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VIABILITY ASSESSMENT OF HONEY BEE, Apis mellifera, SPERM USING DUAL FLUORESCENT STAINING

A. M. Collins[1a] and A. M. Donoghue[2]

[1]Bee Research Laboratory, ARS, USDA, Beltsville, MD, USA
[2]Germplasm and Gamete Physiology Laboratory, ARS, USDA, Beltsville, MD, USA

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ABSTRACT

Since the development of instrumental insemination of honey bee (Apis mellifera) queens in the 1930s, there has been interest in the evaluation and in vitro storage of semen. Several fluorescent stains, when used in combination, have been effectively used to assess sperm viability in mammalian and avian species. Our objectives were to test two combinations of living:dead fluorescent stains, SYBR-14 with propidium iodide (PI), or Calcein-AM with PI , and validate the use of these probes with honey bee sperm. SYBR-14 is a nuclear stain producing green fluorescence of the DNA in living sperm, Calcein-AM is a membrane-permeant esterase substrate staining entire sperm green, and PI is a traditional dead cell stain giving a contrasting red color. Both living stains fluoresced bee sperm, but the SYBR-14:PI produced a clearer distinction between the living and dead sperm. A graduated series of known living:dead sperm proportions was used to validate the accuracy of the stains for determining sperm viability in honey bees.
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Key words: semen, viability, nuclear stain, spermatozoa, instrumental insemination

INTRODUCTION

The honey bee, Apis mellifera L., queen mates with 7-17 drones (the males)(35), in flight, at a distance from the colony, in a "drone congregation area". The week-old queen

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[a]Reprint requests should be directed to A. M. Collins, USDA, ARS, Bee Research Lab, Bldg. 476, BARC-East, Beltsville, MD 20705.

will store sperm from each drone in a storage organ, the spermatheca. Each drone may produce up to 1.25 μL of semen with 10 million sperm (23). From that, a well-mated queen will retain about 4.5 to 5.7 million sperm in her spermatheca (12, 40), and potentially fertilize hundreds of thousands of eggs over her lifetime of several years.

With natural matings, beekeepers have little control over the source of the semen that a queen stores in her spermatheca, unless they use isolated mating yards or flood an area with desirable drone types (16, 17). With the development of instrumental insemination techniques (see 20 for a review), the ability to control the number and genetic stock of drones involved in mating has been achieved. Instrumental insemination has been used primarily for research purposes (single drone inseminations: 31; genetically marked semen: 27), and for the maintenance and selection of stocks with specific traits (15).

There have been some successful attempts to store honey bee semen in vitro for various periods of time. A number of investigators have reported using semen, stored at room or refrigerator temperatures over weeks or months, to successfully inseminate queens (14, 30, 34, 37). Harbo reported on extensive work to preserve honey bee semen in liquid nitrogen (9, 10, 11). He was able to obtain viability of 20-35% in the cryopreserved semen, but queens inseminated with this semen produced insufficient numbers of workers to maintain a functional colony. Various other authors have also reported limited success with cryopreservation of semen (19, 22, 24, 29, 33), including freezing of the intact spermatheca from a mated queen (38).

In much of this work, the viability of the sperm was assessed by the quality of the brood produced by an instrumentally inseminated queen. Since the honey bee is a haplo-diploid organism, fertilized eggs develop into diploid females, the workers or queens, and unfertilized eggs develop into haploid males, the drones, that each produce millions of identical sperm. When laying, the queen controls the release of a small number of sperm as the egg moves through the reproductive tract, where it is fertilized (1,2). Occasionally the queen lays eggs without releasing semen, and these unfertilized eggs develop into the few drones. The two sexes can be distinguished during early pupation by the type of capping that is built over the cell in which they develop. Worker bee cells have a wax cap that is level with the cell edges, the drone cells have distinctly raised caps. However, evaluation of the quality of semen by inseminating a queen and subsequently observing the brood she produces is a very lengthy process. Rearing virgin queens to maturity takes 2.5 to 3 weeks, egg laying does not begin until about a week after insemination, and capping of pupae occurs after another two weeks.

A more direct way to evaluate the viability of semen is clearly needed. A group working with Peng (21, 28) tested a number of dual staining techniques for assessing sperm viability directly from the semen sample. For permanent dry mounts, they reported that eosin yellow gave good discrimination between dead (yellow-green-stained)

and living (blue-purple) sperm. In wet mounts, a pair of DNA binding fluorochromes, propidium iodide (PI) and Hoechst 33342 clearly stained dead sperm red and living sperm green, respectively.

More recent reports have validated the use of fluorescent stains SYBR-14 and Calcein-AM (both from Molecular Probes, Inc., Eugene, OR 97402) in conjunction with PI to assess sperm viability of mammalian (5, 6) and avian species (3). SYBR-14 is a membrane-permeant DNA stain and Calcein-AM is a membrane-permeant esterase substrate, both of which fluoresce bright green in living cells. Propidium iodide is taken up by cells with compromised membranes and fluoresces bright red. We report here the validation of these fluorescent stains for measuring viability in honey bee sperm.

MATERIALS AND METHODS

Semen Collection

Semen was collected from mature honey bee drones produced by queens of several commercially available stocks using the standard technique (12, 20) as described below. Sexually mature drones, approximately 14 days old, were stimulated to ejaculate by pressing on the thorax and/or removing the head. This usually resulted in partial eversion of the penis. Further pressure on the abdomen forced hemolymph into the penis and completed the process. The cream colored semen and a white mucus plug were released on the end of the penis. Under a dissecting microscope, 2.5X, the semen was collected without mucus into a syringe, designed by Harbo (8, 12). The syringe was composed of a tip (made from a capillary pipette drawn to a fine point, 0.17 - 0.21 mm ID), a collection tube (capillary tubes of various sizes), and 0.12" ID tubing filled with 3% NaCl solution connected in that sequence to a 0.2 ml. micrometer syringe (Gilmont Instruments, Barrington IL 60010). The hydraulic system thus created readily drew up the small quantity, <1 μL, of semen from each drone and allowed for a pooled collection from many drones.

Semen was sequentially collected from 40 to 55 drones, and the pooled semen held in a 40 μL capillary tube. The collected sample was expelled into a sterile Eppendorf tube, mixed by gentle stirring, and recollected into sterile 5μL capillary tubes, each with 2 μL of semen. Use of pooled semen was done to avoid the normal drone to drone variation in sperm viability. Small volumes of 3% saline with 0.25% dihydrostreptomycin (9) were collected before and after the semen, separated by a small air bubble. The tube ends were sealed with Critoseal (Sherwood Medical Industries, St. Louis, MO), a vinyl plastic compound.

Evaluation of Sperm Viability with SYBR-14 and Calcein-AM

For staining, 2 μL of semen was diluted in 1000 μL of buffer (26) [glucose, 0.3 g, potassium chloride, 0.41 g, sodium bicarbonate, 0.21g, sodium citrate dihydrate 2.43g, to 100 mL in deionized water]. Two aliquots, 200 μL each, were removed from the diluted semen and stained with 8 μL of propidium iodide and 0.267 μL of either Calcein-AM or SYBR-14 . Stock solutions of SYBR-14 and Calcein-AM were prepared by dissolving the stains in DMSO (Aldrich Chemical Company, Milwaukee, WI) at 1 mg/ml. The stock solution of PI was prepared in SemAid Extra (Poultry Health Lab, Davis, CA) at 4 mg/ml. A final 1:10 dilution in DMSO was made for the SYBR-14 and Calcein-AM stains before adding to the sample. These volumes were based on previous experience with avian sperm (3). After incubation at 35° C or room temperature, respectively, for 15 min, 4.5μL aliquots were mounted on slides with a cover glass. The fluorescent staining of sperm was evaluated and photographed using a Zeiss Axiophote (Carl Zeiss, Hanover, MD) epifluorescent photomicroscope equipped with a fluorescent isothiocyanate (FITC) filter set. One hundred sperm in random fields were scored as red (dead) or green (living), and 4 to 6 subsamples were counted for each sample. A set of 8 samples was counted on the day of semen collection, a second and third replicate of 8 samples each were done a week later with semen in-vitro stored either at room (23° C) or refrigerator temperature (12° C).

Stain Validation

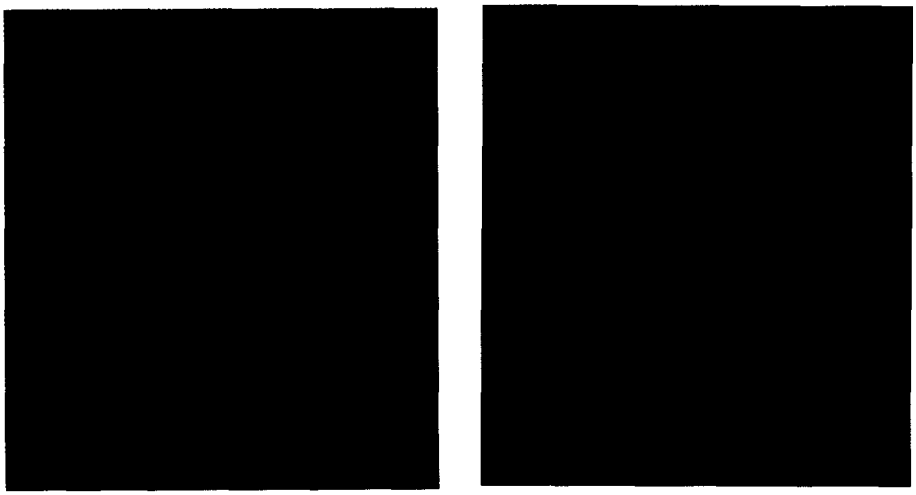
To verify that counts of the stained cells did accurately measure the viability, a series of known proportions of living:dead sperm were made. Thirty μL of semen was collected as described above and added to 1,000 μL of buffer in a sterile Eppendorf tube. It was gently, but thoroughly mixed by inversion. The semen was reconcentrated by centrifugation at <82 xg in a table-top centrifuge, and divided into two portions. Half of the mixed semen was killed by freezing for 2 h at -80° C without cryoprotectant. Separate dilutions of 2 μL of fresh or freeze-killed sperm in 500 μL of buffer were made. From these a sample series of the following living:dead proportions was made: 100:0, 75:25, 50:50, 25:75, and 0:100; then stained with SYBR-14 and counted as above. This process was repeated a second time at a later date with freshly collected semen.

Statistical Analyses

The numbers of living and dead sperm obtained using the two different stains, SYBR-14 or Calcein-AM, were compared using ANOVA with a Statistical Analysis System GLM procedure (32). The model compared the stains, and sample ages (fresh or 1 week) and storage temperature (refrigerator or room), with replication. Correlation coefficients were calculated for the expected and observed proportions of living and freeze-killed sperm (32).

RESULTS

The nuclei of viable sperm from honey bee semen fluoresce bright green when stained with SYBR-14 (Figure 1A). Because esterases are found throughout the sperm, the Calcein-AM (Figure 1B) stains the whole sperm. The green fluorescence of these two stains is in clear contrast to the red, PI-stained nuclei of dead sperm.



A B

Figure 1. Photomicrographs (x 400) of living (green-stained) and dead (red-stained) honey bee sperm. Duplicate aliquots of semen were diluted 1:1,000 in buffer and stained with SYBR-14 plus propidium iodide [A], or Calcein-AM plus propidium iodide [B].

The comparison of the proportions of viable sperm from the 8 paired semen samples stained with either Calcein-AM or SYBR-14 are shown in Figure 2. The values for percent of cells that were living were not significantly different for the two stains ($F=1.48$, $P=0.63$), whether used on fresh semen or semen stored in vitro for one week at two different temperatures. Honey bee sperm are similar in diameter (0.5 μm) throughout their long axis, with 10 μm long heads in contrast to a very long, 250 μm, tail. While the Calsein-AM nicely stained whole cells, the long tails were usually coiled or folded on themselves, which made it hard to distinguish individual sperm. Therefore, staining only the nuclei (SYBR-14) gave much clearer discrimination between sperm and allowed for faster estimation of sperm viability.

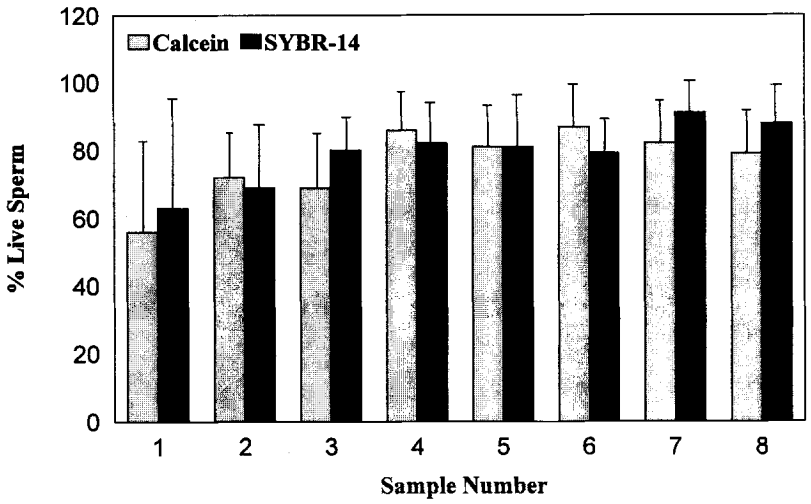


Figure 2. Mean (± Std. Dev.) percentage of living honey bee sperm observed in paired aliquots of 8 different semen samples. One of each sample pair was stained with SYBR-14, a nucleic acid stain, and propidium iodide, the other with Calcein-AM, a non-specific esterase substrate, with propidium iodide.

The counts made from the series of living:dead proportions are shown in Figure 3. The values clearly reflect the actual proportions as mixed. The correlation coefficient between the proportions of expected and actual living, green-stained sperm in the five groups was significant ($r = 0.92$; $P<0.001$) and between expected living and actual, red-stained dead sperm was negative and significant ($r = 0.92$; $P< 0.001$). If the expected living is adjusted for the actual proportions found in the 100% living sample, then both $r = 0.94$.

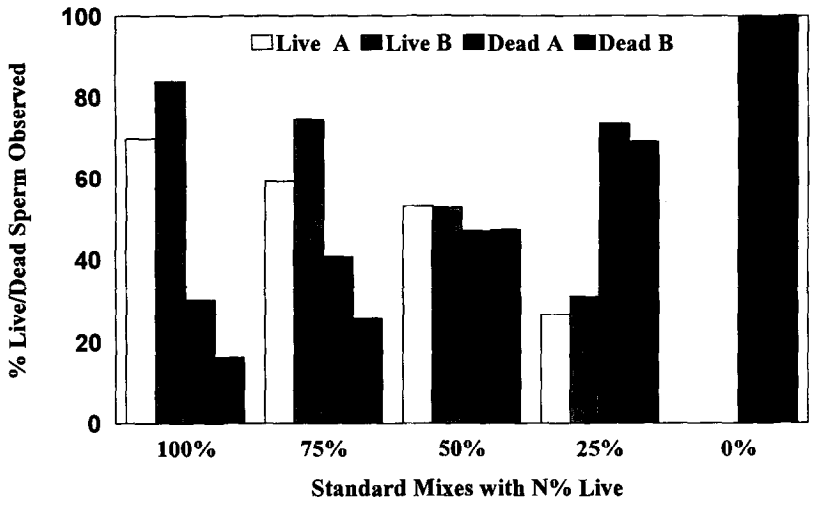


Figure 3. Proportion of living and dead honey bee sperm observed in a replicated [A & B] series of five known proportions of fresh:freeze-killed sperm. SYBR-14 and propidium iodide were used as the differentiating fluorescent stains.

DISCUSSION

Both SYBR-14 and Calcein-AM, when paired with propidium iodide, provided an effective method for differentiating between living and dead sperm in samples of semen. As shown in Figure 3, the relative proportions of living and dead sperm that were identified in the series of known ratios of fresh to freeze-killed cells reflected the expected values. These results validate our method for use in assessing the viability of honey bee semen. Since SYBR-14 stains the nuclei of the sperm, as does PI, this stain pair has more practical application in our work because of the ease of distinguishing between living and dead sperm.

Almost all of the samples stained and counted for these studies had more than ten percent dead sperm. This is probably due to the mixing, by stirring or inversion, of the multiple drone samples. Fresh samples from individual drones not subjected to as much physical stress often gave us values of close to 100% living sperm. The protocol we used for diluting, mixing and reconcentrating semen by centrifugation was based on those reported by Kaftanoglu & Peng (18), Moritz (25), and Harbo (13). However, initial trials

using their higher centrifugation speeds caused too much damage to the sperm, and consequently high levels of dead cells in the living portions of the graded series. Even the gentle handling of the samples that we did, followed by centrifugation at <82 xg was sufficient to kill some of the sperm. Therefore, one use of this staining protocol would be to evaluate various mixing and handling techniques to optimize survival of sperm, as pooling semen from many drones is a tool that will continue to be used in honey bee genetic studies and breeding.

Selective honey bee breeding programs by scientists or commercial breeders would be significantly enhanced by sperm storage techniques that allow instrumental insemination during seasons of low drone availability, reduce the need to synchronize the production of drones and queens, and extend the useful life of queens that are used to produce drones (drone-mothers). A semen viability assay that correlates highly with subsequent fertility would be extremely valuable in evaluating the semen quality of stored semen or of drones from drone-mothers used in an instrumental insemination program. The combinations of SYBR-14 and Calcein-AM with PI were shown to be effective in differentiating cells with different membrane integrities. When known amounts of living and freeze-killed sperm were analyzed, the correlation between the percentage of living sperm added to the sample and the percentage of sperm staining green with either SYBR-14 or Calcein-AM was strong. These data establish the usefulness of these stain combinations to determine the proportion of living sperm in honey-bee drone semen. Ultimately, experiments need to be run in conjunction with sperm assessment by fluorescent staining to determine the quantity of worker offspring (fertilized) vs. drone offspring (unfertilized) produced by inseminated queens to show the usefulness of these stains in predicting fertility outcomes.

Additionally, a small population of dual-stained (red and green) sperm was detected using both SYBR-14 and Calcein-AM with PI. This transitional population of sperm, which can be observed microscopically, is similar to that found for mammalian and avian sperm (3, 5). Dual-staining sperm may not only represent a population unfit for in-vitro storage or fertilization, but reveals that these sperm have lost some functional capacity because they are unable to resist the uptake of PI. If genetic differences in the ability of sperm to withstand handling, storage or cryopreservation are found, selection for this character might also be economically important.

Considerable progress has been made in using dual staining to assess sperm function in mammals (4, 7) and avian species (3). Stain combinations have been used to assess sperm response to stresses such as cryopreservation and osmolality of diluents (4, 5, 36, 39). Our results show that various combinations of fluorescent stains can be used to assess factors that influence insect sperm integrity. Because the spermatheca of the honey bee queen can be recovered after a period of egg laying, and the sperm contents assayed, these stains also show promise for further elucidation of the mating and reproductive functions of the honey bee.

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