Original Article

EFFECT OF POST-THAW DILUTION OF DIALYZED AND CRYOPRESERVED DRONE SEMEN ON QUALITY OF QUEEN HONEY BEES

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Received: 28 October 2025; accepted: 26 November 2025

Abstract

Cryopreservation of honeybee (*Apis melifera*) drone semen remains a significant challenge. Despite decades of research, maintaining sperm viability and functionality after thawing continues to limit the practical use of frozen semen. This study investigated the effect of post-thaw dilution of cryopreserved drone semen with Bee Semen Solution (BSS) on semen viability and queen reproductive performance. Queen bees were instrumentally inseminated with fresh or cryopreserved semen, either undiluted or diluted with BSS after thawing. The impact of these treatments on the number and quality of spermatozoa in the spermatheca and on brood pattern was assessed. Cryopreservation markedly affected sperm quality and the reproductive performance of inseminated queens, resulting in lower sperm viability and reduced fertility compared with fresh semen. However, post-thaw dilution of semen with BSS tended to improve sperm parameters in the spermatheca and queen fertility. The results indicate that post-thaw handling of semen, including the use of appropriate diluents, may play an important role in improving the outcomes of insemination with dialysis method cryopreserved semen. We also emphasize that multiple insemination should be considered as a method of improving spermatozoa number in spermahteca and, consequently, queen fertility.

Keywords: multiple insemination, post-thawed semen, queen performance, spermatozoa, sperm quality

INTRODUCTION

Cryopreserving and storage honeybee (*Apis mellifera*) drone semen remain key challenges in the instrumental insemination of honey bee queens, breeding and research. Despite many years of attempts to develop effective protocols (e.g. Melnichenko & Vavilov 1975, Harbo 1977, Kaftanoglu & Peng 1984, Hopkins et al. 2012; Wegener et al. 2014a; Alçay et al. 2019), there is still no well established widely used method that preserves semen fertility after long-term storage (Cobey, 2016; Smilga-Spalvina et al., 2023). The difficulties associated with cryopreserving drone semen include the freezing and storage of samples and the subsequent effective insemination of queen bees with thawed material. Freezing and thawing clearly increases the occurrence of morphological defects with a higher percentage of sperm with head deformities and twisted tails (Yániz et al., 2024) and lower sperm motility and viability (Hopkins & Herr, 2010; Özkök & Selcuk, 2020).

A number of studies are focused on the medium used to dilute semen prior to cryopreservation and its effect on semen parameters (Harbo, 1979; Taylor et al., 2009; Hopkins & Herr, 2010; Wegener et al., 2012; Alçay et al., 2019; Alçay et al., 2022; Gulov & Laskin, 2021; Aktar et al., 2024; Nur et al., 2024; Mohammandi et al., 2025). Several studies have demonstrated the toxicity of some of the most commonly used cryoprotectants on spermatozoa and/or bee queens (Hopkins & Herr, 2010; Wegener & Bienefeld, 2012; Wegener et al., 2012). A possible

solution to minimising the toxic effects of cryoprotectants would be to dilute or remove a significant amount of the toxic cryoprotectant after cryopreservation or thawing (Wegener, 2014a; Gül et al., 2017; Auth & Hopkins, 2021). Given these limitations, a search for alternative diluents that stabilize sperm cell membranes after thawing without causing excessive damage seems reasonable.

However, few studies have investigated the effect of semen dilution after cryopreservation on its quality in the spermathecae and queen fertility. Wegener et al. (2014a) diluted cryopreserved semen with Bee Semen Solution (BSS), then centrifuged it to remove the cryoprotectant and concentrate the semen. This procedure resulted in reduced sperm motility in the spermatheca and decreased queen fertility, with a 27% worker brood percentage. Similarly, Gül et al. (2017) washed and centrifuged the semen sample with a semen diluent as well as with glucose solutions and fresh ram and drone semen plasma. However, no significant improvement in queen fertility (percentage of worker brood) was achieved. In both cases, centrifugation after dilution may have negatively affected sperm functionality and insemination success (Cobey et al., 2013). According to Wegener et al. (2014a), skipping centrifugation of semen after cryopreservation in favor of gentle dilution of Bee Semen Solution (BSS) and manual mixing of the sample increased semen quality and queen fertility rates. However, only three queen bees underwent this procedure, and the dilution ratio used (1 part semen : 2 parts BSS) differed from the ratio usually used in queen bee insemination (1:1) (Skowronek et al., 1995; Gerula et al., 2016; Kahya & Gençer 2022).

The objective of the study was to determine how semen dillution with BSS after cryopreservation using the prior to freezing dialysis method influences the queen's fertility, as well as the number and quality of spermatozoa in spermatheca.

MATERIAL AND METHODS

The study was conducted in the Apiculture Division of the National Institute of Horticultural Research in Poland in 2024 on Carniolan honey bee (*Apis mellifera carnica*) queens.

Semen for cryopreservation was collected from drones reared in strong Carniolan bee colonies with the use of the standard method (Cobey & Bienkowska, 2025) in July and frozen with the dialysis method (Wegener et al., 2014a). Samples were cooled to -40°C at 3°C/min in an automated freezer (IceCube® 11XS, Sy-Lab Cryobiology) and then transferred directly into liquid nitrogen. The semen was stored in liquid nitrogen for 30 days. Thawing was done by immersion in a water bath at 35°C for one minute (Wegener et al., 2012). Fresh semen was collected on the day of insemination from the same colonies as those used for cryopreservation. Motility was used as a measure of semen quality (Wegener et al., 2012).

Seven-day-old virgin queens lowly related to the semen collection colonies from the same genetic line were inseminated in August twice with doses of 4-µL semen each within a 72-hour period using the Loc device. Fresh undiluted (4 µL semen), fresh diluted (4 µL semen + 4 µL BSS), cryopreserved undiluted (4 µL semen), and cryopreserved diluted semen (4 µL semen + 4 µL BSS) were used to inseminate the queens. As a diluent we used a Bee Semen Solution (Wegener et al. 2014b), which was prepared one day before use and stored at 4°C. We used a different batch of fresh and post-thawed semen for the first and second insemination. During each insemination procedure, the queens were anaesthetized under CO_2 for three minutes. Immediately after insemination, each queen was marked and returned into three frame trapezoid nucleus hives with approx. 1000 young adult bees. A total of 50queens were inseminated.

The brood pattern (proportions of drone and worker brood) were measured 14 days after the queen began laying eggs, and all capped brood cells on both sides of the middle comb were counted. Then the spermathecae of inseminated queens were dissected and measured with the use of KEYENCE VHX-970F to enable a later calculation of their volume. Semen from each ruptured spermatheca was transferred into Kiev solution to evaluate sperm motility, viability, and spermatozoa number (Wegener et al., 2012).

Sperm concentration was measured with the use of a Makler chamber with a depth of 10 µl and the number of spermatozoa in the spermatheca was estimated (Yaniz et al., 2020). Then sperm motility was estimated with the use of the method descibed by Wegener et al. (2012). Sperm viability was determined with the use of a combination of SYBR14 and propidium iodide with the LIVE/DEAD™ Sperm Viability Kit (L7011, Invitrogen™, Thermo Fisher Scientific) (Yániz et al., 2020) with an Axiolab 5 fluorescence microscope.

Statistical analysis

The chi-squared test (χ^2) was used to analyse the difference in the number of dead queens between groups. Due to the small sample sizes in some groups, the Monte Carlo version of this test (with 10,000 random permutations) was used to obtain more robust empirical *p*-values, which are less affected by low expected frequencies.

Differences in brood pattern, number of spermatozoa, sperm motility and viability between groups were tested with non-parametric Kruskal-Wallis test, followed by Mann-Whitney U tests with Bonferroni correction for multiple comparisons. Variables showing strong skewness (number of spermatozoa, sperm motility and viability) were log(1+x) transformed prior to analysis. Brood pattern was analysed without transformation, since the values were concentrated near the maximum value of 100%.

In addition to the significance tests, the medians and means of the characteristics between groups were compared, to assess the strength of differences with the use of Cliff's delta (δ) (Cliff, 1993; Meissel & Yao, 2024). This enabled the direction and potential biological significance of differences to be indicated, even if they did not reach formal statistical significance. Cliff's delta values were interpreted according to the adjusted thresholds proposed by Meissel and Yao (2024) for biological data: 0.11, 0.28, and 0.43 for small, medium, and large effects, respectively.

RESULTS

The motility of freshly collected and thawed semen reached 98% and 62% respetively, in the tested samples. The semen used for insemination (fresh, fresh diluted, cryopreserved or cryopreserved diluted) did not significantly affect queen mortality (Tab. 1). Monte Carlo simulation confirmed that the differences observed between the groups were not significant (χ^2=1.14, df=3, *p*=0.77).

Non-parametric analyses revealed significant effects of semen treatment on all evaluated traits (Kruskal-Wallis test, *p*<0.001 for all traits).

Brood pattern differed significantly among groups (*H*(3)=28.13, *p*<0.001). Queens inseminated with cryopreserved semen (groups 3 and 4) produced less worker brood than those inseminated with fresh semen (groups 1 and 2) (Tab. 1). The effect of cryopreservation was strong (δ=0.94-1.00, large effect), while no difference was found between fresh semen groups (*p*=1.000). The comparison between cryopreserved groups (3 vs 4) indicated a small to medium positive

Table 1.

The mortality and brood pattern (percentage of worker brood) of queens inseminated after different treatments on semen

Semen (no. group)	Number of inseminated queens	Queen mortality		Brood pattern		
		No. of dead queens	% of dead queens	mean ±SEM	Median	Min-Max
Fresh semen (1)	10	3	30.0[a]	98.9 ±1.14	100[a]	92-100
Fresh diluted semen (2)	10	2	20.0[a]	99.3 ±0.33	100[a]	98-100
Cryopreserved semen (3)	15	3	20.0[a]	65.9 ±6.95	74[b]	0-90
Cryopreserved diluted semen (4)	15	5	33.33[a]	78.4 ± 4.89	85[b]	58-95

Table 2.

Number of spermatozoa (million) and quality of semen in spermatheca of queens inseminated after different treatments of semen.

Semen (no. group)	Number of spermatozoa			Semen quality					
				Viability			Motility		
	Mean ±SEM	Median	Min-Max	Mean ±SEM	Median	Min-Max	Mean ±SEM	Median	Min-Max
Fresh semen (1)	4.06 ±0.59	4.01[a]	1.40-5.78	89.0 ±2.98	92a	76-97	89.6 ±2.13	92[a]	79-94
Fresh diluted semen (2)	4.10 ±0.42	3.36[a]	3.18-5.78	90.7 ±0.66	92a	88-92	74.3 ±3.92	76[a]	60-87
Cryopreserved semen (2)	1.86 ±0.41	1.20[b]	0.72-5.09	65.7 ±6.79	72b	7-86	35.8 ±6.33	32[b]	0-63
Cryopreserved diluted semen (3)	2.04 ±0.19	2.28[b]	1.00-2.62	71.2 ±3.43	75b	58-83	54,2 ±4.73	53[b]	31-73

effect of BSS dilution (δ=-0.35), suggesting a slight though non-significant biological improvement (Tab. S1).

The number of spermatozoa in spermathecae showed a significant treatment effect ($H(3)=7.84$, $p=0.0005$) (Tab. 2). Queens inseminated with cryopreserved semen had substantially lower sperm numbers than those inseminated with fresh semen ($p<0.05$, δ=0.72-1.00, large effects). BSS dilution tended to increase spermatozoa number in cryopreserved samples (group 3 vs group 4), indicating a moderate positive effect (δ=-0.30), but not significant ($p=1.000$) (Tab. S1).

Sperm motility also varied significantly ($H(3)=25.60$, $p<0.001$) (Tab. 2). Cryopreservation influenced a decrease in motility, confirmed by large effect sizes (δ=0.73-1.00). Post-thaw dilution in BSS (group 4) resulted in moderately higher motility compared to undiluted frozen semen (group 3; δ=-0.52, large effect), although the difference was not significant ($p=0.26$) (Tab. S1).

Sperm viability was also significantly affected by treatments ($H(3)=23.70$, $p<0.001$) (Tab. 2). Cryopreserved semen exhibited a strong decline in viability compared with fresh semen (δ=0.83-1.00, large effects). Queens inseminated with BSS-diluted post-thawed semen (group 4) showed slightly higher viability than those receiving undiluted semen (group 3), but the difference was negligible (δ=0.03, $p=1.000$) (Tab. S1).

DISCUSSION

Our results clearly show that the cryopreservation process affects the number of spermatozoa and the quality of the semen stored in the spermathecae. This finding is commonly reported in the scientific literature (Harbo, 1983; Kaftanoglu & Peng, 1984; Hopkins et al., 2012; Wegener et al., 2012; Wegener et al., 2014a; Gül et al., 2017; Pallard et al., 2017; Gulov & Laskin, 2020, 2021; Yaniz et al., 2020; Nur et al., 2024; Egyptien et al., 2025; Mohammadi et al., 2025). Nevertheless, only a few studies have examined the fertility of queens inseminated with cryopreserved semen. These studies differ in terms of the freezing method and diluent composition used, both before and, in some cases, after freezing. However, the true measure of the effectiveness and efficiency of cryopreserving drone semen is whether the inseminated queen produces an adequate number of workers to sustain the growth of the bee colony (Bryła & Trzcińska, 2024) or at least enough fertilized eggs to raise new queens, thus preserving genetic lines and supporting selective breeding (Wegener et al., 2012).

Our research and previous studies have consistently shown that queens inseminated with cryopreserved semen are capable of producing fertilized eggs, although overall fertility outcomes have been variable. Early experiments by Harbo (1983) and Kaftanoglu

and Peng (1984) demonstrated successful oviposition following single inseminations (3.3 µL and unspecified volumes, respectively), yet the proportions of worker brood (8-22% and 47% respectively) and spermathecal sperm counts (0.1-0.2 million) remained low.

Efforts to optimize diluents have produced mixed results. Wegener et al. (2012) found that the use of dimethyl sulfoxide (DMSO) during cryopreservation reduced spermathecal sperm counts (0.7-1.3 million vs. 2.4-3.6 million for fresh semen), whereas the addition of cryoprotectant through dialysis of semen improved the percentage of worker brood to 49-79% (Wegener et al., 2014a). Gentle post-thaw dilution instead of centrifugation further enhanced fertility, yielding up to 65.8% worker brood and 1.8 million sperm in the spermatheca (Wegener et al., 2014a). Similarly good results were obtained by Hopkins et al. (2012), who demonstrated that queens inseminated with 5 µL of cryopreserved semen could produce fertile offspring across generations, with fertilization success reaching 95-100% (on average 50% in initial and 46% in first generation). Subsequent studies have sought to improve post-thaw sperm viability and queen fertility through the modification of freezing and insemination protocols. Gül et al. (2017) reported that queens inseminated once with 5 µL of post-thawed semen washed in drone semen plasma achieved worker brood percentages comparable to frozen-thawed controls (48% and 47% respectively) and numer of spermatozoa in spermatheca (0.81-1.6 million, respectively) without significant improvement. Such alternative diluents before freezing as honey-based solutions, have yielded more promising results. Gulov and Laskin (2020, 2021) reported that queens inseminated once with 8-12 µL of cryopreserved semen laid fertilized eggs with 17-99% (average 61%) worker brood and average of 1.98 million spermatozoa in the spermatheca. In turn, Nur et al. (2024) achieved oviposition after single inseminations (8-10 µL) using cryoprotectant-free extenders, though brood and sperm data were not provided. Excessive dilution, as shown by Pallard et al. (2017), reduced spermathecal sperm counts to 0.14-0.15 million regardless of centrifugation. Also, using a simple antibiotic-free freezing procedure, Egyptien et al. (2025) obtained fertilized eggs from a small proportion of queens inseminated once with 5 µL of cryopreserved semen, confirming feasibility but limited efficiency. Concurrently, the present study obtained hight percentages of fertilized eggs, both in the group with undiluted (up to 90%; average 66%) and diluted cryopreserved semen (up to 95%; average 78%), and the number of spermatozoa in the spermatheca was also higher than in results previously reported (1.86 and 2.04, respectively). Collectively, these studies indicate that while cryopreserved semen can support fertilization and worker production, outcomes remain strongly dependent on insemination volume, semen handling (dilution, centrifugation), and the choice of cryoprotectant or diluent. In the presented study, queens were inseminated twice, which may have contributed to the difference in spermatheca filling compared to previous studies. According to Woyke (1960), Mackensen (1964) and Bieńkowska et al. (2011), dividing a single dose of semen into two inseminations increases the number of spermatozoa in the spermatheca compared to a single insemination. Studies conducted on thawed semen have also shown that inseminating a honeybee queen twice with small amounts of diluted semen results in significantly more sperm entering the spermatheca than in the case of single inseminations with the use of the same total volume (Bolten & Harbo, 1982). The second insemination delivers 68% to 77% more spermatozoa to the spermatheca than the first (Bolten & Harbo, 1982).

However, Gąbka (2021) found no difference in the onset of egg laying between queens inseminated with different semen doses. Furthermore, multiple inseminations may increase the risk of semen contamination and queen infection (Gąbka, 2021; Smilga-Spalvina et al., 2023). Nevertheless, multiple inseminations appear reasonable for increasing the number of spermatozoa reaching the spermatheca, particularly when cryopreserved semen is used for insemination in which the freezing process itself affects sperm viability

and motility, consequently impacting the queen's performance (Wegener et al., 2012; Yániz et al., 2024).

The dilution of semen in the appropriate medium could also have influenced our results, although the use of fresh semen dilution did not significantly improve the parameters studied. Previous experiments with fresh diluted semen have shown that dilution has no great effect on the success of inseminations (Mackensen, 1969; Skowronek et al., 1995; Lodesani et al., 2003; Gerula et al., 2016; Gąbka & Cobey, 2018; Kahya & Gençer, 2022). The findings of these studies, irrespective of the utilized diluent or the semen-to-diluent ratio, revealed no significant disparities in the number of sperm stored in the spermatheca of queens inseminated with undiluted and diluted semen. Similarly, dilution of cryopreserved semen did not lead to significant differences compared to undiluted cryopreserved semen. However, the effect of dilution may be greater when it follows dialysis of semen against a hypertonic cryoprotectant solution, as in the present experiment. Dialysis clearly withdraws water from the semen, and post-thaw dilution could potentially re-establish a natural osmolarity and viscosity. This may explain why the analysis of medians, means and size effects (Cliff's delta) revealed a clear trend towards higher values in diluted frozen semen. This was particularly evident in terms of sperm motility, as well as number of spermatozoa and brood pattern. One of the ingredients of the diluent used - BSS (Wegener et al., 2014b) - is trehalose, which is found in the drone seminal fluid (Blum et al., 1962). Many such molecules as proteins, amino acids, and sugars present in drone seminal fluid are known to help sperm viability after storage (den Boer et al., 2009). According to Nur et al. (2020), the addition of 0.1 or 0.05 M trehalose to the freezing media containing 12% DMSO incresed post-thaw cell motility and plasma membrane integrity of spermatozoa. Also, the use of trehalose without DMSO allows for a significantly higher percentage of sperm motility and kinetic parameters to be maintained after cryopreservation compared to DMSO and glycerol (Tsvetkov et al., 2024). The use of BSS with trehalose could mitigate the negative impact of freezing on sperm motility to some extent, which could improve the ability of queens to lay eggs. In honey bees, significant losses of sperm occur naturally in terms of both number and viability at the stage of transport to the oviducts and spermatheca. Under natural conditions, only 3-5% of sperm reach the spermatheca (Yaniz et al., 2020), so any further reduction in motile sperm resulting from cryopreservation could significantly impact the queen's fertility. According to Paillard et al. (2017), queens are at an increased risk of failing to lay fertilised eggs when fewer than 0.5 million sperm reach the spermatheca. In this context, semen dilution can be considered a means of improving sperm dispersion. The reduction in semen viscosity through dilution is also worth noting (Tofilski, 2014; Bratu & Pătruică, 2021). Semen is very dense, tends to clump (Cobey et al., 2013) particularly when collected from older drones (Czekońska et al., 2013), and is more difficult to soak into the needle, especially when a smaller tip diameter is used (Bieńkowska & Panasiuk, 2006). The semen dillution could potentially facilitate both the technical administration of the sample during insemination and the subsequent migration of spermatozoa in the queen's reproductive tract. Further studies on the composition of diluents and insemination protocols are needed to optimize queen fertility and make semen cryopreservation a viable tool for honey bee breeding and genetic conservation.

FUNDING

The study was conducted as part of a research project entitled "Storage of semen of honey bee drones a guarantee of maintaining the gene pool of bees with the highest breeding and utility value" founded by Polish Ministry of Education and Science, CONTRACT No MEiN/2022/DPI/1813

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Supplementary online material:

Table S1. Pairwise comparisons Mann-Whitney U with Bonferroni correction and non-parametric effect size measure Cliff's δ