Cryobiology 102 (2021) 136–139

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

Brief Communication

Cryopreservation of seminal vesicle derived spermatozoa from *Bombus impatiens* and *Apis mellifera* – Implications for artificial insemination of bumble bees

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ARTICLE INFO

Keywords:
Bombus
Apis
Conservation
Cryopreservation
Germplasm
Spermatozoa
Pollinators
Seminal vesicle
Accessory testis

ABSTRACT

This study evaluates the efficacy of a cryopreservation protocol for spermatozoa derived from the accessory testis of male *Bombus impatiens*. It is also the first report of successful cryopreservation of bumble bee spermatozoa. The spermatozoa viability was compared with the similarly treated honey bee spermatozoa derived from its accessory testis. The semen was frozen using a yolk-free non-activating buffer containing dimethyl sulphoxide and stored in liquid nitrogen for 24 h to ~14 days. Thereafter, the frozen samples were thawed rapidly and assessed by staining with live/dead differentiating fluorescent dyes. Semen viability in cryopreserved samples (55.8 ± 14.0%) was significantly different than controls (96.2 ± 10.5%). Similar assessment with *A. mellifera* resulted in 82.2 ± 7.0% viable cryopreserved spermatozoa versus 99.4 ± 0.1% in controls. A similar proportion of the sperm cells were also capable of motility upon dilution of the extender medium with phosphate buffered saline. The proportion of viable accessory testis derived sperm cells obtained post-cryopreservation was estimated to be sufficient to initiate long term storage and artificial insemination programs.

Germplasm cryopreservation is a safe and cost-effective long-term storage technique for the gametes, germinal tissues, stem cells, seeds, pollen, spores, microorganisms, *etc*. [1]. However, germplasm cryopreservation is not a widely adopted conservation method for insects except among a few major pest species [2]. This is despite the enormous impact that insects have on the ecosystem and on human lives in their role as pollinators of critical crops, such as the bumble bees, as crop pests such as the tephritid fruit flies, vectors of diseases such as the mosquitoes, *etc*. While climate change, habitat fragmentation, agrochemical use, importation of diseases and other abiotic factors are assumed to be contributing factors for the declining population of native pollinators [3,4], the dwindling pollinator abundance, and the geographical distribution of bee species have raised concerns as to how best to conserve them [5]. The technique that is used extensively to preserve floral, mammalian, piscine and to some extent the honey bee semen, is cryopreservation. However, cryopreservation is not used as a conservation tool for any other native or non-native hymenopteran pollinators such as the bumble bees, many species of which are considered endangered [6]. Bumble bees are remarkably effective pollinators for numerous species of plants specific to their habitat including those with non-dehiscent anthers such as the plants of Solanaceae family that are a vital source of food and medicine [7]. Cryopreservation of bumble bee germ cells would allow researchers to conserve valuable genetic traits before they are lost as well as aid in reinstating or restocking dwindling pollinator populations.

In most animal species, the semen used for cryopreservation is generally from an ejaculate. However, under certain circumstances, when the ejaculate is not accessible, the spermatozoa can be obtained from either the testicular or male accessory structures (*viz*. epididymis, vasa deferentia, and seminal vesicle) or the female storage organs (spermatheca in insects) depending on the species that is being studied. In the case of insects, it is only among the honey bees that the ejaculate semen is easily available. Prior to the wider adoption of collecting ejaculate semen from the honey bee drones, bee semen was obtained from the drone accessory testis/seminal vesicles for both the academic and applied use. In this study, the term 'seminal vesicle' is used to describe the modified and coiled vas deferens which serves as the accessory testis in the mature male bumble bees and the enlarged vasa deferentia in the case of honey bees. The seminal vesicle could contain up to 0.25 μl of semen per honey bee drone during the bee's peak

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https://doi.org/10.1016/j.cryobiol.2021.06.002
Received 9 April 2021; Received in revised form 3 June 2021; Accepted 5 June 2021
Available online 8 June 2021

maturity [8,9]. A minor drawback of seminal vesicle derived sperm cells from honey bees is that in young drones, the spermatozoa may still be maturing [10]. Despite this and other potential issues, the best conservation strategy for bumble bee male germplasm is to cryopreserve either the testicular sperm cells or the cells derived from the accessory storage organs such as the seminal vesicles in the males or the spermatheca from a mated queen.

Here, we describe the development and effectiveness of a cryopreservation methodology for the seminal vesicle derived honey bee and bumble bee sperm cells. The methodology was initially tested and confirmed to be effective on honey bee semen. The cryopreservation procedure was applied to the bumble bee spermatozoa and the viability was estimated. The primary aim was to establish if the procedure could be used for future conservation and reproductive protocols in *Bombus* species.

The study utilized multiple colonies of *Bombus impatiens* (Koppert Natupol, MI, USA) and *Apis mellifera* (Mann Lake Ltd., MN, USA), that were maintained by the USDA-ARS, Fargo, ND, USA. The drone (male) honey bees were collected from the entrance of the hives. The male bumble bees were either reared in queenless satellite colonies with worker bees or picked up from normal colonies when first noticed during daily observations. The bumble bee males, when first observed in a colony, were marked by painting their thorax to aid tracking their age and to ascertain their maturity. They were brought to the laboratory after seven more days. The seminal vesicles were dissected out from males of both *Bombus* and *Apis*. Seminal vesicles from multiple males were kept on ice during the collection process after which they were gently disrupted to release the spermatozoa [11]. Spermatozoa from the seminal vesicle were pooled into four samples per treatment for each of the species. To each sample, equal parts of 20% DMSO in FEM7 extender medium [12] was added. Diluted spermatozoa were then loaded into a 0.5 ml CBS™ high security straw using a syringe in such a manner that 3/4th of the semen buffer was ahead of the suspended sperm cells, 1/4th after and totaling the volume of the sperm cells in-between. Once straws were loaded with the spermatozoa suspension, the straws were heat sealed and returned to ice for not more than 15 min prior to cropreservation. A programmable cryopreservation freezer (Kryo 10, Series III, Planer, UK) was used to freeze the straws at a rate of 3 °C per minute from 4 °C to −40 °C. After 5 min of soaking at −40 °C, the straws were rapidly sorted, based on treatment groups, into Visotubes™ in a daisy goblet (Cryo Bio Systems, CA, USA). The goblets were kept chilled in liquid nitrogen in a Styrofoam box prior to and during the transfer process. On completion of the transfer, the goblets were sealed and stored in a 33-L liquid nitrogen storage container (MVE, MN, USA) in cryo-canisters. Untreated control spermatozoa were immediately stained using the LIVE/DEAD™ Sperm Viability Kit (Thermo Fisher Sci., USA) and the proportion of viable spermatozoa cells was estimated. The two-dye component kit stained the live cells impermeable to propidium iodide with SYBR-14 (green) and dead cells were differentiated by propidium iodide staining (red). The bumble bee semen was activated to motility by dilution of the sperm suspension to approximately 50% phosphate buffered saline (p3744, Sigma-Aldrich, MO, USA). The motility index was assessed as described in Rajamohan et al. [12].

After at least two weeks of storage in the liquid nitrogen, the straws were removed and rapidly thawed in a 35 °C water bath. Spermatozoa was stained using the LIVE/DEAD™ Sperm Viability kit as described above. Sperm cell viability was visualized and estimated using an automated live cell imager (Biotek Lionheart FX, Biotek/Agilent, USA). The images obtained were analyzed using Fiji/ImageJ software [13]. The viability data was assessed and compared using the non-parametric Conover-Iman test with Benjamini-Yekutieli adjustment [14] (Stata IC/16.1, College Station, TX).

As noted above, honey bee seminal vesicles were the primary source of sperm cells for both academic as well as applied use for over 60 years and prior to the wider adoption of the thoraco-abdominal stimulated ejaculation procedure [8,9]. We have revisited the use of seminal vesicles because both techniques are lethal to the male honey bee, and stimulated ejaculation is unlikely to be applicable to other pollinating species. One concern with collection from seminal vesicles is obtaining sufficient spermatozoa for artificial insemination. In the case of honey bees, our estimated count of spermatozoa per seminal vesicle was 276.7 ± 67.6 × 1000 [Fig. 1]. After mating, spermatozoa are stored in the spermatheca of the queen, from which she is capable of deliberately fertilizing her eggs to produce diploid females or withhold fertilization to produce haploid drones [15]. Queens will lay worker eggs if inseminated with as little as 0.2 μl of semen containing approximately 0.5 million viable sperm cells [16]. The spermathecae of recently mated queens often have more than 3 million sperm cells which reduces with queens' age [17]. This would mean that the spermatozoa collected from as few as two seminal vesicles is sufficient to inseminate and obtain a queen capable of producing diploid worker bees. Spermatozoa insufficiency in the spermatheca could result in the queen laying predominantly haploid drone eggs.

A second concern with collection of sperm cells directly from the seminal vesicle is spermatozoa viability. Post-cryopreservation viability of honey bee spermatozoa directly collected from the seminal vesicles was 82.2 ± 6.9%, compared to 99.4 ± 0.5% for controls [Fig. 3]. To successfully inseminate honey bee queens with cryopreserved semen, the arbitrary acceptable spermatozoa viability has been established at ~45%, based on the previously estimated minimum spermatozoa count [16] after taking into consideration the dilution factor and the amount of semen required per insemination.

There were significant differences noted during the collection and comparative assessment of the seminal vesicles from bumble bees versus the honey bees. The spermatozoa yield was significantly lower in bumble bees. It was 59.9 ± 39.3 × 1000 spermatozoa per seminal vesicle [Fig. 1]. Additionally, this value ranged from 9500 to 100,000, which highlights the fact that while the honey bees' seminal vesicles have been well characterized, further studies are required to evaluate the relationship between spermatozoa numbers and maturity in the seminal vesicle versus the age of the males in bumble bees. These observations have important implications for *Bombus* artificial insemination protocols, since factors such as the *Bombus* spermathecal capacity and the queens' propensity for multiple mating [18] result in a minimal threshold for both spermatozoa count and viability for successful artificial insemination. In the case of a closely related species of bumble bee, *Bombus terrestris*, the estimated spermathecal sperm cell count was reported to be 40,000–50,000, with 90% viability [19,20]. Therefore, to develop a successful cryopreservation strategy for the bumble bee germplasm, a larger number of seminal vesicles must be used to obtain sufficient sperm cells for a single artificial insemination event.

After cryopreservation, bumble bee spermatozoa viability was significantly lower compared to the controls (55.8 ± 14.0% versus 96.2 ± 10.5%), and significantly lower than what was obtained in the case of the honey bees [Figs. 2 and 3]. Assuming a count of ~60,000 sperm cells per seminal vesicle and with 99% viability, the current cryopreservation protocol would yield ~33,000 viable bumble bee sperm cells. Hence, while only one or two male bumble bee donors would be required for successful artificial insemination with fresh and untreated semen, seminal vesicles from at least three drones would be required to effect successful artificial insemination with cryopreserved spermatozoa. These assumptions are yet to be tested as the insemination techniques designed for other bumble bee species have not yet been optimized for *Bombus impatiens* [11].

This study represents the first successful attempt to cryopreserve spermatozoa from a *Bombus* species. The freezing protocol assessed in this study provides a proof of concept for the genetic preservation of *Bombus*. The results also demonstrate that there is a need for further studies on the development and optimization of an insemination procedure for the bumble bees. Alterations to semen diluent to conform it to species specific physiology and to better protect the ionic and osmolarity requisites are being pursued to improve the post-cryopreservation sperm

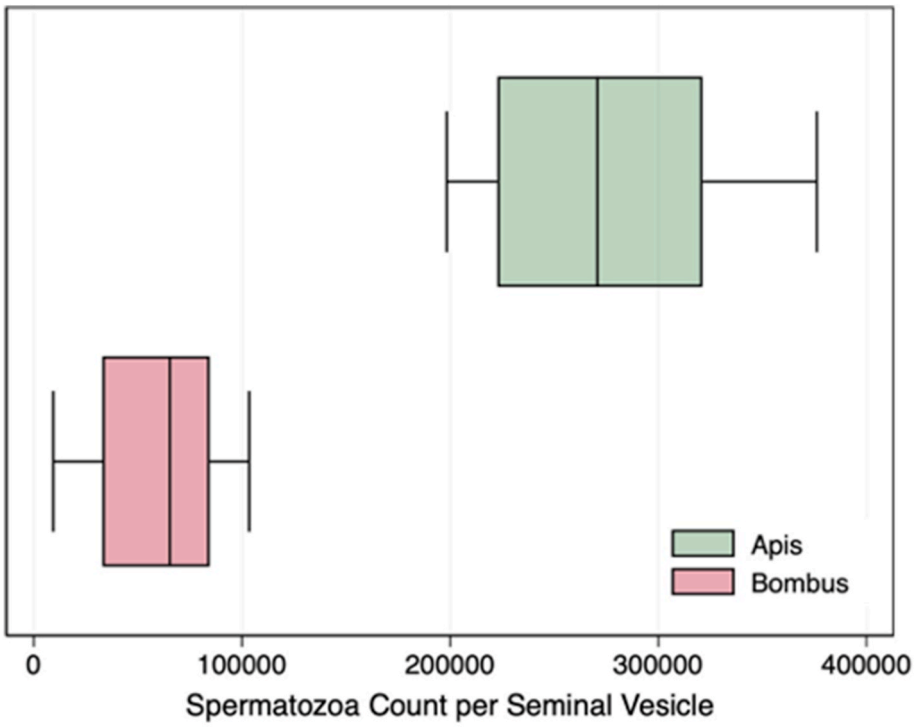

Fig. 1. The spermatozoa count in a seminal vesicle (accessory testis) in the *Apis mellifera* and *Bombus impatiens*. The dissected seminal vesicles were crushed in 50 μl of FEM extender medium and 4 counts was performed with 10 μl loaded on a hemocytometer/cell counter slide (Biotek, USA) using phase contrast optics.

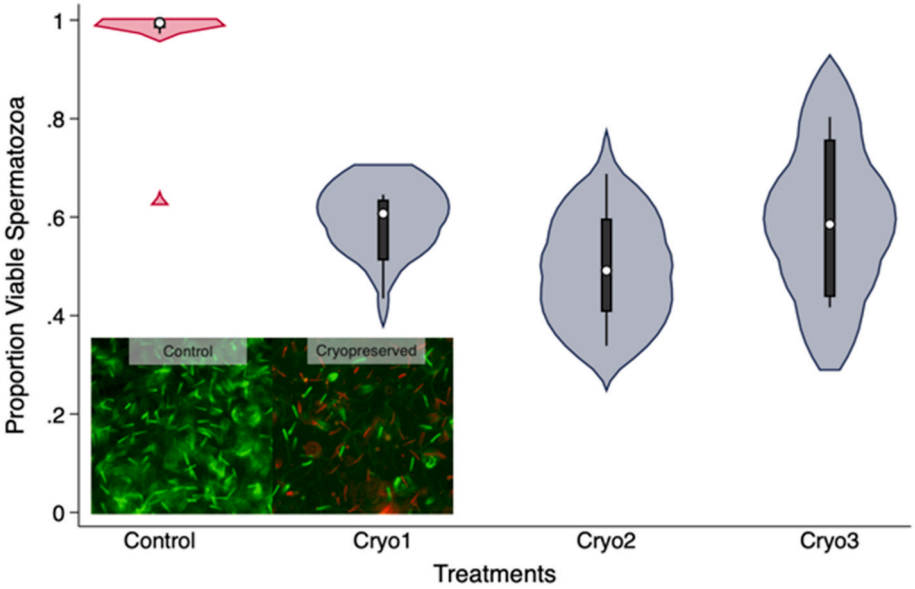

Fig. 2. Cryopreservation of *Bombus impatiens* spermatozoa derived from seminal vesicles. Sperm viability decreased significantly after cryopreservation compared to untreated controls. Variation in viability between three different treatment batches and the controls were significant when compared using Dunn's Test ($\chi^2(3) = 15.6$; $p = 0.0093$, 0.0070 and 0.0107; n = 24).

viability [12,21]. Further research is being conducted to investigate spermatozoa motility and the possibility of genomic fragmentation due to cryopreservation and other ancillary treatments. However, the basic methodology presented in this study could be used as a strategy for the long-term storage of genetic diversity of *B. impatiens,* and when transferred to other *Bombus* species, will facilitate more widespread conservation efforts for these vital pollinators.

Funding

This study was conducted with support from the United States Department of Agriculture - Agricultural Research Service, USA. The materials used and cited in this study does not imply endorsement by the United States Department of Agriculture.

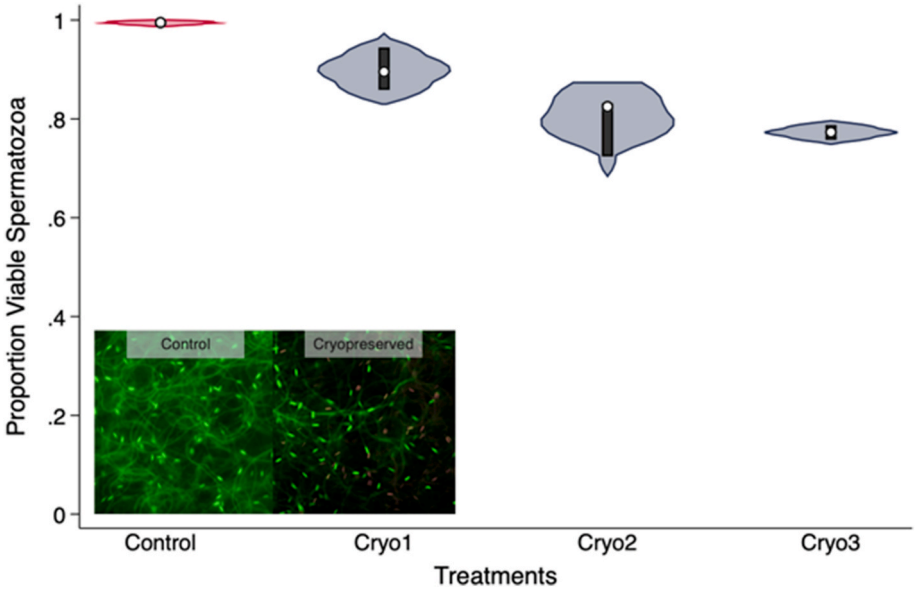

Fig. 3. Cryopreservation of *Apis mellifera* spermatozoa derived from seminal vesicles. Sperm viability was significantly lower after cryopreservation compared to untreated controls. Variation in viability between three different treatment batches and the controls were significant when compared using Dunn's Test ($\chi^2(3) = 11.4$; $p = 0.1428$, 0.0132 and 0.0097; n = 14) with the exception of the treatment 'Cryo1'.

Declaration of competing interest

The authors confirm that there are no conflict of interest with anyone or any organization with reference to this study or the manuscript.

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