Original article

Factors affecting the successful cryopreservation of honey bee (*Apis mellifera*) spermatozoa*

Brandon Kingsley HOPKINS, Charles HERR

Center for Animals Near Biological Extinction, 1016 S. Main Colfax,WA 99111, USA

Abstract – This report is about cryopreservation of honey bee semen. There has been little advancement of this technology over the past 20 years. Cytotoxicity of the cryoprotectants, temperature sensitivity, freezing rate, and cold shock were investigated. The least toxic cryoprotectant was DMSO. Spermatozoa were tolerant to temperatures up to 40 °C. A programmable freezing rate of 3 °C/min proved superior in most treatments when compared to a freezing rate of approximately 28 000 °C/min. Highest viability of spermatozoa (93%) post cryopreservation resulted from the treatment containing a 10% DMSO diluent, slow cooled to just above freezing, and frozen at a rate of 3 °C/min. Spermatozoa frozen in such a manner yielded viability and motility indistinguishable from that of unfrozen semen. Promising results warrant a field study.

cryopreservation / *Apis mellifera* / spermatozoa / cold shock / vitrification

1. INTRODUCTION

There is a need for honey bee semen cryopreservation to counter allelic losses caused by the population declines due to Varroa mites, colony collapse disorder, and other future threats. Maximum storage life at room temperature for honey bee semen is approximately two weeks (Cobey, 2007). Liquid nitrogen (LN$_2$) storage of spermatozoa promises long-term storage capabilities; but success with LN$_2$ storage methodologies are currently inadequate for use in instrumental insemination because of poor brood production (Melnichenko and Vavilov, 1976; Harbo, 1979, 1983; Verma, 1983; Kaftanoglu and Peng, 1984). Kaftanoglu and Peng (1984) reported using cryopreserved semen for instrumental insemination and achieved 47% worker production; lower than the ideal 95–99%, indicating a low fertilization rate. It has been

decades since the last reported study, meaning methods have been shelved for years.

Our research project investigated honey bee spermatozoa cryopreservation by studying factors that might reasonably have affected post-thaw viability. These factors included the type and concentration of cryoprotectant, the effect of cold shock, freezing rates, and general temperature sensitivities. Cryoprotectants are molecules that prevent intracellular ice; ice being the primary damaging agent (Watson and Fuller, 2001). Cold shock is the term used when cells are damaged by too quickly cooling to temperatures just above freezing (Kaftanoglu and Peng, 1984). The cells of some species need to be cooled slowly to just above freezing, whereas the cells of other species can be cooled quickly without injury. This study investigated two freezing rates, programmable freezing and rapid freezing. The rapid freezing method was an attempt to achieve a vitrified amorphous state of water. Programmable freezing is a technique where samples are cooled slowly and ice is encouraged to form outside the cells, effectively dehydrating cells and preventing intracellular

Corresponding author: B.K. Hopkins,
kingsley27@msn.com
* Manuscript editor: Yves Le Conte

ice (Mazur, 1963). In contrast, vitrification is an attempt to cool so fast ice crystals have no time to form either inside or outside cells; the minimum cooling rate has been estimated to be 30 000 °C/min. This minimum rate though depends on cryoprotectant type and concentration (Risco et al., 2007). In order to accomplish this rate, samples must be tiny and have a sufficiently high surface area to volume ratio. The small volume of semen produced by honey bees made it an attractive candidate for the vitrification approach.

2. MATERIALS AND METHODS

2.1. Semen collection

Honey bee semen was kindly collected for us by Susan Cobey, UC, Davis using methods similar to those described by Laidlaw (1977). Semen was collected from multiple drones using a Harbo's syringe (Harbo, 1985). After collection, the semen was held until use in a glass capillary tube sealed with petroleum and stored at room temperature. Spermatozoa were verified viable prior to use in treatments, using the staining procedure described in Section 2.9.

2.2. Diluents

We tested five different diluents each containing one of three different cell -permeating cryoprotectants. All solutions were made using water purified to 18.6 meg-ohms. Semen was mixed 3:2, semen to diluent, except in the case of the modified ram semen diluent as described below.

The first diluent was prepared with 500 µL buffer, 250 µL dimethyl sulfoxide (DMSO) (ICN, Cat# 191418), and 250 µL egg yolk. The buffer was made by adding 239 mg NaH_2PO_4 (Sigma, Cat# S-7907) and 112 mg Na_2HPO_4 (Sigma, Cat# S-8282) to make a final volume of 25 mL and pH adjusting to 7.2 with 6 M NaOH (Harbo, 1983). This diluent, referred to herein as Harbo's diluent, was one of two used in the preliminary experiment and also subsequently in the other experiments.

The buffer is presented here in final concentrations prior to mixing with cryoprotectant, egg yolk and semen (pH adjusted to 7.2 using NaOH) contained:

- 79.7 mM NaH_2PO_4
- 31.6 mM Na_2HPO_4

The second diluent, used only in the preliminary experiment, was a modification of one used for ram semen (Evans and Maxwell, 1987); the modification being the increased glycerol concentration from 4.5% to 9% (v/v). Modified glycerol diluent (Mod. Gly.) was prepared by adding 1.91 g of tris(hydroxymethyl)aminomethane (Tris Base) (Invitrogen, Cat# 15504-020), 263 mg glucose (Sigma, G-8270), 958 mg citric acid (Sigma, Cat# C-2404), 2.96 mL glycerol (BDH, Cat# 101186M), 32,895 IU penicillin (Sigma, Cat# P-4687), 33 mg streptomycin (Sigma, Cat# S-1277), filled to a final volume of 25 mL. The addition of 24% egg yolk was mixed fresh on the day prior to mixing with semen. Semen was mixed with diluent in a 1:1 ratio.

Our Mod. Gly. is presented here in final concentrations prior to mixing with semen or egg yolk (pH adjusted to 7.2 using 6 M NaOH).

- 630.7 mM Tris Base
- 58.4 mM glucose
- 199.5 mM citric acid
- 11.8% glycerol (v/v)

The following four diluents used in this experiment contain the same base solution composed of: 6.08 g trehalose (Sigma, Cat# T-9531), 268 mg glucose, 268 mg fructose (Baker, Cat# M556-05), 879 mg citric acid, 1.662 g Tris Base, plus the addition of one of four cryoprotectants (Tab. I), and filled to a final volume of 25 mL. The addition of 30% egg yolk was mixed fresh on the day prior to mixing with semen. Semen and diluents were mixed at a ratio of 3:2, respectively.

The HH DMSO, HH Extra DMSO, HH Glycerol and Ethylene glycol (EG) diluents are presented here as concentrations prior to mixing with semen or egg yolk (pH adjusted to 7.2 using 6 M NaOH).

- 709.2 mM Trehalose
- 59.5 mM Glucose
- 59.5 mM Fructose
- 183.0 mM Citric Acid
- 548.8 mM Tris Base

Final cryoprotectant concentrations including egg yolk and semen for HH Glycerol, HH DMSO, HH Extra DMSO, and EG are: 8% glycerol, 10% DMSO, 15% DMSO, and 3.7% ethylene glycol, respectively.

Table I. Treatments with respective total cell counts from pooled samples and percentage of viable cells listed in descending order of cell viability. Different letters in the % viable column indicate a significant difference ($P < 0.002$). Vit=vitrified freezing, Pro=programmable freezing, slow vs. rapid cool is treatment of sample prior to freezing.

Treatment	Total cell counts	% Viable (+/−) SD[1]
Pro/Slow cool/Harbo's	1403	92.94 +/− 0.87 (A)
Vit/Slow cool/Harbo's	626	77.48 +/− 1.67 (B)
Vit/Rapid cool/Harbo's	538	75.46 +/− 1.86 (B)
Vit/Rapid cool/HH DMSO	755	52.85 +/− 1.82 (C)
Pro/Rapid cool/HH DMSO	345	48.41 +/− 2.67 (D)
Vit/Slow cool/HH DMSO	748	47.06 +/− 1.78 (E)
Vit/Slow cool/Extra DMSO	1202	42.68 +/− 1.41 (E)
Vit/Rapid cool/Extra DMSO	721	42.44 +/− 1.86 (E)
Pro/Slow cool/Extra DMSO	1160	36.12 +/− 1.38 (F)
Vit/Slow cool/EG	839	27.29 +/− 1.54 (G)
Pro/Slow cool/HH DMSO	566	24.56 +/− 2.34 (G)
Pro/Rapid cool/EG	507	22.29 +/− 2.07 (G)
Pro/Slow cool/EG	896	14.29 +/− 1.19 (H)
Pro/Rapid cool/Harbo's	425	13.41 +/− 1.71 (H)
Vit/Rapid cool/EG	492	2.44 +/− 0.68 (I)

[1] SD is an abbreviation for standard deviation.

2.3. Freezing containers

Semen was frozen in micro glass cryostraws, fabricated by pulling Pasteur pipettes (VWR, cat# 14672-380) to an internal diameter of approximately 150–180 μm. Size of the straws was determined using an ocular micrometer. Fluid is moved in and out of the straws by fitting a short piece of Silastic® tubing (0.062" ID; 0.095" OD; Dow Corning cat# 602–265) over the end of the straw. All straws were siliconized by drawing up and expelling Sigmacote (Sigma, Cat# SL-2) and then left to cure overnight. Straws siliconized to reduce residual loss and aid in drawing and expelling semen.

2.4. Mixing semen and diluent

Semen was mixed with the diluents by drawing samples from the capillary tube containing semen using a pulled capillary tube, siliconized (same as above), and fitted to a Hamilton threaded-plunger syringe (Model #1001TPLT) backfilled with Fluorinert® (Electronic Liquid FC-77, Cat# 98-0204-0701-5). Fluorinert® was ideal because of its non-toxicity, low viscosity and insolubility in aqueous solutions. These properties allowed for easy and accurate control while moving semen. Volumes were controlled using the threaded syringe calibrations (1/4 turn = 3.25 μL). Semen samples and diluents were mixed in separate wells of a Robinson Scientific Low Profile Terasaki 96 well tray (cat# 1007-01-0). Straws were loaded with 3 μL of semen/diluent.

2.5. Cooling samples prior to freezing

Samples from each treatment were cooled from room temperature to ~0 °C by one of two methods prior to freezing. (1) Slow cooled samples loaded in micro glass cryostraws were sheathed in 0.5 mL Cassou Straws (IMV, France) and submerged in 450 mL of room temperature water in a 600 mL beaker and placed in a standard refrigerator for two hours, which is when the water became ~4 °C. After two hours in the refrigerator, samples were removed from the Cassou Straws and plunged into an ice water bath for 4 s, bringing the samples to ~0 °C. (2) Rapidly cooled samples were removed from the Cassou Straws at room temperature just before freezing and plunged in to an ice water bath for 4 s.

Immediately after submergence in the ice bath, all samples were either placed back into the Cassou straw, and then placed in a programmable freezing chamber, or plunged, without a sheath into a LN_2 vortex.

2.6. Vitrification

Three styrofoam coolers were placed side-by-side. The first was a round container placed on a magnetic stirrer (Fisher Sci. Jumbo Stirrer) with a magnetic stir bar on the bottom. Stirring the LN_2 at the highest setting created a vortex that maximized the cooling rate by rapidly removing any vapor formed around the sample as it froze (Stucky et al., 2008). Micro glass cryostraws were removed from their sheaths plunged into the ice bath, held for 4 sec and then plunged into the outer edge of the LN_2 vortex for 15 s. Samples were then rapidly moved from the vortex container into the second LN_2 container in order to be re-sheathed under LN_2. The sheathed samples were then transferred in LN_2 into a third LN_2 container so they could be packed into the LN_2 storage goblet. The goblets were then transferred to a standard LN_2 tank and maintained under LN_2 at atmospheric pressure ranges of our local.

2.7. Programmable freezing

Micro glass cryostraws were loaded into their Cassou sheaths after being submerged in the ice water bath, and loaded into the Freeze Control Cryochamber (CL-3000 CryoLogic PL, Australia), holding at 0 °C. Controlled freezing of all samples was done at a rate of 3 °C/min from ~0 °C to –40 °C. When the temperature reached –40 °C the samples were removed from the cryochamber and plunged into LN_2 where they were packed into a goblet for storage.

2.8. Thawing

We froze spermatozoa using two substantially different freezing rates; consequently, we used two different thawing methods to coincide with the freezing method. Currently accepted thought is that the risk of ice damage to cells also occurs during the thawing process. Vitrified samples need to be rapidly thawed (Rall and Fahy, 1985). Micro glass

cryostraws frozen in the LN_2 vortex were thawed rapidly. Each was removed from its sheath and LN_2, simultaneously, and plunged for 3 s into a 37 °C circulating water bath (VWR, model 1167).

The micro glass cryostraws that were frozen using the programmable freezing unit were air thawed. A hair dryer was fixed to a post and a distance was found at which the air coming from the unit was ~40 °C. Each micro glass cryostraw was removed from its sheath and LN_2, simultaneously, and held at the position providing 40 °C air for 7 s.

2.9. Live/dead cell staining for cell viability

Spermatozoa were stained using a dual fluorescent nucleic acid staining system (Molecular Probes cat# L7011, Eugene, OR) (Collins and Donoghue, 1999). The live stain (SYBR-14) was diluted 1:10 in DMSO. Samples were expelled from their micro glass cryostraws into 1.5 mL Ependorff tubes containing 1 μL of the diluted SYBR-14 (live stain) and 1 μL of propidium iodide (dead stain), and 98 μL of Earls balanced salt solution. Spermatozoa were then incubated at 37 °C for 6 min. Viablity rates were evaluated based on live/dead cell counts. Cells staining red were counted dead, cells staining green were counted alive. Samples were observed in 1 μL drops on an inverted epifluorescent microscope with a xenon light source and required filters.

The few described motility observations were made using an inverted microscope at 320X magnification with phase contrast. Motility was observed as undulating tails after dilution in Earls balanced salt solution including the live/dead cell stain. Motility was noted as either present or absent.

3. EXPERIMENTAL DESIGN

3.1. Preliminary experiment

A preliminary cryopreservation study was conducted to investigate the possibility of vitrification for honey bee semen. Harbo's (1983) diluent and a controlled freezing rate of 3 °C/min was used for comparison. Along with the two different freezing rates, two diluents were tested: "Harbo's diluent" and "modified glycerol diluent" as described above. Semen was frozen and thawed on two separate

occasions. All samples in this experiment received the slow cooling treatment. The first attempt was thawed after storage in LN_2 for 343 days. The second was thawed after 6 days. All samples were stained and counted using the same method. There were not multiple samples within the treatments.

3.2. Cryoprotectant toxicity

In order to investigate the toxicity of the different diluents independent of the freezing and thawing, semen was mixed with each diluent and left to equilibrate for one hour. At one hour, samples were examined for motility; then stained, and subjective live/dead cell counts were performed. Toxicity in this study is defined as the degree to which cryoprotective diluents caused cell death. The one-hour time point was chosen as an estimated room temperature handling time of semen in diluent during the cryopreservation process.

3.3. Temperature sensitivity

To generate useful information for thawing purposes, an experiment was run to determine the highest temperature honey bee spermatozoa could tolerate without causing measurable damage. Four water baths were set up in separate styrofoam containers: 30, 35, 40, and 45 °C. A micro glass cryostraws was taped to the side of each of the containers with the semen/diluent submerged. The straws were left in their relative water baths for 1 hour, and then the spermatozoa within were stained for live/dead cell counts.

3.4. Cryopreservation

3.4.1. Cold shock

Two out of every five micro glass cryostraws per diluent treatment were left at room temperature until plunging into an ice water bath immediately prior to freezing. This lowered sample temperature from room temperature to 0 °C within 1 s. These treatments were performed to test the effects of cold shock. The

other three out of five samples were treated with the slow cooling procedure explained above.

3.4.2. Freezing rate

Five (2 rapid cooled, 3 slow cooled) of every 10 samples for each diluent were frozen using the programmable freezing unit. The other 5 were frozen in the LN_2 vortex.

3.4.3. Diluents

For each of the four diluents used in the main cryopreservation experiments, 10 straws were loaded with the diluent semen mixture. Half the straws for each of the diluents were frozen in the programmable freezing unit, and the other half were frozen in the LN_2 vortex.

3.5. Statistical analysis

Analysis of post-thaw viability ratios involved the large-sample statistical test for estimating differences between binomial parameters of two binomial populations (Mendenhall, 1979). The z statistics calculated according to Mendenhall formed the basis for a two-tailed test for significant difference between the proportions of viable cells measured for each treatment. In the cryopreservation study replicates within each treatment yielded similar results that were not statistically different, therefore the results of the replicates were pooled for statistical analysis between treatments.

4. RESULTS

4.1. Preliminary experiment

For the experiment in which samples were stored in LN_2 for 6 days the greatest proportion of viable cells (93.2%) received vitrification freezing, slow cooling to above 0 °C and were in Harbo's diluent. The proportions of viable cells in the remaining treatments ranked as follows: programmable freezing,

slow cooling in Harbo's (78.8%); vitrification, slow cooling, with HH glycerol (38.9%); and, programmable freezing, slow cooling with HH glycerol (26.0%). Comparisons of viability of spermatozoa between treatments differed significantly ($P < 0.002$).

For the experiment in which samples were stored in LN_2 for 343 days the greatest proportion of viable spermatozoa as determined by live/dead cell stain (54.26%) received programmable freezing, slow cooling, and Harbo's diluent. The proportions of viable cells in the remaining treatments ranked as follows: vitrified freezing, slow cooling with Harbo's diluent (41.46%); vitrification, slow cooling with glycerol (18.77%); and, programmable freezing, slow cooling with glycerol (6.52%).

4.2. Cryoprotectant toxicity

Motility was maintained and 95% of the spermatozoa remained viable after an hour at room temperature in both Harbo's and HH DMSO diluents. Spermatozoa motility was absent in both glycerol and EG containing diluents. Upon dilution of the samples containing EG motility resumed, and 90% of the sperm cells were viable. Spermatozoa in the glycerol diluent never resumed motility, and staining revealed cell viability of just 35%. Only 65% of spermatozoa stained alive after an hour when mixed with the HH Extra DMSO diluent.

4.3. Temperature sensitivity

Figure 1 shows the viability of spermatozoa after submerging samples in water baths at 30, 35, 40, and 45 °C for 1 hour. Samples exposed to temperatures of 30, 35, and 40 °C showed no significant reduction in viability after 1 hour. The sample exposed to the 45 °C water bath showed a substantial decrease in viability after exposure for 1 hour (Fig. 1).

4.4. Cryopreservation

Slow cooling samples prior to programmable freezing using Harbo's diluent resulted in sperm cell viability of 93%, and
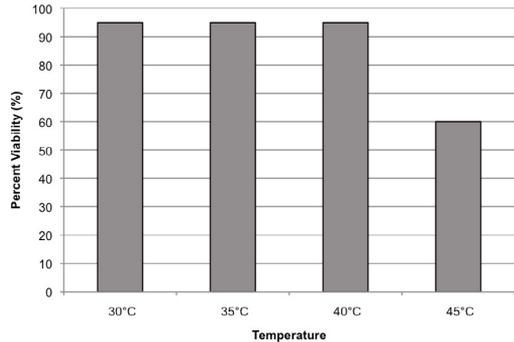
Figure 1. Cell viability calculated as (# viable cells)/(total # cells) using dual fluorescent staining system after exposure to different temperatures for 1 h.

was significantly different than all other treatments ($P < 0.002$) (Tab. I). In a side-by-side comparison with fresh semen motility was indistinguishable. Motility was absent in all other treatments. Rapidly cooling spermatozoa to just above 0 °C prior to freezing and then programmable freezing in Harbo's diluent resulted in 13.41% viability (Tab. I).

A method was considered successful if viability was above 50%. Four methods provided a satisfactory viability by this measure: programmable freezing/slow cooling/Harbo's diluent (92.94%); vitrified/slow cooling/Harbo's diluent (77.48%); vitrified/rapid cooling/Harbo's diluent (75.46%); and, vitrified/rapid cooling/HH DMSO (52.85%).

No samples in the ethylene glycol diluents produced greater than 30% viability. Samples in HH Extra DMSO treatment did not produce viability above 43% (Tab. I).

5. DISCUSSION

Using Harbo's diluent, slow cooling to prevent cold shock, and freezing samples at 3 °C/min produced the greatest spermatozoa viability (93%) (Tab. I). Motility in this treatment was indistinguishable from unfrozen semen. When using the same diluent and freezing rate, but rapidly cooling to just above 0 °C the viability of spermatozoa was just 13%, and motility was absent. Comparing these two

results would suggest that honey bee spermatozoa is susceptible to cold shock when frozen at 3 °C/min in Harbo's diluent. When Harbo (1983) performed his freezing experiments, spermatozoa containing solutions were loaded as 22 μL volumes in capillary tubes and placed in a refrigerator at 5 °C overnight. The cooling rate would be expected to have been more rapid than our slow cooling method and might explain his lower percentage of worker brood (22%). Harbo provided no post-thaw cell viability data that would make possible a comparison with our study. Whereas these results are consistent with honey bee spermatozoa being cold shock sensitive, interestingly, cold shock did not appear to be as important a factor with the other treatments (Tab. I). The other treatments being resistant to cold shock would be consistant with findings in Kaftanoglu and Peng's (1984) report. Harbo's diluent is the only one that lacked Tris base. We observed that at least a portion of the egg yolk in Harbo's diluent formed an insoluble flocculent, whereas, in diluents containing Tris base, the egg yolk and diluent appeared clear, as if the yolk had been solubilized. It has been suggested that egg yolk protects against the effects of cold shock (Amann and Graham, 1993). Maybe the difference in egg yolk solubility is why slow cooling to above 0 °C was such a major factor in Harbo's diluent and not in others. Hopefully, slow cooling might eliminate the need for egg yolk, or make finding a replacement easier, which is one of our future goals.

In the preliminary experiments, viability was poor in treatments using glycerol. Cryoprotectant toxicity tests, using live/dead staining, were done in order to investigate cell viability independent of the effects of freezing and thawing. We found that the glycerol containing diluent caused the greatest percentage of cell death prior to freezing; interesting because glycerol is widely and successfully used for mammalian semen freezing (Curry, 2007). Glycerol was eliminated as a candidate cryoprotectant in this study. However, cryoprotectant toxicity of glycerol was not tested prior to our preliminary experiments and so was used. The lower spermatozoa viability recorded in the glycerol containing treatments in the pre-liminary experiments might have been in part caused by the glycerol toxicity, not freezing. A future study is needed that includes a treatment where glycerol is added to the Harbo's base diluent.

Ethylene glycol was far less toxic to spermatozoa than glycerol, but simple observations of motility could have easily concealed this fact. When placed in a diluent containing EG, all motility ceased. However, when diluted out of the diluent, motility was restored. An interesting possibility seemed that maybe spermatozoa might benefit by having motility inhibited during the freezing process. No results bared this out though. In general, EG performed poorly as a honey bee semen cryoprotectant. However, a future study is needed that includes a treatment where EG is added to the Harbo's base diluent.

Spermatozoa survived well and seem to tolerate both "HH DMSO diluent" and "Harbo's DMSO diluent" absent freezing. To further investigate the limits of tolerance to DMSO, spermatozoa were exposed to an even greater concentration of DMSO in our "HH DMSO diluent" ("HH Extra DMSO") in order to determine the cells' toxicity limit. At the increased concentrations of DMSO (15% v/v), there was a significant drop in cellular viability over an hour at room temperature.

We tested two different freezing rates: 3 °C/min and rapid freezing (vitrification). Future studies that test other freezing rates and methods would seem worthwhile. It would be worth testing some more commonly used freezing methods used in the artificial breeding industry. Freezing in pellets on dry ice and in LN_2 vapor are some of the industry standards for semen freezing. However, it would seem more worthwhile to first field test the method that generated the best results presented within this report, since these results appeared to equal unfrozen semen in terms of viability and motility. Our current concern is for the compatibility of the diluent with queen insemination. Harbo (1976) reported queens produced normal progeny when inseminated with semen containing DMSO at concentrations below 12%. However, egg yolk has not been tested in the insemination of queens.

The tiny volume of semen collected from a drone made it an attractive candidate for the vitrification method. This being the first reported study of honey bee semen vitrification, the method could be improved by reducing the number of transfers between LN$_2$ baths. In preliminary experiments, vitrification showed promising results, but samples thawed after prolonged storage had significantly lower cell viability. Using the same method of freezing and thawing; samples stored for 6, 42, and 343 days resulted in 93.2, 77.5, and 41.5% viability, respectively. Although these comparisons are of data from different experiments, and the experiments were not designed to investigate this unexpected phenomenon; still this decline in post-thaw spermatozoa viability when using the vitrification method warrants concern. Searching the literature revealed a recent study published on cryopreservation of mouse embryos that reported similar findings (Mozdarani and Moradi, 2007). The study included a vitrification treatment where the severity of damage and subsequent failure of embryos was related to the amount of time spent in LN$_2$ storage. Ice crystals forming at LN$_2$ temperatures in the storage straws over time might be the cause of the decline in cellular viability for vitrified samples. This finding would contradict a currently accepted concept within the field of cryobiology, which is that below a temperature called the glass transition temperature; ice fails to form (Giovambattista et al., 2004). We are further researching this phenomenon.

Results using the method of slow cooling semen in Harbo's diluent, and freezing in a programmable freezing unit are promising enough to warrant field trials. Live/dead cell staining is useful but limited in its ability to assay sperm viability. Obviously, the point of the technology is to use it in the field. Next, use of the cryopreserved semen with the instrumental insemination of queens is necessary.

There is an urgent need for the cryopreservation of honey bee spermatozoa. The frightening losses of honey bees and humans' vital link to the species means the ability to properly preserve and distribute honey bee genetics is now crucial. Increased genetic diversity has been shown to decrease severity of infection (Seeley and Tarpy, 2007), and enhance productivity and fitness (Mattila and Seeley, 2007). There are populations of bees that do not require chemical treatment for the control of parasites or disease because of genetic characteristics. With proper cryopreservation methods those valuable alleles can be stored in the form of semen, analyzed and distributed worldwide for the purpose of increasing genetic diversity and selective breeding without fretting time constraints or environmental conditions. Certainly, there are yet to be discovered important alleles, which might disappear in the future without a conscious effort at preserving the currently available genetic diversity.

Facteurs jouant un rôle dans le succès de la cryopréservation de spermatozoïdes de l'abeille *Apis mellifera*.

cryopréservation / *Apis mellifera* / spermatozoïdes / stress thermique / froid / vitrification

Zusammenfassung – Faktoren, die die erfolgreiche Kältekonservierung von Spermatozoen der Honigbiene (*Apis mellifera*) beeinflussen. Honigbienen können instrumentell besamt werden (I.B.). Das Potential der I.B. wird jedoch dadurch begrenzt, dass es unmöglich ist, den Samen über längere Zeiträume hinweg aufzubewahren. Da Einfrieren nicht möglich ist, muss aufgenommener Samen innerhalb von kurzer Zeit verbraucht werden. Geeignete Verfahren der Kältekonservierung würden jedoch die wiederholte Nutzung einer umfassenden Sammlung von Sperma während einer Saison, oder ihre Aufbewahrung zur Konservierung wertvoller genetischer Ressourcen erlauben. Einflüsse, die möglicherweise die Lebensfähigkeit der Spermien nach dem Auftauen beeinflussen könnten, wurden untersucht. Ein Review ergab vier allgemeine Faktoren, auf die wir unsere Arbeit konzentrierten: (1) Zelltoxizität von Kälteschutzmitteln, (2) Temperaturempfindlichkeit von Spermien, (3) die Gefrierrate, und (4) Kälteschock. Samen wurde aufgenommen und vor dem Gebrauch für 7 Tage in einer Glaskapillare aufbewahrt. Der Samen wurde im Verhältnis von drei zu zwei mit verschiedenen Verdünnern gemischt. Die Zellgiftigkeit von Kälteschutzmitteln wurde

getestet, indem der Samen mit Verdünner gemischt wurde, der Kälteschutzmittel enthielt, und dann für eine Stunde in Glaskapillaren aufbewahrt wurde. Das Überleben der Zellen wurde in der gesamten Studie durch eine Lebend-Tot-Fluoreszenzfärbung gemessen. Mit Lösungsmitteln, die 10 % DMSO enthielten, blieb die Beweglichkeit erhalten und 95 % der Spermatozoen überlebten. Wenn das Lösungsmittel jedoch 15 % DMSO enthielt, blieben nur 65 % der Spermazellen am Leben. Die Beweglichkeit der Spermatozoen ging deutlich zurück, wenn das Lösungsmittel Glyzerin oder EG enthielten; die Färbung ergab, dass in diesen Fällen 35 % bzw. 90 % der Zellen überlebten.

Die Temperaturempfindlichkeit wurde gemessen, indem Glaskapillaren mit der Samen/Lösungsmittelmischung für eine Stunde in verschieden temperierte Wasserbäder getaucht wurden. Proben, die Temperaturen von 30 °C, 35 °C, und 40 °C ausgesetzt wurden, zeigten keine signifikante Reduktion der Lebensfähigkeit. Die Probe, die einer Temperatur von 45 °C ausgesetzt wurde, zeigte dagegen einen deutlichen Rückgang der Lebensfähigkeit (Abb. 1).

Die Experimente zur Kryokonservierung wurden so angelegt, dass sechs verschiedene Kälteschutzmittel, zwei Gefrierraten und der Effekt von Kälteschock in einem kombinierten Ansatz untersucht werden konnten. Die Kombination von Faktoren, die zur größten Überlebensfähigkeit (93 %) beitrugen, war: Harbo's Verdünnung mit 10 % DMSO, bei langsamer Abkühlung und Einfrieren in einem programmierbaren Gefriergerät bei 3 °C/min (Tab. I).

Die mit der Methode des langsamen Abkühlens von Samen in Harbo's Verdünnung und Einfrieren in einem programmierbaren Gefriergerät erzielten Ergebnisse sind vielversprechend genug für Feldstudien. Offensichtlich liegt der Nutzen der Technologie in der Anwendbarkeit in der Praxis.

Kältekonservierung / *Apis mellifera* / Spermatozoen / Kälteschock / Vitrifizierung

REFERENCES

Amann R.P., Graham J.K. (1993) Spermatozoal Function, in: McKinnon A.O., Voss J.L. (Eds.), Equine Reproduction, Blackwell Publishing, pp. 715–745.

Cobey S.W. (2007) Comparison studies of instrumentally inseminated and naturally mated honey bee queens and factors affecting their performance, Apidologie 38, 390–410.

Collins A.M., Donoghue A.M. (1999) Viability assessment of honey bee (*Apis mellifera*) sperm using dual fluorescent staining, Theriogenology 51, 1513–1523.

Curry M.R. (2007) Cryopreservation of mammalian semen, in: Day J.G., Stacey G.N. (Eds.), Cryopreservation and Freeze-Drying Protocols, Second Edition, Humana Press, pp. 303–311.

Evans G., Maxwell W.M.C. (1987) Salamon's Artificial Insemination of Sheep and Goats, Butterworths, Australia, pp. 122–141.

Giovambattista N., Angell C.A., Sciortino F., Stanley H.E. (2004) Glass-transition temperature of water: A simulation study, Phys. Rev. Lett. 93, 047801-1-047801-4.

Harbo J.R. (1976) Survival of honey bee spermatozoa in Liquid Nitrogen, Ann. Entomol. Soc. Am. 70, 257–258.

Harbo J.R. (1979) Storage of honey bee spermatozoa at –196 °C, J. Apic. Res. 18, 57–63.

Harbo J.R. (1983) Survival of honey bee (Hymenoptera: Apidae) spermatozoa after two years in liquid nitrogen (–196 °C), Ann. Entomol. Soc. Am. 76, 890–891.

Harbo J.R. (1985) Instrumental insemination of queen bees – Parts 1 & 2, Am. Bee J. 125, 197–202, 282–287.

Kaftanoglu O., Peng Y. (1984) Preservation of honeybee spermatozoa in liquid nitrogen, J. Apic. Res. 23, 157–163.

Laidlaw H.H. Jr. (1977) Instrumental insemination of honey bee queens: pictoral instruction manual, Hamilton, IL: Dadant & Son.

Mattila H.R., Seeley T.D. (2007) Genetic diversity in honey bee colonies enhances productivity and fitness, Science 317, 362–364.

Mazur P. (1963) Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing, J. Gen. Physiol. 47, 347–369.

Melnichenko A.N., Vavilov Y.L. (1976) Long term storage of drone semen by freezing in liquid nitrogen SP S Kennan, Soviet Agric. Sci. 1, 34–36.

Mendenhall W. (1979) Introduction to Probability and Statistics, in: Beal C. (Ed.), 5th ed., Duxbury Press, Belmont, CA.

Mozdarani H., Moradi S.Z. (2007) Effect of vitrification on viability and chromosome abnormalities in 8-cell mouse embryos at various storage durations, Biol. Res. 40, 299–306.

Rall W.F., Fahy G.M. (1985) Ice-free cryopreservation of mouse embryos at –196 °C by vitrification, Nature 133, 573–575.

Risco R., Elmoazzen H., Doughty M., He X., Toner M. (2007) Thermal performance of quartz capillaries for vitrification, Cryobiology 55, 222–229.

Seeley T.D., Tarpy D.R. (2007) Queen promiscuity lowers disease within honeybee colonies, Proc. R. Soc. Lond. B Biol. Sci. 274, 67–72.

Stucky M., Hopkins B.K., Herr C. (2008) Cryopreservation of honey bee spermatozoa, Reprod. Fert. Dev. 20, 127–128.

Verma L.R. (1983) Effect of deep freezing on the survival of the honey bee (*Apis mellifera* L.) spermatozoa, Am. Bee J. 123, 851–852.

Watson P.F., Fuller B.J. (2001) Principles of Cryopreservation of Gametes and Embryos, in: Watson P.F., Holt W.V. (Eds.), Cryobanking The Genetic Resource: Wildlife Conservation for the Future? CRC Press, London, pp. 21–46.