Functional characterisation of semen in honeybee queen (A.m.ligustica S.) spermatheca and efficiency of the diluted semen technique in instrumental insemination

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ABSTRACT

Differences over time in the quality of semen present in the honey bee (Apis mellifera ligustica) queen spermatheca were studied. An increase in the non-vital spermatozoa was shown to be evident (P>0.05) between the 12th and 24th month. The study of semen viability demonstrated that the passage of the semen to the spermatheca is due to sperm motility. In the queen inseminated with non-viable spermatozoa, no semen was detected in the spermatheca. Queens inseminated twice with a Hyes solution/semen mixture (1:1) stored as many spermatozoa in their spermatheca as those inseminated once with the classic technique. Queen replacement, oviposition and other functional characteristics were similar to those observed in the classic insemination procedure.

Key words: Apis mellifera ligustica, Queen honey bee, Spermatheca, Spermatozoa, Instrumental insemination.

RIASSUNTO

CARATTERIZZAZIONE FUNZIONALE DEGLI SPERMATOZOI NELLA SPERMATECA DELL’APE REGINA (A.M.LIGUSTICA S.) ED EFFICIENZA DELLA TECNICA DI INSEMINAZIONE STRUMENTALE CON SEME DILUITO

La fisiologia dell’accoppiamento in Apis mellifera presenta due importanti peculiarità: l’accoppiamento in volo e la polian- dria. La gran parte degli spermatozoi raccolti dall’ape Regina durante il/i voli di accoppiamento (8-16 fuchi), viene espul- sa nelle successive 24 ore. Gli spermatozoi residui (1/15 circa di quelli provvisoriamente inglobati negli ovidotti), reste- ranno quiescenti per tutta la vita dell’ape Regina. La fisiologia degli spermatozoi in spermateca è pertanto molto impor- tante, ma al momento non ancora del tutto chiara: a tal fine sono state condotte due sperimentazioni. I risultati ottenu- ti evidenziano che il passaggio degli spermatozoi in spermateca avviene esclusivamente per l’azione della motilità degli stessi, infatti nelle regine inseminate con materiale seminale non mobile non sono stati ritrovati spermatozoi in sperma- teca. I dati ottenuti evidenziano una riduzione della concentrazione degli spermatozoi (Chi-Square=6.841 P<0.05) ed un aumento di quelli non vitali (Chi-Square=4.665 P<0.05) all’aumentare del tempo intercorso dall’inseminazione. Quest’ultimo dato - seppur non statisticamente significativo - è biologicamente rilevante: è infatti ipotizzabile una sele- zione qualitativa degli spermatozoi contenuti nella spermateca, date le basse percentuali di spermii non vitali presenti nelle spermateche delle regine inseminate da due mesi, inferiori rispetto a quanto evidenziato nel seme prelevato diret- tamente dal fuchi.

L’utilizzo dell’inseminazione strumentale è per ora relegato - almeno in Italia - a ristretti ambiti di ricerca o a produzio- ne di ibridi per il commercio. Le prove di manipolazione e conservazione del materiale seminale proveniente da fuchi di
colonie diverse hanno fornito, a tutt’oggi, risultati spesso contradditori. L’obiettivo che ci si è preposti è stato quindi quel-
lo di mettere a punto una metodologia di raccolta, diluizione e miscelazione del materiale seminale, semplice ma effica-
ce. Nei due gruppi di colonie con regine inseminate oggetto del confronto (controllo= inseminazione classica con 8µl di
seme fresco; diluito= inseminazione con due aliquote (4µl/cad) di seme di diluito 1:1 con soluzione Hyes), sia le carat-
teristiche produttive delle colonie sia quelle anatomo-funzionali proprie delle regine non si sono differenziate in maniera
significativa. Anche la durata dell’attività di ovodeposizione ed il tasso di sostituzione delle regine negli alveari - punto
critico per le regine inseminate con la tecnica della centrifugazione del seme - non sono risultati essere significativamente
diversi. La tecnica sperimentata permette di evitare la fase di centrifugazione del materiale seminale diluito, rendendo
più efficiente e sicura la metodologia della miscelazione del seme.

Parole chiave: Apis mellifera ligustica, Ape regina, Spermateca, Spermatozoi, Inseminazione strumentale.

Introduction

At the end of the mating flight, the honeybee queen (Apis mellifera) goes back to the hive with
an average of 80-90 million spermatozoa in the lateral oviducts; the major portion of the semen is
expelled within the next 24 hours (Koeniger, 1986). Thanks to the motility of the spermatheca
and to the contraction of the longitudinal muscles in the walls of the spermatheca duct that cause a
vacuum pump effect, about 5 million spermatozoa reach the spermatheca (Page, 1986; Koeniger and
Ruttner, 1989). Here the spermatozoa are quies-
cent for the entire life of the queen which no longer
mates. This physical aspect of the spermatozoa is
extremely important, but at the moment not
entirely clear. To evaluate these particular physio-
logical aspects, some preliminary tests have been
undertaken.

Using a mutant marker, Moritz (1983, 1984)
demonstrated that the dilution of drone semen and subsequent centrifugation result in the homo-
geneous mixing of the spermatozoa. The centrifugation process not only places limitations on use of
the technique and increases risk of contamination
(Kuhnert, 1988), but may also alter the composi-
tion of the seminal plasma, thus it could be one of
the causes of queen loss and supersedure
(Volprecht Maul, personal communications and
personal experience) and of the greater number of
drone layer queens (Fisher, 1987) and supere-
sedures in the second year of activity (personal expe-
rience).

Skowronek et al. (1995), building on the work
by Harbo (1990) which describes the possibility of
insemination with diluted semen - thereby avoid-
ing the subsequent separation of the diluent by
centrifugation - evaluated mixing effectiveness
using drones of different races. However, their tests
were concluded once the queens began ovipositing
(7-10 d after oviposition). In contrast, the aim of
our test was to study the long term effect of inse-
mination with mixed diluted semen. We evaluated
the possibility of implementing breeding programs
that could be easily approached by queen bee
breeders who need a whole productive season to
carry out the evaluations.

Material and methods

Functional characterisation of sperms from
individual drones and from queens’ spermatheca

To evaluate two physiological aspects (number
and viability) of the sperm in the spermatheca
over time, the contents of spermatheca from 12
artificially inseminated queens that were 2, 12
and 24 months old (4 queens per age group) were
analysed. Queens were inseminated with 8 µl of
fresh semen and maintained in 10-frame hives
until dissection. semen used to inseminate the
queens came from drones from the same mother
queen in the case of 8 queens dissected at 24 and
12 months; drones from a daughter of the mother
queen were used to inseminate the queens that
were dissected after 2 months. The spermatheca
were removed from the queens according to Dade’s
procedure (1985), opened, and the contents diluted
in Tris-buffer.

The released sperm were counted with a
haemocytometer (Bürker counting chamber) and
viability was evaluated by staining with propidi-
um iodide (Collins, 2000). After dilution of 1µl
semen with 400 µl of Tris-buffer at pH 8.8 (Verma, 1978), 6 µl propidium iodide (1 mg/mL in 0.1M phosphate buffer at pH7, incubated at room temperature for 5 minutes) was added. Red fluorescent DNA-intercalating stain revealed non-viable sperm which were visualised by epifluorescence microscopy (1250X) (Figure 1-d). To determine difference in viability between sperm in spermatheca and in fresh semen, 10 sexually mature drones (16-20 days old) produced by the same queen were used. Approximately 1 µl of semen was collected from each drone and sperm viability was evaluated by staining as described before.

**Role of sperm motility as the sperm passes into the spermatheca**

The role of sperm motility during passage into the spermatheca was assessed by inseminating queen bees with seminal material containing: only live spermatozoa, 50% live sperms and 50% dead sperms, or only dead sperms. Fifty µl of semen were collected from a single colony in a large capacity syringe moving the mass of semen with the pressure mechanism of the syringe and was subsequently separated in two 25µl doses. Both doses were diluted with 5µl of Hyes (Ruttner, 1976) solution to fluidify the semen and facilitate further operations. The two 30µl doses represented the live semen and the dead semen - this dose having been treated with 3 cycles of freezing with liquid Nitrogen and thawing with hot water. By mixing 10 µl of live semen with the same amount of dead semen, we obtained 20 µl of semen containing half dead and half live spermatozoa. Virgin queens from nucs, were inseminated as soon as the semen had been treated and returned to the nucs. Three days after insemination the queens were removed from the nucs to avoid the risk of the worker bees culling the queens containing dead semen and placed in transport cages together with 10 workers. These cages were kept in an incubator at 25°C and 65% RH. For each thesis half of the queens were sacrificed on the day in which they were removed (1° repetition), whereas the other half were removed 3 days later (2° repetition). The decision to diversify the days (repetitions) on which the spermatheca was inspected was taken in order to ascertain whether the filling of the spermatheca occurred more slowly with dead sperm than the normal time span of 24-40 hours after insemination (Woyke, 1983). The content of the spermatheca was diluted with tris-buffer and the number of spermatozoa was counted with a haemocytometer (Bürker counting chamber).

Figure 1. Drone spermatozoa anomalies visualised by interferential contrast microscopy and by epifluorescence microscopy (bottom-right) (x1250). a) with coiled tail; b) with frayed tail; c) with double-ended tail; d) non-viable (arrow indicate DNA damaged - fluorescent nuclear region).
Efficiency of diluted semen method in instrumental insemination

Immediately after collection of the semen from drones of a single colony, the glass-tips were emptied into a sterile Eppendorf tube, to which Hyes diluent (Ruttner, 1976; Verma, 1978) was added in a 1:1 semen/diluent ratio. After careful mixing performed with the sterilized tip of a micropipette by stirring and by repeated suction into pipette tip for 1' - the diluted semen was drawn back into the glass-tips and used to inseminate a group of sister-queens (N=16) with an aliquot of 8 µl. On the following day the operation was repeated, inseminating the same queens with an additional 8 µl. The control group was formed by 16 queens (sisters of the previous queens) which were inseminated with untreated semen (4µL x 2 times). Fifteen days after the beginning of oviposition, the 32 mated queens moved from the nucs and were introduced in queenless colonies, which had previously been equalised in number of brood and honey combs.

The queen parameters used to evaluate the success of the adopted methodology were the following: dead in nucs or never laying; days to lay eggs; supersEDURE in hives at the end of the first year; weight at removal (15 months after insemination); brood viability: proportion of capped cells in a representative section of 100 brood cells at L5 stage; total amount of brood reared in the year following the insemination (from March to August, at regular intervals of 21 days, brood combs were inspected according to the method described by Wille and Gerig, 1976a, 1976b, 1976c and Gerig, 1983); n. of surviving queens at the end of the test; residual sperms in spermatheca; proportions of live and dead spermatozoa (determined by fluorescent staining, as described above).

Data were evaluated by analysis of variance (ANOVA).

Results and discussion

Functional characterisation of sperms

The microscopic study of the spermatozoa revealed some of the most frequent anomalies, shown in Figure 1. Furthermore a significant variability among drones of a same colony was underlined as far as spermatozoa viability is concerned (N=10 mean=32.8±4.06 s.e.) (Figure 2).

As expected, the total number of sperm decreased significantly with the ageing of queens (F = 6.170, DF = 2,9, P = 0.021 by analysis of variance). There were 5.1 ± 0.9 (SE) million sperm in 2-month-old queens, 4 ± 1.1 million in 12-month-old queens and 2.6 ± 0.9 million in 24-month-old queens (Figure 3). Queens aged 24 months held significantly fewer sperm than queens aged 2 months (P < 0.05 by Duncan’s multiple range test).

In contrast to the total number of sperm, the proportion of dead sperm increased from 20.5 ± 2.5% in 2-month-old queens, 4 ± 1.1 million in 12-month-old queens and 2.6 ± 0.9 million in 24-month-old queens (Figure 3). Queens aged 24 months held significantly fewer sperm than queens aged 2 months (P < 0.05 by Duncan’s multiple range test).

In contrast to the total number of sperm, the proportion of dead sperm increased from 20.5 ± 2.5% in 2-month-old queens, 21.5 ± 2.1% in 12-month-old queens and then to 33.5 ± 3.2% in 24-month-old queens (Figure 3). These proportion
differed ($F = 4.741$, $DF = 2,9$, $P = 0.039$ by analysis of variance after the proportion data were arcsine transformed to equalize variances), with queens aged 24 months carrying significantly more dead sperm than the queens of the other two ages ($P < 0.05$ by Duncan’s multiple range test). The relation between dead sperm and time (age of the queens) showed a significant exponential trend ($y = 18.3e^{0.0228x}$, $R^2 = 0.86$, $P < 0.05$). The sperm in the spermatheca apparently underwent an initial selection for quality. This study found a relatively low proportion of dead sperm (20.5%) in the spermatheca of 2-month-old, artificially inseminated queens when compared with the semen collected directly from drones ($32.8 \pm 4.1 \%$ dead sperm). According to Collins (2000), however, not all non-viable sperm is eliminated in the passage of the semen in the spermatheca because live sperm drag others along in the migration.

Figure 3. Average residual amount of semen and viability of spermatozoa in the spermatheca of queens different ages ($n=4$ per group). Vertical lines are one SE

| Treatment of semen | Repetition | Sperm viability (%) | Inseminated Queens (n) | Average n. of sperm/repetition (million) | Average n. of sperm/treatment (million) |
|--------------------|------------|---------------------|-----------------------|------------------------------------------|----------------------------------------|
| Dead               | 1°         | 0                   | 2                     | 0                                        | 0                                      |
|                    | 2°         | 0                   | 1                     | 0                                        | 0                                      |
| Dead/live          | 1°         | nd                  | 2                     | 0.250                                    | 0.285                                  |
|                    | 2°         | 78                  | 2                     | 0.321                                    |                                        |
| Live               | 1°         | 88                  | 2                     | 1.755                                    | 1.997                                  |
|                    | 2°         | 44                  | 2                     | 2.240                                    |                                        |

nd: not determined
Role of sperm motility as the sperm passes into the spermatheca

The queens inseminated with non-treated semen show spermatheca-filling values lower than those normally reached with usual instrumental insemination technique (3-5 million, by Mackensen, 1964) due to the semen dilution during the dose preparation (Table 1). No spermatozoa were found in the spermathecal contents of queens inseminated with only freeze-killed semen (Table 1). This finding is also supported by the different mean numbers of spermatozoa detected in the spermatheca at the two different sampling times (repetitions). For the first treatment the seminal material was of a poorer quality than the one used for the second treatment; this accounts for the lower mean number of spermatozoa at the first sampling. The limited number of individuals for each replica and for each thesis does not, however, allow a statistical comparison between replications of the same thesis.

The results obtained in the test conducted with queens inseminated with various mixes of fresh and freeze-killed semen, confirmed research by other authors (Harbo, 1976; Collins, 2000; Wilde et al., 2001) and demonstrated that passage of the spermatozoa into the spermatheca is attributable exclusively to the action of spermatozoa motility. Collins (2000) stated that when the live proportion of spermatozoa in the seminal material (dose) was reduced to less than 50%, this activity could carry only a portion of dead cells. In the present experiment the low concentration of sperm present in queens inseminated with 50% of dead semen - less than half the concentration present in queens inseminated with 100% live sperm - could be due to the "entrapping" effect of the flow of dead semen which is expelled from the oviduct in the hours immediately following the insemination.

Efficiency of the dilution and mixing of semen from different drones method

In the two groups of colonies with inseminated queens compared in this study, the productive and reproductive characteristics of the two colonies showed no significant differences (Tables 2 and 3). Likewise, duration of oviposition activity and the supersede rate in hives at the end of the second year of activity were also not significantly different in the two groups. The group of queens inseminated with diluted semen started oviposition 5.7 days on average after insemination, about one day later (4.35 days) if compared with the data obtained by Kuhnert (1988) with queens inseminated with homogenized semen technique. The results Kuhnert obtained, on the other hand, 61.84% of survival rate in the colonies after only 8 months from insemination, mean that one third of the queens are lost before a complete evaluation cycle. In the present study, the efficiency of diluted semen technique has been tested in a longer period: the survival rate was greater (71.4%) after 15 months.

Conclusions

If the exponential increase in the proportion of non-viable sperm held by a queen is confirmed, future research should also examine whether there is a relationship between the amount of non-viable sperm and the likelihood that queens become drone layers. If such a relationship is con-

Table 2. Results of queens inseminated with the two techniques (control = two inseminations with 4 µl of untreated semen each; diluted = two inseminations with 8 µl of 1:1 diluted semen).

| Treatment semen | Total inseminated (n.) | Dead in nucs or never laying (n.) | Time to lay eggs (mean±SE) in hives (1st year) | Supersede (n.) | Surviving at the end of the test (n.) |
|-----------------|------------------------|----------------------------------|-----------------------------------------------|----------------|-------------------------------------|
| Control         | 16                     | 3                                | 5.5±0.4                                       | 2              | 11                                  |
| Diluted         | 16                     | 2                                | 5.7±0.5                                       | 4              | 10                                  |
firmed, then improved technology for storing and manipulating (e.g., mixing) semen should be sought. At the current state of knowledge, in experiments in which residual sperm in aged queens is of interest, it would be advisable to evaluate the proportion of viable sperm to get a more complete understanding of the spermathecal contents.

The technique of queen bee insemination with diluted semen, already shown in other studies to be effective in achieving homogenous mixing of the various sperm types, makes it possible to avoid the stage of centrifuging the diluted seminal material. The present study was carried out with the aim to evaluate the efficiency of diluted semen technique in a long period (15 months). This time span is important because Italian queen bee breeders choose the best queens only at the end of the second year, after a whole seasonal evaluation of the colonies. This means they need most of the queens to arrive at the end of the evaluation cycle. The semen mixing methodology is therefore more efficient and reliable and is beneficial from genetic and breeding points of view. The only disadvantage of this methodology concerns the twofold insemination. This is indispensable in order to introduce into the spermatheca the minimum dose of sperms required for the two years of activity of the queen.

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