Baer et al. 2003). We analyzed sperm number and sperm quality (estimated from sperm viability and sperm DNA integrity) in the spermatheca of queens throughout the colony life cycle, from mating to the emergence of males.

Materials and methods

Study species and rearing

A sample of 300 males and 110 virgin females of *B. terrestris* were purchased from Biobest (Westerlo, Belgium). We performed copulation assays following the methodology of Lhomme et al. (2013). Briefly, one virgin queen (3-day old) was placed with three virgin males (7–10 days old), unrelated to the queen and to each other, in a flight cage (35 × 35 × 60 cm) and exposed to natural light at room temperature. Once the queen was copulating with one of the males, the other two males were removed from the cage to avoid disruptions. After mating (± 30 min), the queen was transferred to a nest box (16 × 11 × 9 cm) with *ad libitum* pollen and sugar. One week after mating, the queens were put into artificial hibernation. They were placed in matchboxes (51 × 38 × 13 cm) with a cotton soaked in water and placed at an ambient temperature of 5–6 °C for 2 months. After the 2-month hibernation, the queens were placed in flight cages (30 × 30 × 30 cm) with food (*Salix* sp. pollen, Rucher de Lorraine) and sugar resources (Biosweet, Biobest) *ad libitum*. They were maintained with a light period of 8 h/day at 24–26°C for 1 week, followed by 3 days of full light to completely awake queens. Then, the queens were reared in plastic cages (8 × 16 × 16 cm), in the dark, with a constant temperature of 26 °C and a relative humidity of 50–60% to start their colony (Vanderplanck et al. 2019).

We estimated sperm quantity and quality in the spermatheca throughout the queens' lifespan. We dissected the queen spermatheca at nine time-points after copulation: 7 days after copulation (just before hibernation); 75 days after copulation (when queens were coming out of hibernation); at the emergence of the first workers in each colony; 10, 20, 40, 55, and 70 days after the emergence of the first workers; and, at the emergence of the first adult males ($n = 10$ per time-point) which indicates the queens recently stopped laying diploid-female eggs. Indeed, caste production in *B. terrestris* occurs sequentially, with no reversions (pers. obs.). Our experimental colonies experienced different rates of development. However, for the sake of simplicity and clarity, time-points for all colonies are referred to in the text as follows: D_0; D_7; D_{75}; D_{105} (the average emergence time of the first workers); D_{115}, D_{125}, D_{145}, D_{160}, and D_{175} corresponding to 10, 20, 40, 55, and 70 days after the emergence of the first workers, respectively; and D_{190} (the average emergence time of the first adult males). Note that under natural conditions, the period between mating and hibernation can last up to 2 months, and the duration of hibernation up to 5 months (Lhomme et al. 2013). Our procedure (1 week between mating and hibernation, and 2-month hibernation) therefore shortens the solitary period of the queens before they found their colony, as recommended in bumblebee breeding experiments (Lhomme et al. 2013).

Sperm extraction

The female reproductive tract was dissected in a *semen diluent* saline buffer solution (188.3 mM sodium chloride, 5.6 mM glucose, 574.1 nM arginine, 684.0 nM lysine, and 50 mM tris(hydroxymethyl)aminomethane, pH 8.7; Paynter et al. 2014), which keeps sperm alive during the experiment. The spermatheca was opened, and its content was extracted, collected, and mixed with 100 µl of *semen diluent*. The sperm solution was then transferred equally into two Eppendorf tubes for flow cytometry analyses: 50 µl was used to assess sperm count and viability, and 50 µl was used for sperm DNA fragmentation analyses. This procedure allowed us to determine sperm number and quality for the same biological samples, each sample corresponding to the spermathecal content of a single queen.

Flow cytometry

Sperm number, sperm viability, and sperm DNA fragmentation were analyzed by flow cytometry (CyFlow® Space Sysmex) (Chérasse and Aron 2018; Degueldre and Aron 2023a, 2023c). Sperm count was determined from the actual number of spermatozoa stored in queens' spermathecae. We assessed sperm viability by determining the proportion of live and dead spermatozoa. For both sperm number and viability, we used the Live/Dead Sperm Viability Kit (Life molecular probes; ThermoFisher). Sperm samples (50 µl) were diluted for a final volume of 1 mL with semen diluent. Next, 5 µl of SYBR-14 (100 nM) was added to this solution, gently homogenized, and left at room temperature in the dark for 10 min. Next, 5 µl of PI (12 mM) was added, and the solution was left at room temperature in the dark for 10 min. The samples were then injected into the flow cytometer. SYBR-14 fluorescence was measured between 516 and 556 nm and PI above 630 nm. For each sample, a

total of 1.500.000 cells were counted at a flow rate of 0.6 µl s^{-1}; the stream was allowed to stabilize for 25 s before counting began. Sperm populations were identified based on their characteristic forward and side scatter. We established a gating strategy by analyzing reference samples with known ratios of live to dead sperm (Supplementary material Figure S2; Paynter et al. 2014). The gating strategy and sperm cell counts were carried out using the FlowJo software (v. 10).

The fragmentation rate of sperm DNA was quantified by using the sperm chromatin structure assay (SCSA, as described in Evenson 2022, Degueldre and Aron 2023c). For SCSA, we used acridine orange (AO) as fluorochrome, which emits green fluorescence when associated with double-stranded DNA, and red fluorescence when associated with single-stranded DNA (i.e., when the DNA is damaged). AO fluorescence was measured at 525 nm for double-stranded DNA and at 650 nm for single-stranded DNA. Sperm samples (50 µl) were diluted in semen diluent for a final volume of 166 µl. To this volume, we added 333 µl of acid detergent solution. After 30 s in solution, 1 ml of staining solution (AO + staining buffer) was added, and the sample was run through the cytometer after 3 min at room temperature. For each sample, a total of 1,500,000 cells were counted at a flow rate of 0.1 µl s^{-1}; the stream was allowed to stabilize for 40 s before counting began. We carried out the gating strategy and sperm cell counts using FlowJo software (v. 10.9.0). The DNA fragmentation index (DFI) corresponds to the ratio of red fluorescence (fragmented sperm) to total (red and green) fluorescence.

For each queen, the number of spermatozoa, sperm viability, and sperm DNA integrity (DFI) in the spermatheca reported correspond to the mean of the three pseudo replicates measured with flow cytometry. To minimize observer bias, blinded methods were used when all data were analyzed using FlowJo software (v. 10.9.0).

Statistical analyses

Flow cytometry data were analyzed using R 4.0.3 (R Core Team 2022). Graphs were produced using the "ggplot{ggplot2}" (Wickham 2016) package within R. Descriptive statistics (mean ± SD) were calculated by "describeBy{psych}" (Revelle 2016). Sperm count, viability, and sperm DNA fragmentation (DFI) were analyzed independently after arcsin transformation of the data expressed as a percentage to normalize residuals. For each statistical test, we tested the normality of data (Shapiro-Wilk test) and homoscedasticity (Bartlett's comparison of variances test). In cases where the transformed data satisfied the conditions for using parametric tests, an ANOVA test was used with a Bonferroni correction (p_{adj}) to compare the different age groups. Otherwise, the Kruskal-Wallis's test was performed for group comparisons based on non-transformed data, followed by Dunn's post hoc test for multiple comparisons. The statistical association between the sperm viability and sperm DNA fragmentation was determined by the Kendall rank correlation coefficient *t* (for data that do not come from a bivariate normal distribution).

Results

From the 110 virgin queens placed with males in mating cages, 91 (83%) mated and 80 of these survived the artificial hibernation and successfully founded their colony. From these, 10 randomly selected colonies were reared until the emergence of the first males, at which stage they reached on average (mean ± SD) of 298 ± 41 workers. Raw data on sperm number, sperm viability, sperm DNA fragmentation (DFI), and colony size (at emergence of the first males) are given in Supplementary material S1.

Sperm count

We observed a gradual but significant decrease in the number of spermatozoa stored in the queens' spermatheca over time (Kruskal–Wallis: $\chi^2 = 84.763$; df = 8, $p < 0.001$; Dunn's post hoc test: D_7–D_{190}, $p < 0.01$; Fig. 1A). Seven days after mating (D_7), the number of spermatozoa in the spermatheca was on average 46,762 ± 3938 (mean ± SD). This number reached 6489 ± 1141 on D_{190}, when the colonies started producing males. This reduction in sperm count was clearly visible when observing the spermathecae during the queen's life (Fig. 1B).

Sperm viability

Sperm viability also decreased significantly over time (Kruskal–Wallis: $\chi^2 = 91.135$; df = 8, $p < 0.001$; Fig. 2A). The proportion of live spermatozoa in the queens' spermatheca on D_7 was 87.67 ± 3.07%; from the remaining spermatozoa, 10.32 ± 3.22% were dead and 2.01 ± 1.80% were moribund (i.e., in the process of dying). Sperm viability did not differ before (D_7) and after hibernation (D_{75}: alive 85.25 ± 3.27%, dead 11.08 ± 2.46%, dying 3.67 ± 2.16%) (Dunn's post hoc test: $p > 0.05$ for live, dead, and dying spermatozoa, respectively; Fig. 2A). Then, sperm viability significantly declined in stages (Dunn's post hoc tests: $p < 0.05$): a first drop occurred on D_{105} which corresponds to the emergence of the first workers (live spermatozoa 68.41 ± 3.87%), a second drop on D_{145} (54.13 ± 6.61%), and a third sharp drop on D_{175} (19.53 ± 7.80%). At D_{190}, at the emergence of the first males, 13.66 ± 8.83% of the spermatozoa were still alive, 54.02 ± 14.51% were moribund, and 32.32 ± 13.93% were dead. The decrease in sperm viability over time was reflected in the increase in

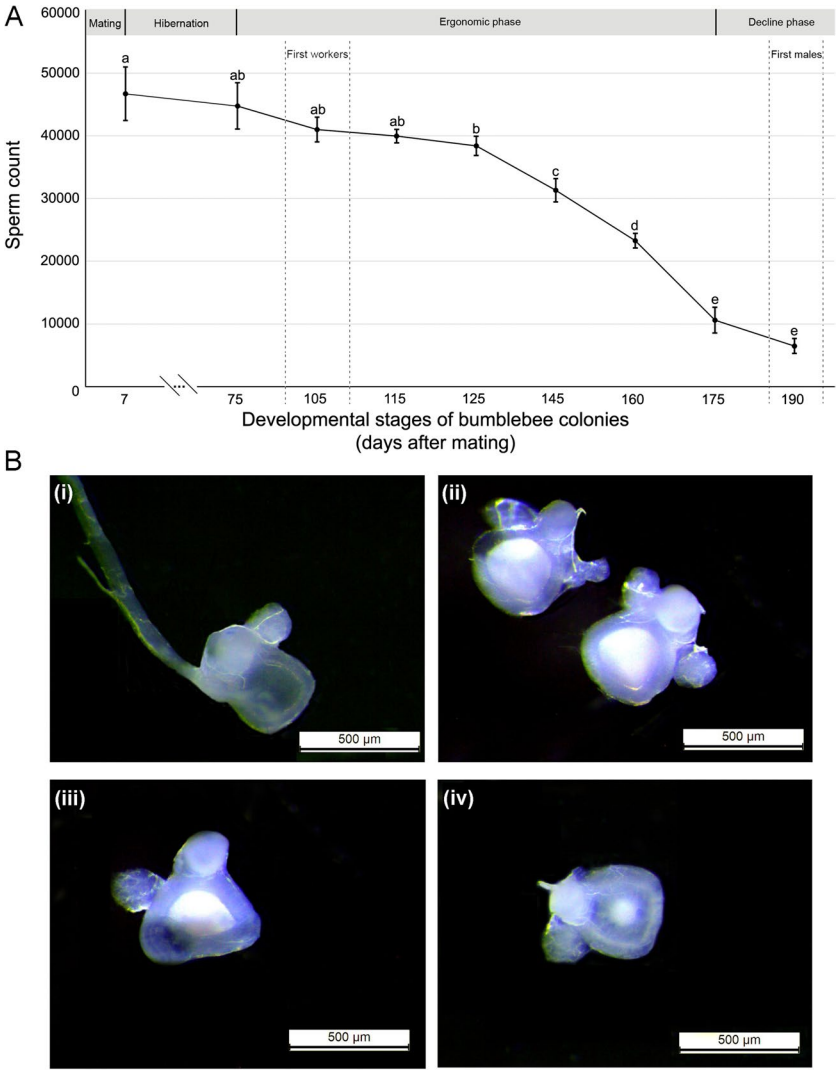

Fig. 1 Sperm quantity (mean ± SD) in the spermatheca of queens at different developmental stages of *B. terrestris* colonies. **A** Number of spermatozoa in the spermatheca at nine time-points after mating (D_0): 7 days (before hibernation, D_7), 75 days (when queens are removed from hibernation, D_{75}), 105 days (which corresponds to the average emergence time of the first workers), 115 days (D_{115}), 125 days (D_{125}), 145 days (D_{145}), 160 days (D_{160}), and 175 days (D_{175}) corresponding to 10, 20, 40, 55, and 70 days after the emergence of the first workers, respectively; and D_{190} (the average emergence of the first adult males). Letters indicate statistically significant differences (Dunn's post-hoc tests: $p < 0.05$). $n = 10$ queens per time-point. **B** Representative photos of bumblebee spermathecae during queen ageing. Spermatheca of (i) a virgin queen (empty), (ii) a queen after mating (D_7), (iii) a queen 145 days after mating (D_{145}), and (iv) a queen 190 days after mating (emergence of first males, D_{190}) showing the reduction in the spermatheca content over time

the proportion of dead and moribund spermatozoa during the decline phase of the colony (Fig. 2A).

Sperm DNA fragmentation (DFI)

There was a significant increase in sperm DNA fragmentation over the 190 days of the experiment (Kruskal–Wallis: $\chi^2 = 86.817$; df = 8, $p < 0.001$, Fig. 2B). DFI was on average 12.68 ± 1.02% (mean ± SD) after mating (D_7). It remained relatively stable until D_{145}, with DFI= 15.79 ± 1.40%. Then, DFI significantly increased, reaching 19.12 ± 1.14% at D_{160} and 34.71 ± 1.71% at D_{190} (emergence of first males) (Dunn's post hoc tests: $p < 0.05$, Fig. 2B). This sharp increase in sperm DNA fragmentation from D_{160} was concomitant with the drop in sperm viability (Kendall's rank correlation t, $z = -9.4839$, correlation coefficient tau = −0.637, $p < 0.001$).

Discussion

This study is the first to precisely determine the variation in sperm number and quality over the queens' lifespan in bumblebees. Our results show that the transition in the production of diploid-female offspring to haploid-male offspring during the decline phase of bumblebee colonies is correlated with a reduced sperm count in the queens' spermatheca and a decline in sperm quality.

These findings add to our understanding of the mechanisms underlying the switch point (SP) characteristic of bumblebee colonies. They support that the transition from diploid to haploid egg production by queens is primarily linked to failed egg fertilization due to sperm quality decrease. Depletion of viable sperm greatly reduces the laying of diploid eggs, hence workers' and/or queens' production. The last diploid eggs laid by the queen during its

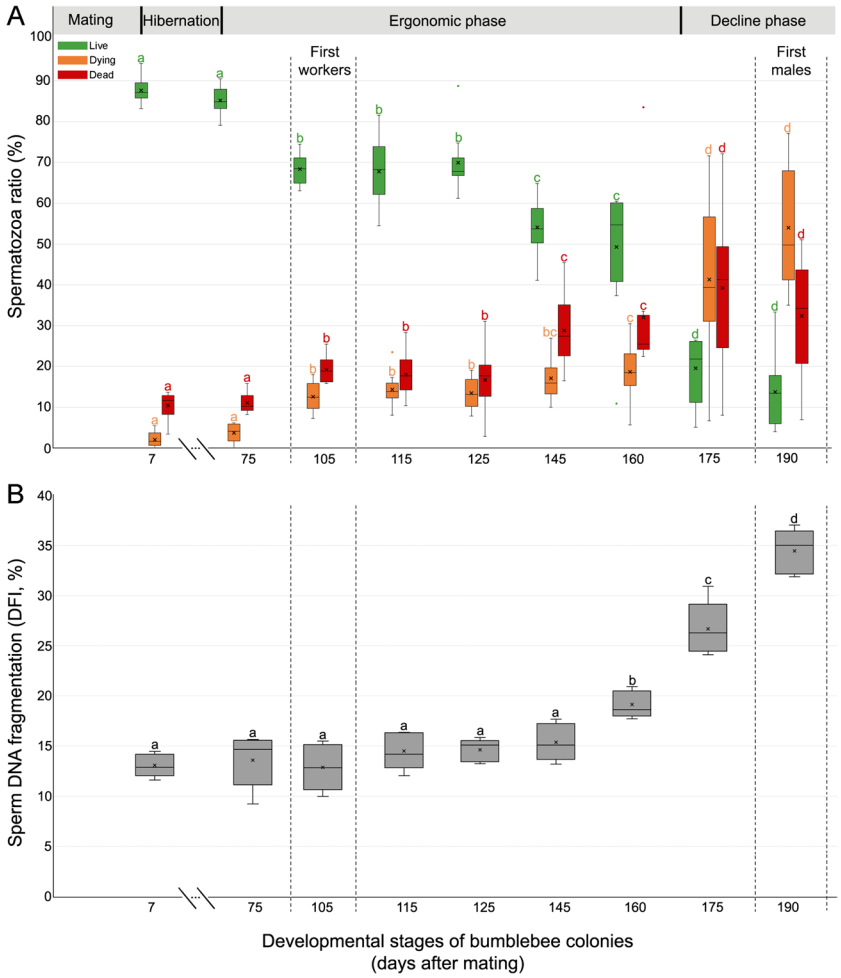

Fig. 2 Sperm viability (**A**) and DNA fragmentation (DFI) (**B**) in the spermatheca of queens at different developmental stages of *B. terrestris* colonies. Sperm viability and DNA fragmentation are given for nine time-points after mating (see legend of Fig. 1 for details). The black lines within each box correspond to the medians and the crosses to the mean. The first and third quartiles are represented by the lower and upper ends of the boxes, respectively. The upper and lower ends of the whiskers denote the smallest and largest values. Different letters above the boxes indicate statistically significant differences (Dunn's post-hoc tests; $p<0.05$). $n = 10$ queens per time-point

life are reared in new virgin queens by workers (Duvoisin et al. 1999; Aron and Passera 2000). The drop in sperm quality during the SP could be part of the initiation of the competitive phase (CPh), at which time colonies enter a degenerative phase. The onset of queen-worker conflict cannot be attributed solely to a single factor such as the queen's switch to male production or a decrease in queen inhibition (Bloch 1999). In normally developing colonies, the processes of queen ageing, increased worker number, and the switch from female to male rearing are interconnected (i.e., degenerative stage) and associated with seasonal environmental changes (Wilson 1971; Michener 1974). As a result, it is challenging to disentangle the relative significance of each of these factors in the initiation of queen-worker conflict. Nevertheless, changes in the worker population size (possibly due to an excess of workers beyond the queen's control) and shifts in the brood sex ratio (i.e., male production) both influence the timing of queen-worker conflict and the transition to queen production in bumblebees (Bloch 1999).

One week after mating, *B. terrestris* queens stored ~46,700 spermatozoa in their spermatheca, most of which (87%) were alive and supposedly fertile. Sperm count progressively decreased over time to reach ~6500 spermatozoa when the first males emerged (D_{190}). Of these, only 10–20% were found alive, the remaining 80–90% were dead or moribund. Simultaneously, sperm DNA fragmentation rose from 13% to over 30%. The limitation of live spermatozoa available to fertilize eggs could therefore explain the increase in male production towards the end of the colony cycle. So far, few studies have explored the number of spermatozoa required to fertilize an egg in social hymenopterans, and none in bumblebees. In the leaf-cutter ant *Atta colombica*, queens use around 2–3 sperm per egg (den Boer et al. 2009). In this species, queens are long-lived, reaching up to 20 years, and form colonies of over a million workers. Colony maintenance depends on sperm management: queens using more sperm to fertilize their eggs have a shorter colony lifespan. In the honeybee *Apis mellifera*, the number of sperm used to fertilize an egg ranges from 4 to 100 (Ruttner 1975; Yu and Omholt 1999). Queens live an average of 4 to 5 years, and colonies may contain several thousands of workers. Compared to these species, *B. terrestris* queens live for 1 year, and societies have an annual life cycle. Our data

indicate that queens used ~40,200 spermatozoa to develop colonies of 250–350 workers, which amounts to a rough estimate of 135 spermatozoa per egg. Given the low number of spermatozoa still alive in the queen spermatheca when the first males emerge (650–1300 spermatozoa, i.e., 10–20% of 6500), the number of eggs that can be fertilized is highly limited (max. 10), which may account for an increase in the number of unfertilized, haploid eggs laid and the production of males by the colonies. This may also contribute to explaining the highly male-biased sex ratio reported in bumblebees (Beekman and Van Stratum 1998).

The decline in sperm number and quality in the spermatheca of *B. terrestris* queens over the year also differs greatly from the results reported in the black-garden ant *Lasius niger*, where 1-year-old queens experience a decrease < 20% in their sperm stock. Furthermore, old queens have significantly higher sperm viability in their spermatheca than incipient queens (Chérasse and Aron 2018), suggesting that ant queens may be able to remove dead sperm cells from storage, as documented in stingless bees (Da Cruz-Landim 2002). Such a discrepancy between these two species may stem from major differences in life history. While *B. terrestris* queens establish annual societies of a few hundred workers, *L. niger* queens store approximately 2.6 million sperm cells (Cournault and Aron 2008; Chérasse and Aron 2018), are extremely long-lived (29 years; Kutter and Stumper 1969), and can produce colonies of up to 55,000 workers (Fjerdingstad et al. 2003). Thus, selection on sperm use and conservation must be much stronger in ants, enabling queens to preserve large amount of good-quality sperm for several years, than in bumblebees which must maintain a limited stock of sperm for a maximum period of 11 months (see "Materials and methods").

Notably, we found no significant differences before and after hibernation in sperm viability and DNA integrity in the spermatheca of queens. This suggests a significant investment by bumblebee queens (e.g., accumulation of nutritive reserves) to maintain sperm quality during this critical period of the life cycle (Colgan et al. 2019). In line with this, Schoeters and Billen (2000) showed that the cells lining the reproductive tract of *B. terrestris* queens contain high reserves of glycogen, which could serve as an energy source for the sperm. Furthermore, a recent study revealed that queen survival during diapause is negatively affected by mating (Bogo et al. 2017), with mated queens experiencing lower survival than non-mated queens. This suggests that keeping sperm alive during the hibernation phase entails high energy costs, and that these costs are traded off against queen survival (Baer and Schmid-Hempel 2005; Greeff and Schmid-Hempel 2008a). Such costs likely come from energy supply, protection against oxidative stress, production of antimicrobial peptides, and regulation of the immune system.

Our findings that sperm number and quality decreased in the spermatheca of *B. terrestris* queens over the year contrast with previous studies on male production in the buff-tailed bumblebee. Röseler (1973) first claimed that queens do not hold significantly less sperm at the end of the colony cycle (spring) despite high data variability and argued that the production of males is a strategy of the queen rather than due to sperm limitation. In his study, however, sperm count in the queen's spermatheca was estimated in autumn, at the mating time, and in spring. In *B. terrestris*, spring corresponds to the ergonomic phase of the colony when workers are produced. Our flow cytometry data and the pictures of the spermatheca at different time-points of the colony cycle (see Fig. 1) clearly show a significant drop in sperm number, with only a small amount (6489 ± 1355 sperm cells) remaining in the spermatheca at the end of the colony cycle (D_{190}). Thus, the discrepancy between the results of Röseler (1973) and ours probably lies in the timing of the sperm count. On the other hand, Greeff and Schmidt-Hempel (2008) reported that sperm viability in the spermatheca was not significantly different between young, freshly mated queens and old queens. These authors did not specify, however, the age of the old queens; yet, as shown in the present work (Fig. 2A), queens' age and the developmental stage of the colonies deeply affect sperm viability in this species. Moreover, the differences found in sperm viability might in part be due to the different methods used. Although both studies rely on fluorescence labelling to identify live/dead spermatozoa, Greeff and Schmid-Hempel (2008b) used epifluorescence microscopy and counted 100 spermatozoa per sample, while we used flow cytometry which allowed us to determine sperm viability for the entire spermathecal content. Our data also showed that sperm DNA integrity (not assessed by Greeff and Schmid-Hempel (2008a)) decreased over time until the emergence of the first males and was significantly associated with a drop in sperm viability.

In conclusion, this study shows that both sperm cell viability and DNA integrity in the spermatheca of queens decrease rapidly after hibernation in the bumblebee *B. terrestris*. More generally, it suggests that sperm conservation is limited and likely adapted to the relatively short lifespan of bumblebee queens (i.e., 1 year), which produce small and annual colonies. Further studies of sperm gene expression (transcriptomic/proteomic analyses) involved in energy metabolism and DNA repair are needed to explore the molecular causes associated with the decrease in sperm viability and DNA integrity.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00265-023-03410-x.

Author contribution Came up with and designed the experiments: BM and SA. Sampling: BM, KP, and CM. Conducted the experiments: BM, KP, and CM. Analyzed the data: BM, KP, CM, and SA. Wrote

the paper: BM, KP, and SA. All the authors helped draft the article, approved the version to be submitted, and agreed to be held responsible for this study.

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Data availability All data are available in Supplementary File 1.

Declarations

Competing interests The authors declare no competing interests.

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