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Original article

Relationship between semen quality and performance of instrumentally inseminated honey bee queens

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Abstract – Techniques to effectively store honey bee semen must meet some minimally acceptable level of spermatozoa survival. To determine this level, honey bee queens were inseminated using various mixes of fresh and freeze-killed semen, and were allowed to lay eggs in small colonies for three weeks. The queens receiving all freeze-killed spermatozoa (0% fresh) had no spermatozoa in their spermathecae, and produced only drone pupae (unfertilized eggs). The proportions of live and dead spermatozoa (determined by dual fluorescent staining) in the spermathecae of queens receiving 25 to 100% fresh semen were not significantly different at 27 days post-insemination. Queens receiving 50% fresh semen or more produced only worker pupae (all eggs were fertilized). Therefore, a program to improve storage of semen should only have to reach survival levels of 50% of the spermatozoa to have functional semen.

***Apis mellifera* / spermatheca / brood pattern / spermatozoa viability / sperm storage**

1. INTRODUCTION

The development of a practical means to store honey bee (*Apis mellifera* L.) semen would enhance our ability to select and maintain superior honey bee stocks. A number of storage techniques have been tried with some success, using both ultra-cold freezing and non-frozen approaches (reviewed in [22]). Various assays were used to evaluate the success of the storage including: assessing semen quality based on spermatozoal motility [12, 21], counting the number of spermatozoa in the spermatheca (semen storage organ) of an instrumentally inseminated queen [2, 6, 9, 11, 12], and comparing the proportion of worker pupae (from fertilized eggs) to drone pupae (unfertilized eggs) produced by an inseminated queen [6, 9, 18, 23]. Harbo [7] also measured egg hatching rate.

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More recent research raises the possibility that previous assessments of storage success may be misleading. Stains that directly assess viability of the spermatozoa have now been applied to the honey bee [3, 14, 17]. Use of such stains indicated that lack of motility was not necessarily an indication of dead spermatozoa [15], nor did presence in the spermatheca ensure that a spermatozoan was living (pers. obs.). In relation to the third assay that has been used, Harbo [8] estimated that 20–35 spermatozoa are released from the spermatheca when a queen lays an egg, many more than are needed for successful fertilization. So the ratio of worker to drone pupae may not actually reflect the proportion of live spermatozoa in the spermatheca. If the presence of even a reduced proportion of viable spermatozoa can ensure that the majority of eggs are fertilized, this would have a profound effect on the goals of any storage methods program. Since the success of any technique for storing honey bee semen will ultimately be measured by the ability of queens to produce sufficient quantities of normal females (workers or, more importantly, daughter queens), in this study the relationship between semen viability and the performance of instrumentally-inseminated queens has been examined.

2. MATERIALS AND METHODS

2.1. Semen collection

Mature drones were obtained from a single colony in our apiary in Beltsville, MD. A total of 100 µL of semen was collected from multiple drones using a Harbo syringe [10]. This system allows for the inclusion of a collection tube to hold large volumes of semen, in this case a sterile, 100 µL capillary tube. Sterile saline solution with antibiotic (sodium chloride 1%, dihydrostreptomycin 0.25% in boiled water) was used to lubricate the tip, but was not collected with the semen. The 100 µL of semen was added to 1.4 mL buffer (D+ glucose: 0.3 g; potassium chloride: 0.41 g; sodium bicarbonate: 0.21 g; sodium citrate–2 hydrate: 2.43 g, in 100 mL deionized, sterilized water) [16] in a sterile Eppendorf tube, and gently mixed by inversion. The tube was then spun at $100 \times g$ for 30 min to reconcentrate the spermatozoa. The spermatozoa in buffer and seminal fluid, approximate volume of 200 µL, was recollected in sterile capillary tubes and capped with plastic seals. Half of the capillary tubes were stored overnight at room temperature; the other tubes were frozen at –80 °C to kill the spermatozoa.

2.2. Evaluation of spermatozoa viability

Because the collection, mixing and handling of semen is damaging to the spermatozoa, it was necessary to check the viability at several stages. Two 2 µL fresh samples were stained with SYBR-14 and propidium iodide using the procedure of Collins and Donoghue [3]. This dual fluorescent staining process colors the DNA present in the heads of living spermatozoa green, and in dead cells red. Four aliquots of each of the two samples were mounted on slides and 100 cells in each were classified by stain color, at 400 × magnification using a fluorescent light source with an isothiocyanate (FITC) filter.

The fresh and frozen semen were used to make five treatment groups for instrumental insemination (II). Virgin queens from a commercial breeder were each inseminated with 8 µL either of 100% fresh; fresh:frozen, 75:25%; fresh:frozen, 50:50%; fresh:frozen, 25:75%; or 100% frozen semen. Six queens were used for each treatment group. All queens were marked on the thorax with a different numbered colored tag, and one wing was clipped to prevent flying. Two samples from the unused portions of each mix and one from the 100% fresh semen used for II were evaluated for percentage live spermatozoa, as before. Three aliquots from each were counted.

2.3. Evaluation of brood

The day after insemination, all queens were narcotized with CO_2 to induce egg laying and introduced into five-frame nucleus colonies with entrances covered by zinc queen-excluder material. Each colony had a minimum of three full frames of bees. Queens were self-released from cages sealed at one end with confectioner's sugar candy, and at three days after introduction, any queens still caged were released. Colonies were fed with sugar syrup (50% by volume in water) and pollen supplement (made with processed yeast).

At 24–25 days after insemination, the colonies were checked for the presence of the marked queens and evaluation of brood. The presence of developing worker and drone offspring was determined by visual inspection. To verify this assessment, a representative section of 100 cells within a patch of brood was outlined with a cardboard template, and the cells were scored as being either worker or drone, based on the form of the capping; drone pupae have wax caps on their cells that are distinctly raised, versus flat caps on worker pupae cells. Ten cells out of the 100 cells were uncapped to check pupal eye formation; drones have much larger eyes. With the same 100 cells, the number of empty cells was counted to determine spottiness. The size of a brood patch was rated as either none, small, medium, large, or very large, with 'very large' being equivalent to an area 2/3 of a standard (9 × 18.5 in) Langstroth frame, and small less than 4 square in.

2.4. Queen dissection

Twenty-seven days after insemination, the spermatheca was dissected from each queen and crushed in 500 μL semen buffer solution using individual sterile Eppendorf tubes with pestles. The semen released was stained for viability, and counted (number live per 100 cells) as previously. Two aliquots were counted for each queen. A control group of five naturally-mated queens from the same breeder were also dissected, and semen from their spermathecae evaluated for viability.

2.5. Statistical analysis

Results were analyzed using a general linear models (GLM) procedure [20]. Samples were classified by treatment group and aliquot sequence number. For number of live cell data, the model consisted of aliquot sequence within treatment group; for percentage worker pupae and percentage empty cells, it was treatment group only (one measurement taken per queen). Aliquot sequence number was not a significant factor, so was removed from the final model statement. The level of viable spermatozoa in the semen used for instrumental insemination and the semen from the spermathecae was also analyzed by GLM, using source by mix level as the model. Appropriate comparisons of means were carried out by the method of least significant differences (LSD).

3. RESULTS

3.1. Semen quality

One queen was lost during the introduction, three died, and four were superseded during the experiment. These were distributed across all treatment groups, and were not included in the results.

The proportion of live spermatozoa in the semen collected from the spermathecae of the inseminated queens 27 days after insemination was not significantly different for the treatment groups that received any live spermatozoa (df = 4; $F = 4.08$; $P < 0.01$) (Fig. 1). No spermatozoa were ever seen in the spermathecal contents of queens inseminated with all freeze-killed semen. All treatment classes were signifi-

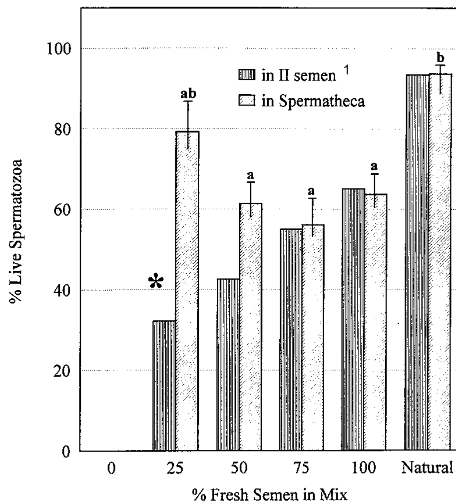

Figure 1. Viability of spermatozoa (mean ± SE) in five mixes of fresh and freeze-killed semen used for instrumental insemination and freshly collected (natural) semen, and in semen retrieved (27 days post-insemination) from the spermathecae of queens inseminated with the mixes, or naturally mated queens. Columns with the same letter are not significantly different (df = 4, F = 4.08, P < 0.01); * indicates that these two levels of viability are significantly different (df = 9, F = 3.47, P = 0.014). [1] Values for natural semen are taken from another study. Viability is expressed as the number of live spermatozoa per 100 counted, using a dual fluorescent staining technique [3].

cantly lower for percentage live spermatozoa than the naturally mated queens, except for the 25% fresh-semen group.

The pooled semen which was used to make the five mixes of fresh and freeze-killed semen had only 65.1% live spermatozoa (treatment group: 100%), based on the assessment of the samples taken prior to insemination (Fig. 1). The values for the other mixes were 55, 42.6, 32.2, and 0%, respectively. This loss of viability was due to handling of the semen during collection, mixing and insemination. Freshly collected unmixed semen, assayed immediately, generally gives values of 90% or more live spermatozoa.

The surprising result of these inseminations, however, was that the mixes with less fresh semen (25 and 50%) pre-insemination, were associated with spermathecal contents that had higher proportions of live spermatozoa, 27 days post-insemination (Fig. 1). Only the 25% level was significantly different (df = 9, F = 3.47, P = 0.014), but the trend was consistent across the treatment groups.

While the number of spermatozoa in the spermatheca of each queen was not counted, relative concentrations could be noted by the number of cells found in the stained viability samples. Two of the three queens in the 25% live spermatozoa group and three of the five queens in the 50% live spermatozoa group had less than 100 cells found and counted on a slide. Therefore, these queens probably had very low numbers of spermatozoa present in their spermathecae.

3.2. Brood quality

At the time of brood evaluation, each queen had one frame in the nuc with sealed pupal cells. Queens inseminated with 50% or more fresh semen produced more brood (all but one had large or very large patterns) than queens in the 25 and 0% fresh groups (Tab. I). All of the brood from the former group consisted of 100% worker pupae, and on average, about 20% of the cells were empty. The queens that received all freeze-killed sperm (0% live) produced only drones (in worker cells), usually in smaller patches, and with twice the number of empty cells. The queens receiving only 25% fresh semen (32.2% live spermatozoa) had brood patches that were generally smaller, but like the first group, on average about 20% of the cells were empty. However, while queen 1 produced 100% worker pupae, queen 2 had 80% worker pupae, and queen 3 had only drone pupae. The two queens laying drones had very low concentrations of spermatozoa in the spermatheca, with 70 and 75% live spermatozoa, respectively. Queen 1 had

Table I. Evaluation of brood from honey bee queens artificially inseminated with varying mixes of fresh and freeze-killed semen.

	Semen mix[1] Group				
	100 (65.0)	75 (55.0)	50 (42.6)	25 (32.2)	0 (0.0)
No. of queens surviving	5	5	5	3	4
No. of queens producing a brood patch (size)					
None					1
Small				1	1
Medium	1			2	1
Large	2	1	3		1
Very large	2	4	2		
Percentage of pupae that were workers in a representative section of 100 cells	100	100	100	60[2] (100) (80) (0)	0
Percentage of cells that were empty in a representative section of 100 cells	18.6	20.6	18.8	18.7	40.5

[1] Mix is expressed as the percentage of fresh semen in a mixture of fresh and freeze-killed semen. Number in parentheses is the actual proportion of live spermatozoa in the semen mix used for artificial insemination, assessed using a dual fluorescent staining technique.
[2] These three queens were very variable in brood quality; see Results, 3.2., for details.

many more spermatozoa in the spermatheca, with a 95% level of viability.

4. DISCUSSION

Although very different mixes of semen were used for the instrumental insemination of the queens, the proportions of live spermatozoa in their spermathecae at 27 days post-insemination were not significantly different for the four treatment groups that had any live spermatozoa present. In some way, live spermatozoa were being concentrated in the spermatheca, although not all dead sperm were eliminated. The shift in proportion of live spermatozoa was greatest in the queens inseminated with semen having the least viable spermatozoa.

Our current understanding of honey bee reproductive behavior (reviewed in [13]) is that the spermatozoa orient to the walls of the oviduct after deposition by the drone. Then contraction of the oviduct and abdomen transport some of them to the orifice of the spermaduct. Once there, the spermatozoa actively migrate through the narrow spermaduct to the spermathecae. A majority of the semen is actually discarded by the queen [19].

All of the queens that were inseminated with only dead spermatozoa had no sperm cells present in the spermatheca, and the spermathecae appeared as crystal clear as those of virgin queens. This agrees with previous observations on frozen semen by Harbo [5]. Although semen was present in the vagina, none of the dead spermatozoa were transported to the spermatheca. This supports the conclusion that the activity of the spermatozoa themselves is critical to their migration to the spermatheca. However, Bresslau [1] suggested that the sperm

pump, a muscular arrangement around a bend of the spermaduct, supported migration to the spermatheca as well as the release of spermathecal contents for fertilization. And Gessner and Ruttner [4] reported that when plastic granules of 7 µm diameter were used, 0.3% of the number predicted if all the movement by the spermatozoa was passive on their part did reach the spermatheca. While dead spermatozoa in the spermathecae were found in the present study, this only occurred in queens where there were live spermatozoa present to 'drag' the others along in the migration. In fact, when the proportion of live spermatozoa was high (56 or 62%), most of the dead spermatozoa were carried along. When the live proportion was reduced (42.6 or 32.2%), their activity could carry only a portion of the dead cells. The net result is that we can not make the assumption that all spermatozoa found in the spermatheca are viable.

Secondly, all of the queens that received 42.6% live spermatozoa (50% fresh semen) or more produced brood that was entirely worker in caste. This ability of these queens to produce only workers (fertilized eggs) is facilitated by the fact that the queen releases 1/153 000th of the spermathecal contents with each egg laid [8]. This volume contains 30–35 spermatozoa initially, and after significant egg laying, falls to about 20 spermatozoa. For the average viability of 64.7% in the four treatment groups, this means 13–26 sperm would be available for fertilization. As it is estimated that 0–10 spermatozoa penetrate the egg [13], this should be sufficient. However, for queens inseminated with lower viability semen, we might expect them to become 'drone layers' more quickly than usual.

The fact that even 42.6% live spermatozoa in the semen could result in high quality worker brood is very encouraging to the prospects for the successful cryopreservation of semen. Harbo [6] reported spermatozoal survival of around 25%. If that survival rate can be increased by only 1-fold, we would have semen that is capable of producing primarily fertilized eggs (workers, or potential queens) for some period of time following instrumental insemination. Since it is not feasible to sex 2–3-day-old larvae, trying to rear daughter queens from a queen laying a mixture of fertilized and unfertilized eggs would be inefficient, because efforts would be wasted in grafting the drone larvae present, but which have no potential to become queens. Future research needs to focus on improved storage technology for increasing the survival rate, and also on assessments of how long a queen inseminated with reduced-viability semen might continue to produce acceptable brood.

Résumé – Relations entre la qualité du sperme et la performance des reines d'abeilles (*Apis mellifera* L.) inséminées artificiellement. Des techniques efficaces pour conserver le sperme des abeilles domestiques faciliteraient la sélection et la maintenance de lignées supérieures. Cette étude a été faite afin de déterminer un niveau acceptable de survie des spermatozoïdes nécessaire pour une telle technique de conservation. Le sperme (100 µL au total) a été prélevé chez de nombreux mâles, dilué dans un même volume de tampon de Kiev modifié [16], mélangé doucement et centrifugé à 100 *g* durant 30 min. Le mélange a été recueilli dans des tubes capillaires stériles ; la moitié a été congelée à –80 °C pour tuer les spermatozoïdes, et l'autre moitié conservée pendant une suite à la température de la pièce. La viabilité des spermatozoïdes a été déterminée à partir de l'échantillon frais à l'aide d'une technique de coloration par double fluorescence.

Les cinq mélanges suivants ont été constitués à partir du sperme frais et le sperme congelé : 1 : 100 % frais ; 2 : 75% frais–25 % congelé ; 3 : 50 % frais–50 % congelé ; 4 : 25 % frais–75 % congelé ; et 5 : 100 % congelé. Six reines ont été inséminées avec chacun de ces mélanges. La viabilité réelle des spermatozoïdes a été déterminés par les

colorants fluorescents ; elle était respectivement de 65,1, 55, 42,6, 32,3 et 0 %. Les reines ont ensuite été introduites dans des nucléi, où elles ont pu pondre.

Vingt-quatre à 25 jours plus tard, on a évalué la proportion de nymphes d'ouvrières et de nymphes de mâles dans le couvain produit par chaque reine, ainsi que la taille relative du nid à couvain. Vingt sept jours après l'insémination, la spermathèque de chaque reine a été disséquée et la viabilité des spermatozoïdes mesurée. Cinq reines issues du même éleveur et s'étant accouplées naturellement ont servi de témoins.

La proportion de spermatozoïdes vivants présents dans le sperme prélevé dans la spermathèque des reines 27 jours après l'insémination n'est pas significativement différente entre les groupes traités qui ont reçu des spermatozoïdes vivants (df = 4, $F = 4{,}08$; $P < 0{,}01$) (Fig. 1). Aucun spermatozoïde n'a été vu dans le contenu spermathécal des reines inséminées avec du sperme tué par congélation. Le pourcentage de spermatozoïdes vivants chez toutes les reines traitées était significativement inférieur à celui des reines qui s'étaient accouplées naturellement, sauf pour le groupe 4 (mélange à 25 % de sperme frais). Dans ce groupe 4 il y avait dans la spermathèque significativement plus de spermatozoïdes vivants que dans le sperme utilisé pour l'insémination. Les reines des groupes 1 à 3 (inséminées avec au moins 50 % de sperme frais) ont produit plus de couvain que celles des deux autres groupes, et uniquement du couvain d'ouvrières (Tab. I). Les reines du groupe 5 (0 % de sperme frais) n'ont produit que du couvain de mâles en petites taches clairsemées et celles du groupe 4 (25 % de sperme frais) du couvain de mâles et d'ouvrières en taches petites à moyennes (Tab. I).

Le fait que 42,6 % des spermatozoïdes vivants et présents dans le sperme aient donné du couvain d'ouvrières de grande qualité est très encourageant pour les perspectives d'amélioration de la cryoconservation du sperme. Harbo [6] a mentionné un taux de survie des spermatozoïdes d'environ 25 %. Si l'on peut seulement doubler ce taux, on disposerait alors de sperme capable de produire principalement des œufs fécondés (d'ouvrières ou de reines potentielles) pendant une certaine période suivant l'insémination artificielle. Puisqu'il n'est pas possible de déterminer le sexe des larves âgées de deux à trois jours, il ne servirait à rien d'essayer d'élever des reines à partir d'un mélange d'œufs fertilisés et d'œufs non fertilisés. La recherche à venir doit se concentrer sur l'amélioration de la technique de stockage pour augmenter le taux de survie et doit déterminer la durée pendant laquelle une reine inséminée avec du sperme à viabilité réduite peut produire du couvain de qualité acceptable.

Apis mellifera **/ spermathèque / surface couvain /spermatozoide / viabilité / conservation sperme**

Zusammenfassung – Die Beziehung zwischen Spermaqualität und Legeleistung von instrumentell besamten Königinnen. Geeignete Techniken zur Lagerung von Honigbienensperma könnten die Selektion und Erhaltung von guten Zuchtlinien erheblich vereinfachen. Diese Studie sollte untersuchen, welche Überlebensrate der Spermazoen bei einer solchen Lagerungstechnik akzeptabel ist. 100 µL Sperma wurde von mehreren Drohnen gesammelt, in einem gleichen Volumen von Kiev Puffer [16] verdünnt, vorsichtig vermischt und bei 100 × g 30 min lang zentrifugiert. Diese Mischung wurde in sterilen Kapillarröhrchen aufgenommen. Die Hälfte wurde bei –80 °C eingefroren, um die Spermatozoen abzutöten, eine weitere Hälfte wurde über Nacht bei Raumtemperatur gelagert. Die Lebensfähigkeit der Spermatozoen wurde aus der frischen Probe mit einer doppelten Fluoreszenzfärbetechnik bestimmt [31].

Aus dem frischen und dem gefrorenen Sperma wurden fünf Mischungen zur künstlichen Besamung von Königinnen

hergestellt: 100 % frisch, 75 % frisch zu 25 % gefroren, 25 % frisch zu 75 % gefroren, und 100 % gefroren. Aus jeder Mischung wurden sechs Königinnen besamt. Die mit der Fluoreszenzfärbung ermittelten tatsächliche Anteile lebender Spermatozoen in den Mischungen waren 65.1, 55, 42.6 32,2 und 0 %. Die Königinnen wurden zur Eilage in Begattungskästen eingeweiselt.

Vierundzwanzig bis 25 Tage nach der Besamung wurde an der von den Königinnen erzeugten Brut das Verhältnis zwischen Drohnen- und Arbeiterinnenpuppen und die relative Ausdehnung des Brutbereichs bewertet. Sieben und zwanzig Tage nach der Besamung wurde die Spermatheka der Königinnen präpariert und die Lebensfähigkeit der Spermatozoen untersucht. Als Kontrollen dienten 5 natürlich begattete Königinnen von dem gleichen Züchter.

Der Anteil lebender Spermatozoen in dem 27 Tage nach der Besamung aus den Spermatheken gewonnenen Sperma unterschied sich zwischen den Gruppen, die überhaupt lebendes Sperma erhalten hatten, nicht signifikant (df = 4, $F = 4,08$, $P < 0,01$, Abb. 1). In der ausschlieβlich mit durch Einfrieren abgetötetem Sperma besamten Gruppe wurden bei keiner der Königinnen Spermatozoen in der Spermatheka gefunden. Alle Gruppen hatten einen geringeren Anteil lebender Spermatozoen als die natürlich begatteten Königinnen, mit Ausnahme der mit 25 % frischen Sperma besamten Gruppe. Diese Gruppe wies zusätzlich einen signifikant höheren Anteil lebender Spermatozoen in der Spermatheka auf, als er in dem für die Besamung verwendeten Sperma gewesen war. Die mit 50 % oder mehr frischem Sperma inseminierten Königinnen erzeugten mehr Brut als die anderen beiden Gruppen, die Brut bestand vollständig aus Arbeiterinnenpuppen (Tab. I). Die nur mit abgetöteten Sperma besamten Königinnen erzeugten nur kleine Ansammlungen von ausschlieβlich Drohnenbrut; die mit 25 % frischem Sperma besamten Königinnen waren unterschiedlich und erzeugten kleine bis mittlere Ansammlungen von Brut mit gemischten Anteilen von Drohnen – und Arbeiterinnenbrut (Tab. I).

Die Tatsache, dass sogar aus einem Anteil von nur 42.6% lebender Spermatozoen im Sperma Arbeiterinnenbrut von hoher Qualität resultieren kann, ist ermutigend für die Aussichten der Entwicklung einer Methode zur Kryopreservation von Drohnensperma. Harbo [6] hatte von einer Überlebensrate des Spermas von etwa 25 % berichtet. Wenn es gelänge, diese Rate auf das Doppelte zu steigern, wäre dieses Sperma geeignet um zumindest über einige Zeit nach der künstlichen Besamung primär befruchtete Eier (Arbeiterinnen oder potentiell auch Königinnen) zu erhalten. Da das Geschlecht von 2–3 Tage alten Larven nicht leicht bestimmt werden kann, wäre die Aufzucht von Königinnen aus einer Mischung befruchteter und unbefruchteter Eier allerdings wenig effizient. Zukünftige Forschung sollte sich auf die Verbesserung der Lagerungstechnologie zur Erhöhung der Überlebensrate der Spermatozoen konzentrieren, sowie weiterhin auf die Ermittlung des Zeitraums, über den mit Sperma reduzierter Lebensfähigkeit besamte Königinnen Brut in akzeptablen Ausmaβ erzeugen.

***Apis mellifera* / Spermatheka / Brutmuster / Spermatozoenlebensfähigkeit / Spermalagerung**

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