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Original article

The insemination of queen honeybees with diluted semen

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Summary — The aim of this study was to assess the possibility of queen honeybee insemination with a semen–diluent mixture and to evaluate the rate of insemination and the accuracy of stirring. Queen honeybees inseminated twice with a Hyes solution/semen mixture (1:1), given every other day at 8 µl per dose, stored as many spermatozoa in their spermatheca as those inseminated once by a classical technique. The onset and percentage of oviposition were similar to those observed in other procedures. The diluted mixture showed complete mixing of the semen collected from several drones.

queen honeybee / instrumental insemination / diluted semen

INTRODUCTION

During natural mating the queen honeybee collects the semen from 8–10 drones in the spermatheca. In the case of instrumental insemination, the semen is collected from a similar number of drones. An increase in this number may be indicated for certain genetic and breeding purposes. Use of a washing procedure for semen collection has substantially increased the number of drones involved (Kaftanoglu and Peng, 1980): semen is washed directly from the bulbs of the drone's endophallus, and after centrifugation of the diluent is used for queen insemination. A similar procedure is used for the semen collected by the classical technique. However, the semen is diluted with a several-fold volume of diluent and centrifuged (Moritz, 1983). This technique was adopted after modification and introduced into practice (Moritz, 1984; Fisher, 1987; Cobey and Lawrance, 1988; Kühnert *et al,* 1989; Dustmann *et al,* 1991; Kühnert, 1991a,b). However, its practical use is limited by the centrifugation process.

Using genetically marked sperm, Harbo (1990) found that careful stirring of the semen with diluent (1:1) renders queen insemination with this mixture possible without the above-mentioned centrifugation process.

The aim of this investigation was to study the effect of queen insemination with semen mixed with diluent on the number of sper-

matozoa entering the spermatheca. Moreover, both the percentage and rate of oviposition by queens were estimated *via* several procedures. The accuracy of sperm stirring was also evaluated.

MATERIALS AND METHODS

Hyes solution was used as sperm diluent as indicated in several reports (Ruttner, 1976; Verma, 1978). Immediately after collection, the semen was diluted (1:1, semen/diluent) and the total volume stirred for 2 min with a sterilized needle. In the first step this solution was used for insemination of Carniolan and Caucasian queens with 2 doses, 8 µl per dose given every other day, or a single 16 µl dose. Control queens of the same origin were inseminated with 8 µl fresh semen. After insemination, the queens were placed in an incubator at 30°C together with 10–15 workers. Two to four days after insemination the queens were anaesthetized, the spermatheca removed and the number of spermatozoa counted using a Fuchs–Rosenthal haemacytometre (Woyke, 1960).

In the second step 1- or 2-d-old queen sisters (Caucasian) were placed in a one-comb nucleus together with about 2 000 workers. All the queens were divided randomly into 3 groups and treated as follows at age 7–10 d.

Group 1 queens were inseminated every other day with an 8 µl dose of sperm diluted with Hyes solution (1:1) prepared as described above. The semen for the insemination of 15 queen honeybees was collected from about 50 Caucasian (45 µl) and 15–20 Italian (15 µl) drones on the day of insemination. After dilution and stirring, the semen was used to inseminate queen honeybees with 50% of the standard dose of semen.

Group 2 queens were inseminated with 8 µl sperm diluted 30-fold with Hyes solution after collection and careful hand shaking. The solution was centrifuged at 2 500 rpm for 10 min (Dustmann *et al,* 1991). Samples of 90 and 30 µl semen from the Caucasian and Italian drones, respectively, were used for insemination of 15 queens.

Group 3 queens were inseminated with 8 µl fresh sperm collected separately (6 µl from Caucasian and 2 µl from Italian drones).

The diluents used were sterile-filtered before use. After insemination, the wings were clipped by 1/3 and queen excluders were fixed to the entrances to prevent natural mating. Two days after insemination, the queens in *Groups 2* and *3* were treated with CO_2 for about 3 min.

The onset of oviposition was examined daily and the queens were collected 7–10 d after oviposition. The day before bee emergence, the combs with sealed brood were placed in an incubator and the emerging workers were counted and divided into batches of yellow-banded and dark progeny. A hundred or more workers were counted for each queen. The data were evaluated statistically by ANOVA. Differences between mean values were evaluated *via* Duncan's test. The number of dark and yellow-banded workers and the correlation with the theoretical distribution pattern was estimated by a χ^2 test.

RESULTS

The number of spermatozoa in the spermatheca of queens (table I) inseminated

Table I. The number of spermatozoa in the spermatheca of queens inseminated with fresh semen or with semen diluted in Hyes solution (1:1).

Procedure	*Number of queens*	*Mean ± SE*	*Range*
Diluted, 2 x 8 µl	11	5 025 ± 573[b]	2 720 – 7 800
Diluted, 1 x 16 µl	14	3 125 ± 409[a]	1 480 – 5 520
Classical; fresh semen, 1 x 8 µl	12	3 48 ± 551[ab]	1 560 – 7 600

[a,b] Duncan's test: means folllowed by the same letter are not significantly different; $\alpha = 0.05$.

with the semen-diluent solution or with fresh semen were found to be substantially different, and ranged from 1.5 to 7.8 million.

Queens inseminated twice with 8 µl semen diluted in Hyes solution had a significantly higher number of spermatozoa than those inseminated once with 16 µl diluted semen. The number of spermatozoa in the spermatheca of the queens inseminated with 8 µl fresh semen appeared lower than the number in queens inseminated with 2 doses of diluted semen, but higher than that in queens inseminated once with 16 µl diluted semen. However, these differences were non-significant.

The 3 insemination methods (table II) showed no substantial effect on the percentage of laying queens. Queens inseminated *via* the classical technique were found to have the lowest percentage of successful insemination (73%), followed by those inseminated with the semen/diluent solution and with centrifuged semen (86%).

The absolute number of non-laying queens differed by only one; this is therefore unlikely to indicate the most efficient method. The mean time of onset oviposition was similar in all the groups examined.

The distribution of dark and yellow-banded progeny differed from normal (3:1). The smallest differences (4%) were noted in queens inseminated with the sperm-diluent solution, and the highest (> 11%) in those inseminated with the centrifuged semen. However, from these data it was not possible to evaluate the accuracy of stirring in the case of sperm originating from drones of 2 different races. This may be evaluated when inclusion of progeny from individual queens is taken into account.

Table III indicates that the numbers of dark and crossed progeny were more closely correlated with the theoretical distribution (3:1) in queens inseminated with the semen/diluent mixture. The values calculated by χ^2 test exceeded the threshold (3.84) in only 2 out of the 11 queens examined. Consequently, the goodness-of-fit between the observed and the theoretical distribution at the 95% level was not found in 2 cases. Furthermore, lack of correlation was noted in the progeny from 5 out of 12 queens inseminated with the centrifuged semen. In 5 cases out of 6, queens inseminated with fresh unmixed semen collected from Caucasian (6 µl) and Italian drones (2 µl), the progeny showed distribution independent of the amount of semen injected.

Table II. Effect of queen insemination with semen mixed by different procedures.

Procedure	*Number of queens*	*Laying queens*		*Time of oviposition (d)*		*Progeny (%)*	
		Number	*%*	*Range*	*Mean*	*Dark*	*Yellow-banded*
Semen/diluent 1:1, 2 x 8 µl	15	12	80	5–12	7.7[a]	70.9	29.1
Centrifuged semen 8 µl	15	13	86	4–13	7.7[a]	86.6	13.4
Classical; fresh semen	11	8	73	6–10	7.2[a]	68.7	31.3

[a] Duncan's test: means followed by the same letter are not significantly different; $\alpha = 0.05$.

Table III. Results showing the number of dark and yellow-banded workers originating from queens inseminated with the semen of Caucasian and Italian drones (3:1), according to semen preparation procedure and goodness of fit (χ^2).

Number of queens	*Progeny from queens inseminated with semen/diluent mixture (1:1)*			*Progeny from queens inseminated with diluted centrifuged semen*			*Progeny from queens inseminated with fresh unmixed semen*		
	Dark	*Yellow-banded*	χ^2	*Dark*	*Yellow-banded*	χ^2	*Dark*	*Yellow-banded*	χ^2
1	99	39	0.61	275	15	48.37	69	37	5.55
2	133	48	0.27	98	28	0.38	105	59	10.53
3	97	43	1.92	120	31	1.29	121	16	12.90
4	67	24	0.06	90	7	13.67	67	35	4.72
5	95	26	0.71	128	16	14.81	86	65	26.10
6	129	57	2.27	269	7	74.28	89	33	0.27
7	93	33	0.04	109	22	4.90			
8	58	58	38.66	52	14	0.33			
9	176	47	1.93	86	20	1.83			
10	144	68	5.69	86	25	0.43			
11	62	30	2.84	150	30	7.43			
12				93	14	8.37			

$P_{0.05} = 3.84$.

DISCUSSION

The number of spermatozoa in queens inseminated with a mixture of semen/Hyes solution (1:1) given twice at 8 µl per dose were not significantly higher than the number of spermatozoa found in the spermatheca of the queens inseminated with a single dose of 8 µl semen. An increase in the number of spermatozoa in queen spermatheca after treatment with 2 doses of semen has been found by several authors (Mackensen, 1955; Woyke, 1960). The mean number of spermatozoa obtained in the present experiment (5 million) resulted in normal oviposition by the queen and was in accordance with the results found by others (Mackensen, 1955; Woyke, 1960).

The percentage of queens initiating oviposition (table II) was relatively high. Only 8 queens (20%) did not lay eggs and 3 of them died while passing queen excluders and when attempting to fly, possibly to attempt natural mating (Woyke and Jasinski, 1992). The remaining dead queens were found in the nuclei, *ie* 2 from *Groups 1* and *2*, respectively, and one from *Group 3*. Three further queens died 1–2 d after the second insemination or from narcosis and 2 non-laying queens died after 2 weeks.

During insemination of queens with centrifuged semen, the following was found:

i) centrifugation was an inconvenient process; and ii) diluent residues appeared in the semen as a result of incomplete separation using the procedure of Dustman *et al* (1991).

The selection of drones for semen collection was made at a ratio of 3:1. However, the sum of the totally dark and the yellow-banded progeny contrasted with this ratio (table II). In the controls, the sperm was collected separately for each queen; thus, the sperm volume collected per needle may not have been the same. Moreover, sperm mixing in the queen's oviducts might be incomplete (Taber, 1955; Woyke, 1960). However, in the case of queens in *Groups 1* and *2*, the semen allotted for all queens was taken in a single dose. Thus, the above consideration does not apply.

In the present queen insemination technique, improvement in semen/diluent stirring is required. The mechanical stirring proposed by Harbo (1990) failed to produce satisfactory results. The semen/diluent mixture (1:1) was found to be too thick to be stirred by mechanical agitator.

CONCLUSIONS

A 1:1 mixture of sperm/Hyes diluent may be used for instrumental insemination of queen honeybees. Queens inseminated with this mixture at 2 doses of 8 μl per dose stored as many spermatozoa in their spermatheca as those inseminated once by a classical method, and the number of spermatozoa was sufficient to initiate normal oviposition.

The rate of oviposition by queens inseminated with the semen/Hyes solution mixture was similar to that resulting from insemination with diluted and centrifuged semen or fresh semen. Moreover, all the procedures examined demonstrated a similar onset of oviposition. In the semen collected from several drones and diluted with the same volume of diluent, stirring effectiveness was similar to that in the case of a higher volume of diluent.

Résumé — L'insémination des reines d'abeilles avec du sperme dilué. L'insémination de reines d'abeilles avec un mélange de sperme préparé selon la procédure habituelle présente l'inconvénient de nécessiter des centrifugations. Le but de ce travail est d'étudier la possibilité d'inséminer des reines avec du sperme dilué dans une solution, et d'évaluer le nombre de spermatozoïdes présents dans la spermathèque des reines inséminées. Le pourcentage et le taux d'oviposition des reines inséminées ont été évalués ainsi que l'homogénéité du mélange de spermes. Le sperme a été dilué dans une solution de Hyes. Les reines ont été inséminées avec une solution de sperme dilué (1:1) avec 2 doses de 8 μl ou une dose unique de 16 μl et placées dans une étuve pendant 2 à 4 j. Des reines témoins ont été inséminées avec du sperme non dilué. Le nombre de spermatozoïdes dans la spermathèque a été calculé pour 8 à 14 reines dans chaque groupe. Des reines caucasiennes ont été inséminées de la façon suivante : i) avec du sperme dilué dans une solution de Hayes (1:1) ; ii) avec du sperme dilué 30 fois et centrifugé ; iii) avec du sperme frais. Le sperme a été prélevé chez des mâles caucasiens (75%) et italiens (25%). Onze à 15 reines ont été inséminées dans chaque groupe. Le début de l'oviposition et le nombre de descendants de chaque couleur (ouvrières à bandes jaunes et ouvrières noires) ont été déterminés. Dans tous les groupes, le contenu moyen en spermatozoïdes dans la spermathèque a varié de 3,125 à 5,025 millions. Une augmentation significative du contenu en spermatozoïdes a été trouvée chez les reines inséminées 2 fois avec du sperme dilué dans une solution de Hayes, comparativement à celles inséminées avec une seule dose de 16 μl.

Ces reines stockent davantage de spermatozoïdes dans leur spermathèque que celles inséminées une seule fois avec du sperme frais. La date du commencement de l'oviposition a été identique pour tous les groupes. Le pourcentage de reines initiant une oviposition a varié de 73 (témoins) à 86% (sperme centrifugé). La distribution de la progéniture a été différente de la normale (3:1). Les différences dans l'homogénéité du mélange de sperme (tableau III) ont été moins marquées chez les reines inséminées avec une solution de sperme que chez celles inséminées avec du sperme frais ou centrifugé.

reine d'abeilles / insémination artificielle / sperme dilué

Zusammenfassung — Insemination von Königinnen mit verdünntem Sperma. Die übliche Praxis der Besamung von Königinnen mit durchmischtem Sperma bedeutet erhebliche Zusatzarbeit durch das Zentrifugieren. Deshalb wurde die Möglichkeit geprüft, die Königinnen mit verdünntem Sperma zu besamen. Es wurde untersucht, wieviele Spermatozoen nach solch einer Besamung in die Spermatheka gelangen, und es wurde der Prozentsatz und die Eilegerate der inseminierten Königinnen und der Erfolg der Vermischung bestimmt. Für die Verdünnung wurde eine Lösung nach Hyes benutzt. Die Königinnen wurden mit einer Sperma/Hyes Mischung von 1:1 besamt, entweder zweimal mit 8 µl oder einmal mit 16 µl. Sie wurden zusammen mit den Kontrollköniginnen, die mit unverdünntem Sperma besamt waren, 2 bis 4 Tage in einem Wärmeschrank gehalten. Die Anzahl der Spermatozoen in der Spermatheka wurde für jede Gruppe bei 8–14 Königinnen bestimmt. Es wurden kaukasische Königinnen wie folgt besamt: i) mit einer Sperma/Hyes Mischung (1:1); ii) mit zentrifugiertem Sperma nach 30facher Verdünnung; iii) mit frischem unbehandelten Sperma. Das Sperma wurde zu 75% von Drohnen der kaukasischen und zu 25% der italienischen Rasse gewonnen. Für jede Gruppe wurden 11 bis 15 Königinnen besamt. Der Beginn der Eiablage und die Anzahl der gelbgestreiften bzw der dunklen Nachkommen wurde bestimmt. In allen Gruppen lag die mittlere Anzahl der Spermien in der Spermatheka zwischen 3,125–5,025. Im Vergleich zu Königinnen, die nur einmal besamt waren, wurde eine signifikant höhere Spermienzahl bei Königinnen gefunden, die zweimal mit 8 µl Sperma/Hyes Lösung besamt wurden. Königinnen hatten nach einer einmaligen Besamung mit frischem, unverdünnten Sperma mehr Spermatozoen in der Spermatheka als nach einmaliger Besamung mit verdünntem Sperma. Dieser Unterschied war jedoch nicht signifikant. Der Beginn der Eiablage war in allen 3 Gruppen ähnlich, er lag bei 73% bei den Kontrollköniginnen und bei 85% bei den Königinnen mit zentrifugiertem Sperma. Die Verteilung der Nachkommenschaft zwischen Bienen mit gelben Abdominalstreifen und dunklen Bienen unterschied sich in allen Gruppen von der Norm 3:1. Die Abweichungen reflektieren den Erfolg der mechanischen Durchmischung des Sperma (Tabelle III) und war bei den mit verdünntem Sperma besamten Königinnen geringer als bei den mit unbehandeltem und zentrifugiertem Sperma besamten Königinnen.

Honigbiene / Königin / instrumentelle Besamung / verdünntes Sperma

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