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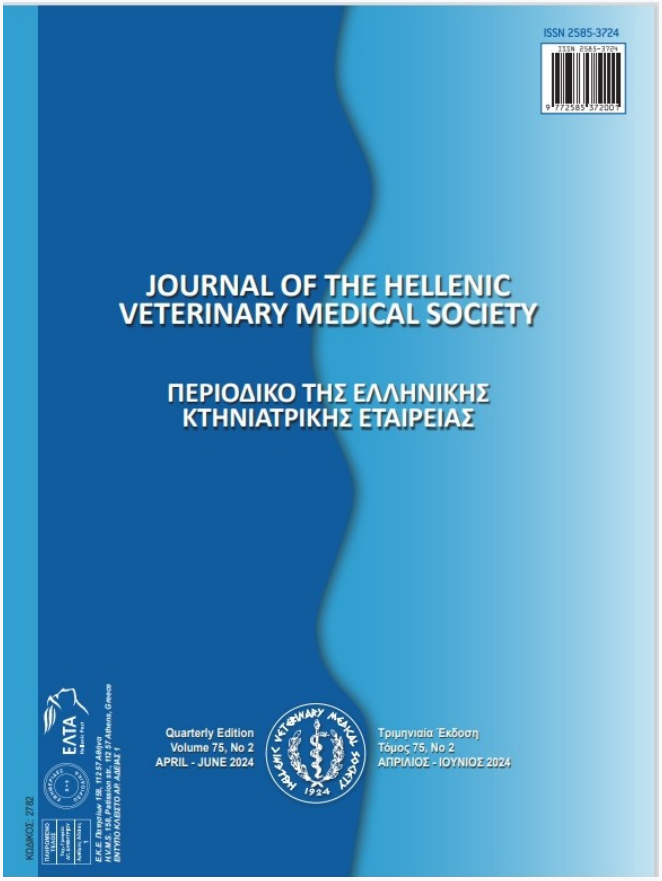

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Effect of partial replacement of dimethyl sulfoxide with dextran, polyvinylpyrrolidone or polyethylene glycol on post-thaw drone semen quality and queen fertility

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ABSTRACT: The aim of this study was to elucidate the toxicity effect of widely used penetrating cryoprotective agent dimethyl sulfoxide (DMSO) on frozen drone semen and fertility. DMSO was replaced totally or partially with either dextran, polyvinylpyrrolidone (PVP), or polyethylene glycol (PEG), which are non-penetrating cryoprotectants. The freezing extender with 12%, 8%, 4% and 0% DMSO were supplemented with 0%, 4%, 8% and 12% of dextran, PVP, or PEG, respectively. For all treatment groups, the final cryoprotectant concentration was 12%. Dextran4, PVP4, and DMSO12 groups with good post-thaw motility, plasma membrane functional integrity that evaluated throughout the study and fresh semen were used for queen instrumental insemination (n=46). Two queen bees (4.4%) died due to manipulation. Alive queens [24 (55%) of 44] had been observed to survive after a week, and 17 (71%) of these queens

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continued to survive after three months. At the end of three months, 15 (88%) of the alive queens continued to produce worker bees. There were no differences among insemination groups (P>0.05) for the rate of survival for three months and presence of worker broods. After overwintering, there were two queens from the PVP4 and three queens from the Dextran4 group that survived and one queen from PVP4 and two queens from Dextran4 group that continued to produce worker bees. In conclusion, addition of 4% Dextran or PVP as an extender containing 8% DMSO solution improves queen bees' worker egg-laying period, but the same results were not observed for the PEG containing groups.

Keywords: *Apis mellifera;* cryoprotectants; honey bee; semen freezing; queen fertility.

INTRODUCTION

Honey bee queens mate with 8-10 drones during mating flights with unknown genotypes (Richard et al., 2007). Proliferation, growth, yield, disease resistance, aging and death are all quantitative and qualitative phenotypic features of colonies which are determined by genotypes of the queen bees and mated drones. Mainly, the sustainability of genetic diversity, protection of desirable genetic lineage and colony loss prevention are only possible with controlled mating of the queen. Moreover, keeping mutant lines, selecting behavioural strains, testing maternal effects and producing inbred lines are only possible with controlled mating through Instrumental Artificial Insemination (IAI) with fresh or frozen-thawed sperm (Cobey, 2007; Moritz, 1984; Smilga-Spalvina et al., 2023)

In natural breeding program, the queen's lifespan must be sufficient to produce enough diploid offspring to sustain functional hives. Still, in the IAI program, the oviposition's onset and laying enough diploid eggs to produce viable daughter queens are not sufficient (Cobey, 2007; Hopkins et al., 2012). Many published studies deal with survivability and queens' ability to produce diploid broods after IAI using frozen or fresh drone semen (Cobey, 2007; Cobey et al., 2013; Collins, 2000; Hopkins et al., 2012; Paillard et al., 2017; Richard et al., 2007). The short lifespan and diploid worker brood laying period were reported after IAI because of inadequate care of queens, poor insemination procedures, insufficient semen dosage and low quality of used semen (Cobey, 2016, 2007; Richard et al., 2007; Wegener and Bienefeld, 2012).

Semen cryopreservation has been used to maintain valuable genetic material for a long time in many mammalian species. Cryopreservation has only been attempted in very few insect species such as honeybees, *Apis mellifera* (Wegener and Bienefeld, 2012). The first presentation of honey bee queen IAI was performed by Watson (Watson, 1929, 1928), which resulted in an increased interest in drone sperm cryopreservation and assisted reproductive techniques (Moritz, 1984)**.** Various mammalian and non-mammalian semen extenders and protocols have been widely used to improve honeybee sperm quality in the cooled or frozen state (Alcay et al., 2019, 2021; Alçay et al., 2019; Hopkins and Herr, 2010; Kaftanoglu, O., Peng, 1984; Nur et al., 2020a; Paillard et al., 2017; Taylor et al., 2009; Wegener and Bienefeld, 2012).

Semen cryopreservation includes cooling, freezing, storing at -196°C in liquid nitrogen and thawing stages. However, these cryopreservation processes cause a reversible and irreversible decrease in sperm motility, viability, and fertility (Soylu et al., 2007; Watson, 2000). Irreversible reduction of sperm metabolism originates from cold shock, the formation of intracellular ice crystals, osmotic stress and lipid peroxidation that occurs throughout the cryopreservation process. Extender composition, cryoprotective additives, pH, cooling rates, storage temperature and warming rate have an important effect to overcome the deleterious effects of ultra-low temperatures and sustaining the fertilization ability of cryopreserved semen (Alcay et al., 2015, 2019; Alçay et al., 2019; Cobey et al., 2013; Hopkins et al., 2012; Nur et al., 2020a; Soylu et al., 2007; Wegener et al., 2014).

Post-thaw sperm survivability typically requires the presence of cryoprotectants (CPAs) that prevents intracellular ice crystallization. CPAs are classified as internal or penetrating and external or non-permeating compounds (Akcay E., 2023). A wide variety of membrane-penetrating (glycerol, ethylene glycol, DMSO, propylene glycol) were reported successfully used in various mammalian sperm cryopreservation (Alcay et al., 2021; Alcay S., 2023; Nur et al., 2020b; Paillard et al., 2017). Polysaccharides, mannitol, sorbitol, dextran, hydroxyethyl starch (HES), methyl cellulose, albumin, gelatine, other proteins, polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), polyethylene oxide (PEO), or polyvinyl alcohol which are all classified as non-penetrating CPAs (Hubálek, 2003) that cause extracellular cryoprotection when present at concentrations of 10-

40% (Hubálek, 2003; Paillard et al., 2017). Melnichenko and Vavilov (Melnichenko, A. N., & Vavilov, 1975) have reported successful drone sperm cryopreservation (VERMA, 1978). Even though DMSO is suspected of causing functional and morphological damage in sperm in published reports (Kaftanoglu, O., Peng, 1984; Paillard et al., 2017; Wegener and Bienefeld, 2012). It has become the preferred choice in many studies because of the high quality post-thaw recovery (Alcay et al., 2019; Alçay et al., 2019; Dadkhah et al., 2016; Nur et al., 2020a; Smilga-Spalvina et al., 2023; Taylor et al., 2009). Yet, there have been limited studies that combined DMSO with different cryoprotectants (Wegener and Bienefeld, 2012).

The aim of this research is to find ways to reduce cryotoxicity of frozen drone semen from DMSO and to extend the queen's fertile life expectancy. To accomplish this, we replaced DMSO totally or partially with non-penetrating cryoprotectants such as Dextran, Polyvinylpyrrolidone (PVP), and Polyethylene Glycol (PEG).

MATERIALS AND METHODS

The experiments were performed in the Laboratory of Andrology in the Department of Reproduction and Artificial Insemination at the Faculty of Veterinary Medicine and in Beekeeping Development-Application and Research Center (AGAM), Bursa Uludag University, Bursa, Türkiye.

Chemicals

NaCitrate, $NaHCO_3$, KCl, Amoxicillin and Catalase, Polyvinylpyrrolidone (PVP), and Polyethylene Glycol (PEG), Dextran and Dimethyl Sulfoxide (DMSO) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All other chemicals were purchased from Merck (Merck & Co., Inc. Germany).

Production of drones and queens

Apis mellifera anatoliaca drone colonies were reared within the foundation of drone wax. Drones (approx. 16 days or older after emergence) were obtained from 5 different colonies and moved to the laboratory.

Queen bees were reared from worker larvae according to the Doolittle grafting method (Doolittle, 1899). The larvae were 4 - 5 days old when grafted into artificial queen cell cups and were introduced into queenless colonies. The queenless colonies were fed with bee candy, or if the pollen was abundant, sugar syrup was given instead. The capped queen cells were moved into an incubator (34°C and 60 - 80% relative humidity) before emergence. One day before the emergence of the queens, each cell was placed in the queenless mating colony and stayed there until insemination day, with a queen screen in the entrance so that virgin queens can be kept inside the mini colony for artificial insemination process.

Semen collection and handling

Semen samples were collected by holding the head and thorax with a gentle squeeze of the abdomen (Collins, 2000). 0.8 mL of saline solution with a ratio of 1/1 was drawn into a syringe (Schley Instrumental Insemination equipment, Lich, Germany) to facilitate suction and avoid drying for sperm collection. The procedure was continued by approximately 0.8 mL of semen (one drone) and visual aid was provided with the aid of a stereo microscope. This process was repeated until the targeted volume of semen was obtained (3 mL for approximately 3 - 4 mature drones) for all freezing groups.

Sperm motility

Sperm motility was evaluated subjectively by with a drop of diluted pooled ejaculates. Samples were studied under a phase-contrast microscope (400x magnification) with a coverslip (Taylor et al., 2009). Sperm motility was scored on a scale of 0 to 5 corresponding to 0%, 20%, 40%, 60%, 80% and ≥95% of the observed population being motile, respectively (Nur et al., 2012; Taylor et al., 2009). Post-thaw sperm motility was also evaluated.

Plasma membrane integrity

Water test was used to evaluate the sperm plasma membrane integrity (Nur et al., 2012). Total of 1.0 µL diluted semen was added to 250 µL of distilled water and incubated at room temperature for 5 min. Immediately after incubation, one drop of semen was placed on a glass slide, covered and evaluated with a phase-contrast microscope (400×). Microscope fields were selected randomly. At least 200 spermatozoa were evaluated per slide, and the percentages of swollen tail spermatozoa were calculated (Nur et al., 2012). Sperm plasma membrane integrity was also assessed at post-thaw stage.

Experiment 1: Determination of post-thaw better groups

The extender compositions were NaCitrate 2.43g,

NaHCO3 0.21g, KCl 0.04g, Amoxicillin 0.03g and Catalase 200μL in 100mL of distilled water (Taylor et al., 2009). Extender pH was adjusted to 8.1 and separated into 10 groups. The freezing extender with the 12%, 8%, 4% and 0% DMSO were supplemented with 0%, 4%, 8% and 12% of Dextran, PVP or PEG, respectively. The control group contained only 12% DMSO. For all treatment groups the final cryoprotectant concentration was 12%.

Pooled semen (3 μL) was diluted with one of the extenders with pre-decided semen/extender ratio (1/10). One-step dilution method was applied at room temperature. Each group was filled with the suitable extender into one end of the straw, followed by an air space (~10 mm). After that the sample was placed into the center of the straw then, followed by another similar-sized air space. Finally, the very same extender was added to the other end of the 0.25 mL straw. Straws were sealed with various colors of the polyvinyl alcohol (PVA) powder for identification. Then the straws were cooled to 5°C within 1 h in a cold cabinet and then equilibrated at 5°C for 2 h. Equilibrated semen was frozen with a programmable gamete freezer (Dadkhah et al., 2016) and then plunged into liquid nitrogen (-196°C) to store. The used freezing curve temperature changes were measured in the freezing chamber above straw level by thermometers (Hanna Inst. HI 9056) (Fig.1). Post-thaw sperm parameters were evaluated with at least two straws from each group that were thawed (at 37°C for 30 s) in a water bath. The procedure was repeated 5 times for each group. The groups with good post-thaw sperm motility and plasma membrane functional integrity were determined. At least 10 insemination doses from the most superior groups of each post-thaw were frozen for instrumental insemination.

Experiment 2: Instrumental insemination and fertility monitoring

Post-thaw motility and plasma membrane functional integrity of frozen semen for artificial insemination were also evaluated and then diluted (1/1) with a cryoprotectant free extender and centrifuged at 700 g for 10 min before insemination. Total of 8 - 10 μL of centrifuged frozen semen was loaded into the insemination syringe.

The queens reared in queenless colonies were inseminated at 7 - 9 days (after emergence from the queen cups) of age (Cobey, 2007; Cobey et al., 2013) with 8 - 10 μL of centrifuged frozen semen from each group that yielded good post-thaw results or was freshly collected semen. All inseminated queens were labelled on the thorax with a different numbered colored tag, and part of one wing was clipped (as 1/3) to prevent flying. These were then transferred into the same mini hives with queenless bee colonies. All inseminated queens were subjected to CO_2 for egg-laying stimulation after insemination. At 3 - 7 days after insemination, the presence of the marked queens was checked visually. Later on the presence of worker bee

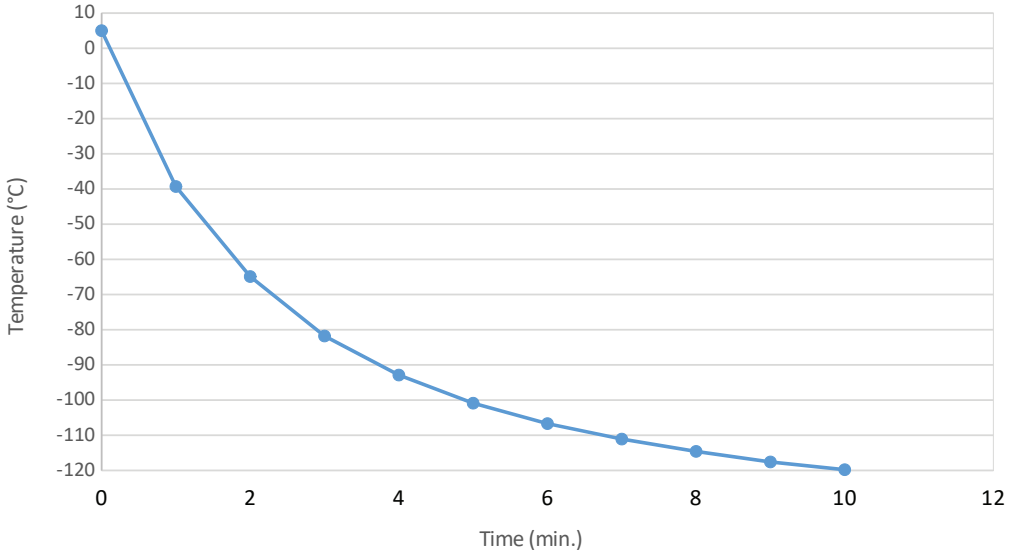

Figure 1. The freezing curve used to cryopreserve honey bee (*Apis mellifera* L.) sperm for all treatments. The indicated temperatures were measured in the freezing chamber above straw level by a thermometer (Hanna Inst. HI 9056)

pupae (after 10 days of egg-laying) was observed and queens were transferred to a queenless Langstroth hive and followed for 3 months. Once worker bee pupae appeared, we determined that artificial insemination was successful (Cobey, 2007; Cobey et al., 2013). The queen bees that overwintered for approximately four months were considered to have survived the treatment.

RESULTS

Experiment 1

The mean observed semen motility and plasma membrane functional integrity relative to DMSO, Dextran, Polyvinylpyrrolidone (PVP), and Polyethylene glycol (PEG) content can be found in Table 1.

The diluted semen at room temperature and the extender composition affected sperm motility dramatically. This dramatic negative effect of the extender was not observed on plasma membrane functional integrity. Extended semen motility of PEG supplemented groups performed the worst out of the treatment groups ($P < 0.05$). Diluted semen plasma membrane functional integrity-related results were generally similar to each other.

The DMSO12, PVP4 and Dextran4 were found to be superior to the other treatment groups in terms of post-thaw motility and plasma membrane integrity ($P < 0.05$). The highest post-thaw sperm membrane functional integrity was observed in the groups containing PVP4 and DMSO ($P < 0.05$). In terms of motility and plasma membrane integrity the DMSO, PVP4 and Dextran4 groups were found to be acceptable for the queen instrumental insemination among the treatment groups ($P < 0.05$).

Experiment 2

Fresh semen motility and plasma membrane integrity that was used for insemination were 67% and 92%, respectively. The post-thaw sperm motility and plasma membrane integrity of DMSO, PVP4 and Dextran4 of insemination groups are 61%, 28% and 32%; 87%, 85% and 81%, respectively.

Obtained frozen and fresh sperm fertility-related findings after instrumental insemination and queen survivability are presented in Table 2. In total, 46 queens were instrumentally inseminated at 7 - 9 days of age. Two of them (4.4%) died due to manipulation. A total of 24 (55%) of 44 live queens were observed to survive after a week, 17 (71%) live queens survived more than three months. Unfortunately, most of the instrumentally inseminated queens with fresh semen were lost due to the stormy weather conditions in mini styrofoam hives. During the study, at least once, 18 (40.9%) of the inseminated queens were observed to produce worker bee pupae. At the end of three months, 88% of the live ($n = 17$) queens continued to produce worker bees and 12% produced drone pupae. In terms of queen survival, for three months, there were no differences among insemination groups ($P > 0.05$). There were no differences in the presence of worker bee pupae cells among groups, two queens (12%) of the DMSO group began to produce drone pupae only in this period.

The survived queen bees over 90 days overwintered successfully for approximately 4 months. The queens inseminated with Dextran4 and PVP4 presented a higher fertility lifespan than the other groups. At

Table 1. The mean observed semen motility and plasma membrane functional integrity from Dimethyl Sulfoxide (DMSO), Dextran, Polyvinylpyrrolidone (PVP), and Polyethylene glycol (PEG) content at two stages.

Group	Extended semen			Post-thaw semen		
	n	Motility (%) ±S	Water (+) (%) ±S	n	Motility (%) ±S	Water (+) (%) ±S
DMSO12	5	76.0±2.5^{a}	92.0±2.0^{a}	13	54.6±2.7^{a}	81.8±2.5^{a}
PEG12	7	54.3±2.0^{abc}	90.1±1.8^{a}	17	0.18±0.1^{c}	13.2±3.0^{e}
PEG8	8	48.6±7.0^{bc}	88.6±1.6^{a}	11	0.0±0.0^{c}	18.1±3.7^{de}
PEG4	5	32.0±3.7^{c}	86.8±0.8^{a}	13	0.0±0.0^{c}	34.7±6.7^{dc}
PVP12	5	68.0±2.0^{a}	93.0±1.6^{a}	10	0.0±0.0^{c}	21.7±1.6^{de}
PVP8	5	74.0±6.0^{a}	91.6±2.0^{a}	11	4.6±4.5^{c}	52.2±4.1^{bc}
PVP4	5	66.0±4.0^{a}	89.2±0.8^{a}	10	32.0±2.0^{b}	81.2±3.4^{a}
Dextran12	5	56.0±6.8^{ab}	92.4±2.0^{a}	9	0.0±0.0^{c}	25.6±6.2^{de}
Dextran8	5	70.0±6.3^{a}	93.2±1.4^{a}	10	0.0±0.0^{c}	35.7±2.2^{dc}
Dextran4	5	60.0±6.3^{a}	92.4±1.1^{a}	12	33.2±5.8^{b}	70.3±6.3^{ab}

Table 2. Frozen and fresh sperm fertility related findings after instrumental insemination and queen survivability.

Group	n	1. Control (3-7 days)		2. Control (>7-90 days)		Drone pupae (%)
		Living (%)	Egg laying (%)	Living (%)	Worker pupae (%)	
DMSO	13	8(62)	2(25)	5 (62)	3 (60)	2 (40)
PVP4	10	6(60)	1 (17)	3 (50)	3 (100)	0 (0)
Dextran4	10	6(60)	0 (0)	5 (83)	5 (100)	0 (0)
Fresh	11	6(54)	2 (33)	4 (67)	4 (100)	0 (0)
Grand mean	44	24 (60)	5 (23)	17 (71)	15 (88)	2 (12)

the end of 10 months post-insemination, 5 queens (2 from PVP4 and 3 from Dextran4 group) were alive after over-wintering and 1 queen from PVP4 and 2 queens from Dextran4 groups continued to produce worker bee pupae.

DISCUSSION

Extenders and cryoprotectants that were used for semen cryopreservation include a variety of simple and more complex chemical compounds. These chemicals interact with different parts of the cells and some extra- and intracellular factors that influence intracellular ice formation (Hubálek, 2003). These chemicals lead to stress and cause biochemical changes in cell metabolism immediately after dilution (Aisen et al., 2001; Ak et al., 2010) and during the post-thaw stage (Aisen et al., 2001; Ak et al., 2010; Alcay et al., 2019; Alçay et al., 2019; Nur et al., 2020a; Soylu et al., 2007). For the diluted semen at room temperature, the extender composition affects sperm motility dramatically. This dramatic negative effect of the extender was not observed on plasma membrane functional integrity.

DMSO is the most frequently used cryoprotectant with satisfactory post-thaw drone semen quality, but suffers from poor queen fertility results (Kaftanoglu, O., Peng, 1984; Paillard et al., 2017; Wegener and Bienefeld, 2012). DMSO is a sulfur-containing organic molecule, which easily crosses cellular membranes, causes membrane lipid and protein rearrangement, increases membrane fluidity, greater dehydration at lower temperatures, and reduces intracellular ice formation (Holt, 2000; Hubálek, 2003). The highest post-thaw motility was obtained in the group containing 12% DMSO only ($P < 0.05$).

Cryoprotectants are classified as penetrating and non-penetrating agents. The permeable cryoprotectants that penetrate the cell membrane dehydrate the spermatozoa and protect intracellular compartments. Also, it decreases the freezing point of the intracellular contents. The non-permeable cryoprotectants that cause extracellular cryoprotection may alter the plasma membrane, act as a solute, lower the medium's freezing temperature, and decrease extracellular ice volume expansion during water solidification (Elliott et al., 2017; Kundu et al., 2002). The non-permeable cryoprotectants (PEG12, PVP12 or Dextran12) that were used in this study were ineffective for the cryopreservation of drone spermatozoa at a 12% concentration. PEG was extremely toxic to drone semen compared to DMSO, PVP and Dextran.

The cryoprotectants can be used alone or combined with a freezing extender, the combination of cryoprotectants can lead to new effects that differ if they were used on their own. The combination of various cryoprotectants with different classes (e.g., alcohols, sugars, diols, amides, and large polymers), molecular weight and cell permeability exhibit additive, synergistic, or antagonistic interactions (Hubálek, 2003). The substitution of DMSO with different concentrations of PEG, PVP or Dextran was not successful for the protection of post-thaw motility ($P < 0.05$). The 4% PVP and 4% Dextran exhibited additive effect with 8% DMSO on post-thaw plasma membrane functional integrity, only. DMSO combined with PEG resulted in reduced post-thaw motility and plasma membrane functional integrity of drone semen. The exact mechanism involved in the cryotoxic effect of PEG-DMSO combination on the post-thaw drone semen warrants further investigation.

Two (4.4%) of the inseminated queens (n = 46) died due to manipulation at 7 -9 days of age. Rearing conditions, IAI or mating age, treatment of queens before and after insemination, semen dosage and handling, pheromone development, effects of CO_2 treatments and environmental conditions can affect artificial inseminated queen's survivability and performance (Cobey, 2016, 2007; Cobey et al., 2013; Richard et al., 2007; Wegener and Bienefeld, 2012).

The success of instrumental insemination with frozen semen should not be measured by the ability to sustain functional hives, but rather the ability of such queens to produce enough worker offspring to produce viable daughter queens (Hopkins et al., 2012). The onset of oviposition and queen survivorship for the production of worker brood is the main criteria for the success of instrumental insemination (Cobey, 2007; Hopkins et al., 2012). Hopkins et al., (Hopkins et al., 2012) reported that queens inseminated with frozen semen were absent after approximately 2 months, whereas queens inseminated with fresh semen continued to produce fertilized pupae for a longer time. It was observed that a total of 40.9% of the inseminated queens produced at least once worker bee pupae. There were no differences between fresh and frozen semen on diploid egg laying duration for three months. At the end of three months, 88% of the queens continued to produce worker bee pupae ($P > 0.05$).

Instrumental insemination with frozen drone semen results in insufficient worker-to-drone ratios to maintain functional colonies (Harbo J.R., 1983, 1977; Kaftanoglu, O., Peng, 1984). The survived queen bees (n = 17) overwintered approximately for four months. Five queens (2 queens from PVP4 group and 3 queens from Dextran4 group) were alive after overwintering. The queens that were inseminated with Dextran4 (n = 2) and PVP4 (n = 3) had a higher fertile lifespan (Approximately 8-10 months' post-insemination) compared to the other groups. One queen from the PVP4 and two queens from the Dextran4 group continued to produce worker bee pupae and exhibited a longer fertile lifespan.

CONCLUSIONS

The results of this study suggest that drone semen cryopreservation could be a way to help preserve bee diversity, and speed up the artificial selection process. The queens inseminated with frozen semen may lay diploid worker eggs for a limited period, but this is adequate to generate a substantial number of functional daughter queens (F1, F2,….). The presence of 12% DMSO as a freezing extender is sufficient for good post-thaw semen motility, membrane integrity and fertilizing ability. The external cryoprotectants (PEG, Dextran and PVP) are not sufficient alone as a 12% freezing extender of bee semen. The addition of Dextran or PVP as a 4% extender containing 8% DMSO improves the worker bee egg laying period, but not in PEG containing groups. PEG exhibited a cryotoxic effect on post-thaw drone semen motility and plasma membrane integrity in all combinations. Our results suggest that future studies are needed so that the worker egg-laying period of queens after insemination with frozen semen can be improved.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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