Cryobiology 92 (2020) 124–129

Contents lists available at ScienceDirect

Cryobiology

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

A non-activating diluent to prolong *in vitro* viability of *Apis mellifera* spermatozoa: Effects on cryopreservation and on egg fertilization

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ARTICLE INFO

Keywords:
Apis
Honey bee
Drone
Spermatozoa
Semen
Cryopreservation
Extender
Diluent
Activation
Capacitance
Long-term storage

ABSTRACT

A non-activating semen diluent does not cause motility or acrosomal reaction or capacitate the sperm cell. The effects of such a diluent on the viability of honey bee spermatozoa stored in ambient conditions were assessed 60 days pre-cryopreservation and 24 h post-cryopreservation. Seven variations of a Tris-based non-activating diluents (FEM1 – FEM7) were compared to samples treated with conventional activating diluent and untreated semen. Semen viability (membrane integrity) was assessed after short- and long-term storage at 14.0 ± 0.2 °C. The non-activating medium FEM7 contained more viable spermatozoa than the activating medium, 24 h after cryopreservation (67.6 ± 10.9% and ~4%, respectively). After 60 days, 22.0 ± 7.8% of spermatozoa was viable in non-activating medium versus 0.0 and 60.8 ± 12.3%, in conventional media and untreated controls, respectively. Hence FEM7 was used to cryopreserve bee semen and subsequently inseminate honey bee queens. The quality of brood produced by the queens was assessed 30–60 days after insemination. The percentage of worker-bee offspring (produced from successfully fertilized eggs) was ~75% for both the non-activating medium and the conventional extender medium. Our results indicate that a non-activating medium possesses significant advantage over the conventional activating medium if the semen requires storage after treatments such as cryopreservation. The percentage of female offspring (from fertilized eggs) produced by queens inseminated with semen diluted in either the activating or non-activating medium did not differ from one another.

1. Introduction

In the honey bee, *Apis mellifera*, spermatozoa are stored in the queen bee's spermatheca after a successful mating flight and can remain viable *in vivo* for several years [21]. Even at ambient conditions *in vitro*, spermatozoa can remain viable for many days or even months [4]. Protection for the spermatozoa is derived from the seminal plasma components, spermathecal accessory gland secretions post-mating and even the phospholids in the spermatozoa plasma membrane [2,16,24,27]. During *in vitro* processing of the semen, the seminal plasma components are either diluted or completely replaced with balanced salt solutions, cell culture media, extenders or diluents which lack many or all of the properties of the seminal plasma and the spermathecal secretions. The nature of seminal plasma support towards the longevity of the spermatozoa is not currently understood [16,20,24]. Spermatozoa under ambient conditions are primed to fertilize while utilizing nutrients and other support systems, such as the antioxidants and antimicrobial agents available to them in the seminal plasma [16,25,31], and accessory gland secretions during storage in the spermatheca. During the period of transfer and migration of spermatozoa to the spermatheca, the biochemical properties of the seminal plasma are irreversibly altered, as has also been noted in studies on vertebrates [8].

Semen sometimes is artificially diluted for purposes such as assaying seminal characteristics, carrying additives such as cryoprotectants, or for extended use. This enables small quantities of semen that is genotypically unique to be used for a large number of inseminations. Development of improved honey bee semen handling (collection, *in vitro* storage, artificial insemination, cryopreservation etc.) is critical to support efforts in curtailing the currently unsustainable colony losses as revealed in surveys conducted within the United States [17,23]. Advances in artificial breeding techniques that improve breeding practices have potential to make immediate impacts and provide future security

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https://doi.org/10.1016/j.cryobiol.2019.11.045
Received 25 September 2019; Received in revised form 28 November 2019; Accepted 29 November 2019
Available online 30 November 2019

for the species and industries that depend on honey bees. Since the intention of this work is to improve *in vitro* storage of the bee semen, the study develops a method to store semen in ambient conditions even after removal from cryostorage. This allows for the transportation of cryopreserved semen to locations where cryogens could not be obtained or transported. In this study, honey bee semen was diluted with various components that partly replace the native seminal plasma. The diluent which sustained the spermatozoa for the longest storage period was used to cryopreserve bee semen and inseminate virgin queens.

This study evaluated diluents for their ability to simulate the reversible arrest of spermatozoa motility. This capability might be a key component to confer longevity to spermatozoa in honey bees and other hymenopteran social insects (ants, bees and wasps). The media reported here also reduce the loss of viability at non-freezing and ambient temperatures. We characterize the viability of spermatozoa and quality of brood produced by queen bees inseminated with the semen treated with a variety of non-activating diluents with and without chelating agents to mask the effects of metal ions such as calcium that play an important role in spermatozoa activation and fertilization [11,12]. In addition to offering another method to improve *in vitro* storage of honey bee semen, the study develops a method that allows for cryopreserved semen to be transported to locations where cryogens cannot be obtained or transported. The study examines the following issues: (a) Effects of activating and non-activating medium during ambient storage; (b) Effects of chelation during long-term ambient storage; (c) Effects of chelated-non-activating diluent on cryopreservation; and (d) The quality of brood produced by queens inseminated with chelated non-activating diluent.

2. Materials and methods

2.1. Semen collection and pre-experimental storage

Spermatozoa were collected from multiple male honey bees (drones) at the USDA-ARS facility in Baton Rouge, LA and shipped in glass capillaries to USDA-ARS, Fargo, ND. When the capillary was filled, semen was diluted using one-part HHBSE saline diluent [3] per 20 parts semen. On receipt, the semen from the capillaries was transferred to 0.2 ml thin walled Eppendorf vials, stirred gently with a blunt glass capillary and stored until use in at 14.0 ± 0.2 °C (EchoTerm in30, Torrey Pines Sci., CA, USA). Prior to semen transfer, the vials were pretreated for 30 min with a solution of Tris:EDTA:EGTA (50:2.5:2.5 mM, respectively; pH 8.9), containing a mixture of antibiotics (penicillin, streptomycin and amphotericin B) at the concentrations shown in Table 1. Prior to the use of semen for any assays, a microliter of the semen was mixed in 250 µl of saline consisting of 0.55% sodium chloride and 0.31% potassium chloride. Ten microliters of the diluted semen was assessed for motility using darkfield microscopy [9], and for viability using SYBR14/propidium iodide based membrane integrity assessment. If both the viability and motility parameters were satisfactory (see section 2.5), 5 µl of the untreated semen was aliquoted into 0.2 µl pretreated vials for all *in vitro* assays. Semen stored in Eppendorf vials at ambient temperatures was topped with 2 µl of halocarbon oil HC-56 (Halocarbon Corp., USA).

2.2. Diluent formulation

The non-activating base medium is Tris buffered and consists of 50 mM Tris, 151 mM of potassium chloride and 150 mM of sodium chloride and is a close approximation of the hypertonic medium described by Wegener et al. [28]. The seven media that were tested in this study were derived from the base medium designated as Fargo Extender Medium 1 (FEM1) to FEM7 (Table 1). All diluents prepared were adjusted to pH 7.2 (±0.3) with 0.1 N hydrochloric acid. The basal medium (FEM1) was designed to be hypertonic and therefore all the media studied were hypertonic. FEM1 was amended with various sugars, chelating agents and antibiotics to create FEM2-FEM7 (Table 1). To assess the efficacy of the designed working extender medium (termed henceforth as FEM7 or as 'non-activating medium'), it was compared against untreated spermatozoa and against semen treated with conventional 'activating medium' [10,13]. The activating medium contains 30 mM of Tris, 82 mM pf KCl and 83 mM of NaCl in addition to sugars (glucose, sucrose and trehalose), amino acids (arginine, glycine and proline), catalase, bovine serum albumin, *etc*.

2.3. Diluent treatment and ambient storage

The plasma in the semen was diluted by washing with an equal volume of one of the experimental media, and centrifuged (Sorvall™ Legend™ Micro 21R, Thermo Fisher Scientific, USA) at 1000×*g* for 12 min [28]. The supernatant was discarded. The semen was stored at 14 °C in the dark as described in section 2.1 until further assessment for up to 60 days.

2.4. Effect of diluents on cryopreservation and recovery

All samples in the study not used for direct insemination were

Table 1
Composition and the component combinations in the medium (FEM) tested in this study. The base non-activating medium (Base A) was tested with and without various components (B – E).

Component Class	Composition Concentration (mM or % or Units)	FEM1	FEM2	FEM3	FEM4	FEM5	FEM6	FEM7
A	TRIS (50 mM)	■	■	■	■	■	■	■
A	NaCl (150 mM)	■	■	■	■	■	■	■
A	KCl (151 mM)	■	■	■	■	■	■	■
B	Trehalose (50 mM)					■	■	■
B	Fructose (50 mM)		■	■	■	■	■	■
C1	EGTA (2.5 mM)			■	■	■	■	■
C2	EDTA (2.5 mM)				■	■	■	■
D1	Penicillin (10 kU)						■	
D2	Streptomycin (0.01%)						■	
D3	Amphotericin (0.0025%)						■	
D4	Gentamycin (0.005%)							■

cryopreserved in 0.2 ml sterile PCR vials as 5 µl samples of semen combined with components shown in Table 1 plus 10% dimethyl sulphoxide as the cryoprotective agent. The vials were frozen in a visotube (Cryo Bio System) at a rate of 3 °C/min to −45 °C using a programmable freezer (Kryo 10/Series III, Planer Biomed, UK). Thereafter, the visotubes were placed in a daisy goblet and transferred into liquid nitrogen until further assessment. Recovery was accomplished by rapidly thawing the semen vial in a water bath at 37 °C for 1 min.

After cryopreservation and thawing, the semen was held at 4 °C for 10 min. Five µl of the non-activating medium (FEM7) was added to 5 µl of semen and gently resuspended by mixing with a blunt capillary. The suspension was then centrifuged at 1000×*g* for 12 min, the supernatant was discarded, and the semen was resuspended in 5 µl of non-activating medium. Immediately upon resuspension, 1 µl of semen was assessed for viability and motility parameters. The rest of the semen was incubated in dark at 14 °C for 24 h as described in section 2.1 until the viability and motility assays were repeated.

2.5. Viability and motility assays

Membrane integrity was used to estimate viability of the spermatozoa. One microliter of the semen suspended in 200 µl of the diluent was treated 10 min apart with 0.02 µM SYBR14 and 12 µM of propidium iodide (Molecular probes, Thermo Fisher Scientific, USA) per mL of semen suspension, and incubated in the dark for 10 min at 35 °C. Ten µl of the stained cells were loaded on a hemocytometer (**Biotek Instruments, Vermont, USA**) and counted using an automated cytological microscope (**Lionheart, Biotek Instruments**) using appropriate filters. Emmens motility scores (Emmens Index, [9]) were noted for each sample before the viability was estimated. Only samples with an Emmens index of 2.5 and above were assessed for viability. Treatment and storage protocols often contained non-activating components and, in such cases, 1 µl of deionized water (>18 mOhm) was added to 1–5 µl of semen suspension and mixed gently with the pipette tip to activate the cells.

2.6. Cryopreservation for insemination assessments

Samples meant for insemination were frozen in 0.25 ml Cassou cryostraws (IMV technologies, France) instead of the 0.2 ml sterile vials to aid in the quick transfer of semen to the insemination device. Equal volumes of semen and either activating diluent [13] or non-activating diluent (FEM7) containing 20% dimethyl sulphoxide were gently combined to minimize osmotic shock and stirred with the pipettor tip. Straws were placed in a programmable freezer (Cryologic Freeze Control CL-3300, Victoria, Australia) and the temperature was lowered at 3 °C/min to −45 °C. Thereafter the straws were stored under liquid nitrogen until required for insemination. Semen straws were thawed at 37 °C in a water bath for 1 min and mounted on an insemination device for virgin queen insemination [**3**].

2.7. Insemination of queen honey bees and assessment of brood

Honey bee queens each were instrumentally inseminated with 5 µl of FEM semen or semen with activating diluent, or 3 µl of untreated (control) semen, using standard procedures [3]. The volumes were varied to equalize the amount of semen provided to each queen. Queens then were established in small colonies in 5-frame hives in either Baton Rouge, LA, or Pullman, WA. One to two months later, up to 200 pupal cells (n = 168 ± 60, mean ± SD) in each colony were evaluated to determine the proportion of cells that contained worker bees, *i.e.*, females that arose from fertilized eggs versus males (drones) that arose from unfertilized eggs. The proportion of worker bees is representative of the quality of the spermatozoa used to inseminate the queen. Data were obtained from 43 queens: 16 inseminated with FEM semen (9 in LA, 7 in WA), 20 inseminated with semen plus activating diluent (12 in LA, 8 in WA) and 7 inseminated with control semen (4 in WA, 3 in LA).

2.8. Data analysis

All treatment effects were analyzed and compared with Conover-Iman pairwise comparison test (conovertest ver.1.1.5) consisting of Kruskal-Wallis non-parametric multiple comparisons with Benjamini-Yekutieli adjustment in Stata IC/16.0 (StataCorp LLC, College Station, TX) [1,6,7]. The viability of the spermatozoa over time was modelled using logistic regression and the models were compared in Stata using 'suest' [30](www.stata.com/manuals15/rsuest.pdf). All graphs were created in Stata/IC 16.0.

3. Results

3.1. Effects of activating and non-activating medium on ambient storage and longevity

The difference between the activating and non-activating media on the spermatozoa longevity was very distinct (Fig. 1). The use of activating diluent resulted in rapid loss of viable spermatozoa within 24 h. Among the non-activating diluents, FEM1 supported viable spermatozoa for up to 7 days. FEM1 is a basal non-activating medium without sugar (s), chelating agent(s) or antibiotics. FEM2, which included 50 mM of fructose, was able to sustain semen viability for 30 days. FEM3 – FEM7 diluents also sustained viability for at least 30 days. The results show that the non-activating medium FEM7 could sustain higher seminal viability than the untreated samples over a period of 60 days (untreated versus non-activating; 22.0 ± 7.9 and 60.8 ± 12.3 ($p = 0.009$)). However, an activating medium could not sustain the spermatozoa viability beyond 24 h at 14 °C (21.3 ± 10.7; $p < 0.001$ versus untreated).

3.2. Effects of chelating agents

The addition of EGTA to the medium (FEM3) allowed the sample to

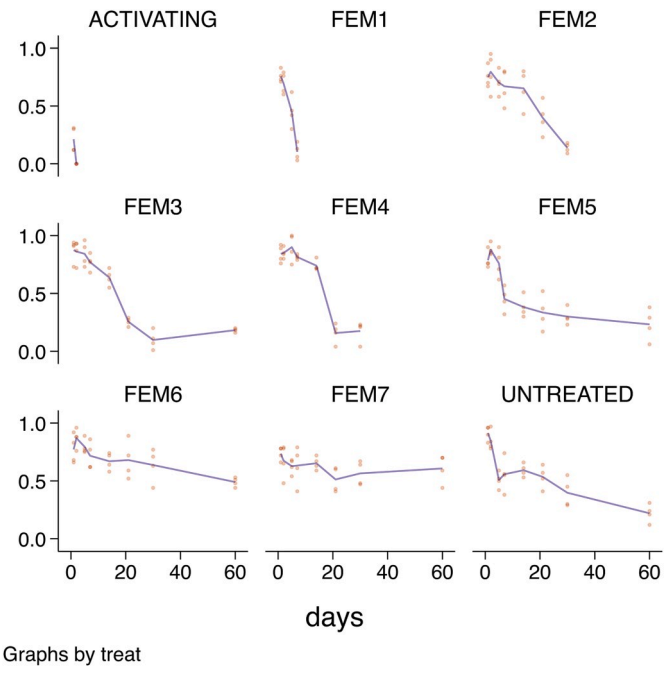

Fig. 1. Sixty-day assessment of spermatozoa viability after washing with 8 different diluents. Activating diluent refers to a phosphate-buffer-derived medium and the untreated refers to control semen that was not extended with any extraneous medium including antimicrobials. FEM1 – FEM7 are described in Table 1.

remain viable until day 60 (18.3 ± 1.7%) versus untreated (22 ± 7.9%) (p = 0.463, Fig. 2). However, the presence of both EGTA and EDTA significantly reduced sperm cell viability (p = 0.019). With the addition of EDTA to FEM3 resulting in FEM4, the semen viability did not extend beyond 30 days (17.5 ± 9.0%). This was ameliorated marginally by the addition of trehalose to FEM4 resulting in FEM5 which after 60 days of storage contained 23.3 ± 13.6% viable cells.

3.3. Effects of antibiotics

Only FEM6 and FEM7 sustained viability at ≥50% at 60 days. The differentiating factor was the presence of antibiotic(s) in these two diluents. FEM6 contained a mixture of an anti-bacterial and anti-mycotic agent (amphotericin B). FEM7 contained only a single antibiotic, *viz.* 5×10^{-3}% gentamycin sulphate. The 60 day assessment showed that FEM6 had approximately 10% fewer viable cells than FEM7 (49.0 ± 3.9% versus 60.8 ± 12.3%; p = 0.543).

3.4. Effects of activating versus non-activating medium on cryopreservation

Samples cryopreserved with non-activating diluent FEM7 containing 10% DMSO and the samples cryopreserved with activating diluent were thawed and diluted with equal volume of the same medium. Five ul of the sample was assessed immediately and after 24 h with membrane integrity staining. Propidium iodide positive cells were significantly higher in the activating media after 24 h, indicating loss of viability (<p = 0.001). The non-activating media did not cause significant loss of viability (p = 0.452; Fig. 3).

3.5. Honey bee brood quality from queens inseminated with chelated-non-activating medium

Insemination with semen in the activating diluent resulted in 73.8 ± 35.9% worker bee offspring and 26.4 ± 35.1% drone offspring (Fig. 4a and B). With the non-activating diluent 76.8 ± 35.0% were workers and 23.4 ± 33.3% were drones. These outcomes for the two diluent types were similar (p = 0.8786). The worker proportions differed for both non-activating and activating diluents (p = 0.0009 and 0.0012, respectively) compared to the untreated semen; untreated semen resulted in 100% worker offspring.

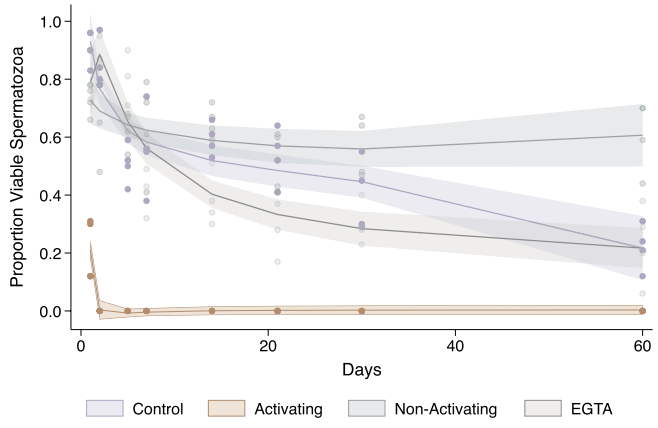

Fig. 2. Means of spermatozoa viability over 60 days. The band tracks the confidence intervals of fractional polynomial logistic regression of the data points. Control (purple) refers to the untreated samples, Activating (light red) is the conventional phosphate buffer-based diluent, Non-Activating (light green) is diluent FEM7 and EGTA (grey) is diluent FEM3.

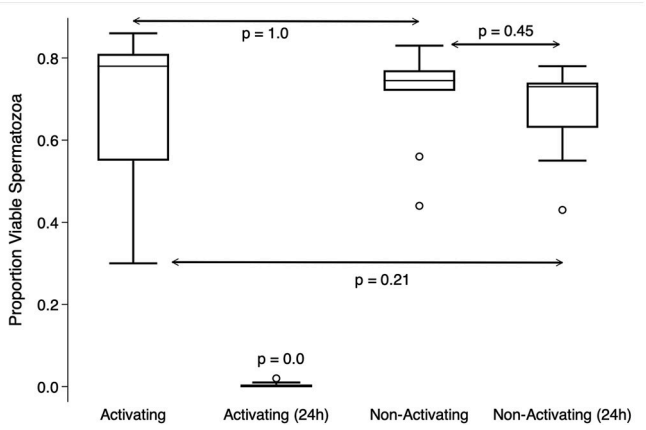

Fig. 3. The effects of activating and non-activating (FEM7) diluents on the viability of spermatozoa 24 h post cryopreservation. The treatments were compared by Conover-Iman post-hoc test as described in the methods.

4. Discussion

Spermatozoa of insects is maintained in a non-capacitated state by the secretions of the accessory glands until they are transferred to the female reproductive tract [18]. However, the presence of other factors of the accessory glands appear to activate the spermatozoa. This process ensures *in vivo* longevity of the spermatozoa in and out of the spermathecae among many of the social hymenopterans. However, in *Apis mellifera,* observation of the dense ejaculate shows no initiation of motility in individual spermatozoa and raises the possibility that factors that are required to move the semen to the spermatheca originate in the female reproductive tract. Pending a better understanding of the various factors involved, this study investigates chelation, sugars, antibiotics and cryopreservation on the spermatozoa in a hypertonic extender medium. **Verma**[25], **Williams**[26] and **Wegener et al.** [28] noted that a simple Tris-based K+ and Na + hypertonic medium could act as a reversible inhibitor of motility. This diluent is not a replacement of the seminal plasma but has an ionic content similar to constituents in the spermatheca [24,25].

We evaluated whether the components of base FEM are sufficient to sustain longevity of spermatozoa in lieu of the complex accessory gland secretions of male and the female honey bees [2]. The complexity of these secretions is evident from the inability of previous attempts to improve sperm survival in instrumental inseminations with extended semen [2,16,18]. This indicates changes to the spermatozoa that the queen's secretion could not reverse prior to or during storage in the spermatheca. In addition, in the current study the treatments are compounded by an additional factor – cryopreservation.

If the seminal plasma is not contaminated or replaced, semen samples survived for up to 200 days (u**npublished results**), which is well beyond the reported 60 days of data in this study. Seminal plasma contamination or replacement reduced viability within 7 days in samples suspended in FEM1. This was surprising considering that the buffer is very similar to the one tested by **Verma** [25] and was shown to contain motile spermatozoa even after 9 months of storage (proportion viability was not reported in the study). One additional factor might be the container effect and oxygenation of the sample. Verma [25]stored the samples in capillaries while in this study the samples were stored in 0.2 ml sterile vials. Also results not shown here indicate that isotonic and hypotonic media will result in loss of viability in a matter of 6–24 h even at 14 °C and higher. This is most probably due to the sperm cell exhaustion due to their motility rather than activation [18]. We believe that this could be a reason for reduced viability in the control sample compared to the sample washed in non-activating diluent after 60 days.

Chelating agents such as EDTA and EGTA reduce the effect of metal ions by binding and modulating their ion-channel activity which

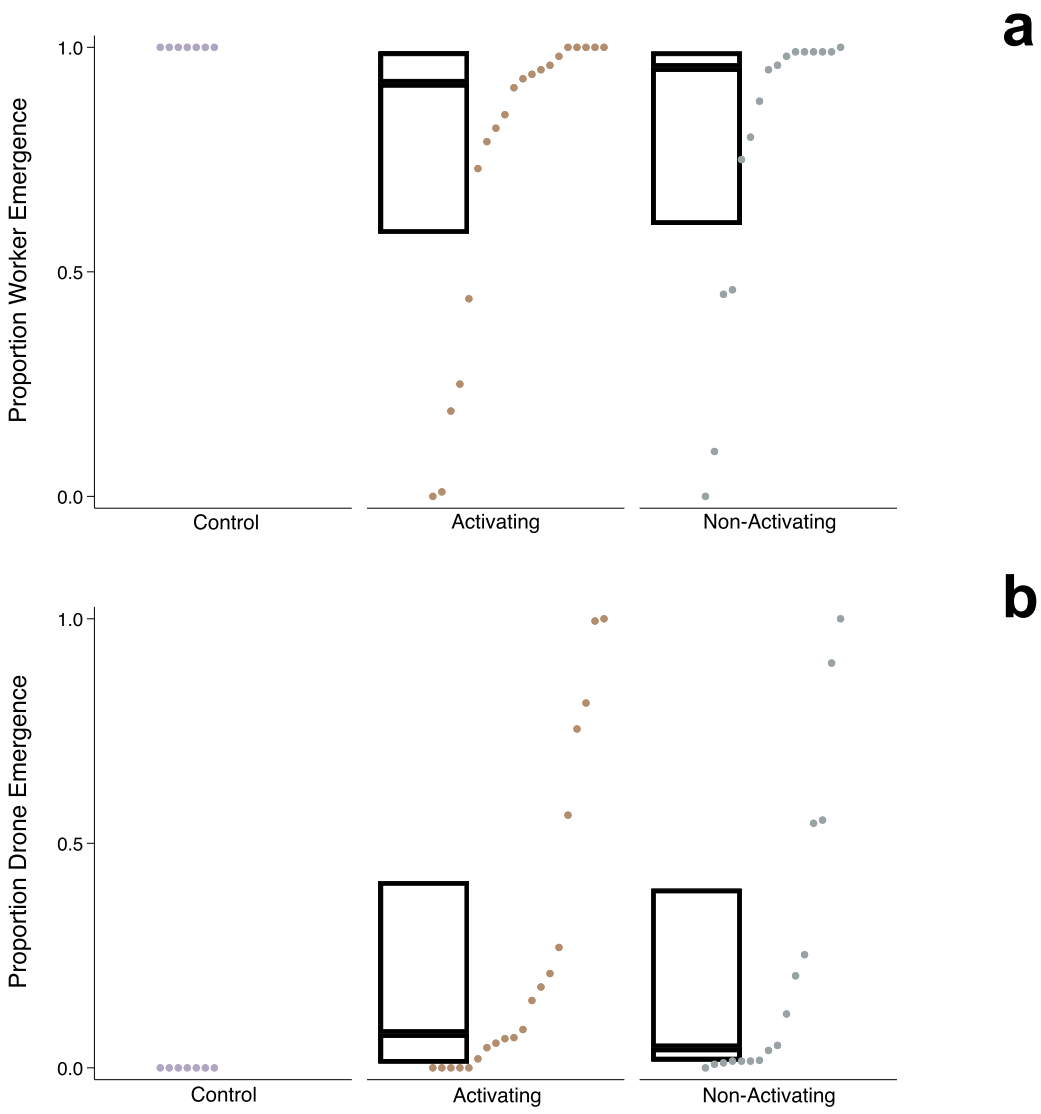

Fig. 4. A strip plot (categorical scatterplot with minimal distribution and density characteristics of the data) with comparison of the results from brood patterns (4a – Worker emergence; 4b – drone emergence) from honey bee queens inseminated with non-cryopreserved (untreated/control), conventional activating and the non-activating (FEM7) diluents.

accounts for sperm cell activation and capacitation [11,12]. EGTA has high specificity for alkaline earth metals such as calcium and magnesium, and its presence in the medium curtails Ca^{++} activity. The role of calcium in spermatozoa activation, acrosomal reaction and fertilization in the vertebrates and invertebrates has been studied to some extent [11,12,29]. Interestingly in some animals, the extracellular calcium has been reported to be inhibitory to spermatozoa capacitation [5]. In the current study, EGTA in the diluent (FEM3) extended the spermatozoa viability. However, addition of EDTA inexplicably cancelled this advantage (see data for FEM4 in Fig. 1). The longevity was reinstated by the addition of trehalose to FEM4 resulting in FEM5. Although further assessment is required to determine the reasons, we believe it might be either due to the protective role of trehalose in cell membrane and protein scaffolding [15] and/or the mere change in tonicity of the medium.

The cryopreservation process entails dilution of the seminal components, and this might reduce the inherent antimicrobial activity of the semen. This is important because of the threat from genitalia-associated microbes [19]. FEM6 and FEM7 that contained antibiotics and/or an antimycotic agent, significantly improved spermatozoa viability compared to the untreated samples. However, we also noticed a tendency for diseased brood to occur after queens were inseminated with these diluents (**unpublished results**). This is perhaps due to differential tolerance of the spermatozoa to various antibiotics [4] and the deleterious effects of the antibiotics on the symbiotic flora in the reproductive tract[32]. There is some evidence that *in lieu* of a diluent, the storage container itself could be impregnated with antibiotics to result in longer storage periods [14,22].

Beyond the rigorous testing to 60 days in this study, preliminary results (**unpublished results**) show that FEM6 and FEM7 can maintain viable semen for significantly longer periods. The longest we have maintained viable spermatozoa with FEM7 is 640 days without cryopreservation as a pre-treatment (45.5 ± 32.1% viability) and 180 days with post-cryopreservation washing treatments (37.0 ± 29.21% viability).

This work provides clear evidence for the requirement of an improved diluent/extender media for the collection and storage of

honey bee semen intended for use in the instrumental insemination of queens. The study also highlights the role of a proper diluent in prolonging the seminal viability after cryopreservation and thawing. Unlike other cell types, spermatozoa can quickly deteriorate if the seminal plasma support system that chaperones the spermatozoa is removed. This is the case during pre- and post-cryopreservation processing. While replicating the complexity of natural seminal plasma is not a reasonable goal, the fundamental requirements for a functional diluent/extender are presented here. The fact that semen could be stored for up to 180 days after thawing and resuspended in FEM7 has important ramifications for honey bee industry if semen must be shipped over long periods in ambient conditions. Furthermore, the results presented here emphasizes the importance and the role of antibiotics in spermatozoa storage and instrumental insemination. Extender media such as these that contain chelating agents, sugars and antimicrobials thus show good potential to significantly improve storage of honey bee semen. Currently this study is being extended to include the *in vivo* longevity of the semen in the queen bee, the colony quality and therefore the queen's health over multiple seasons.

Acknowledgements

This project was made possible by the crucial expertise and timely assistance with queen rearing, insemination and hive management by Garrett Dodds (USDA-ARS, Baton Rouge, LA). Support from the USDA-ARS Innovation Fund to [JR, RD and Harvey Blackburn, NGPBR, USDA-ARS, Fort Collins, CO] is acknowledged. Mention of commercial product names in this manuscript does not constitute endorsement by the institutions or the authors of this study.

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