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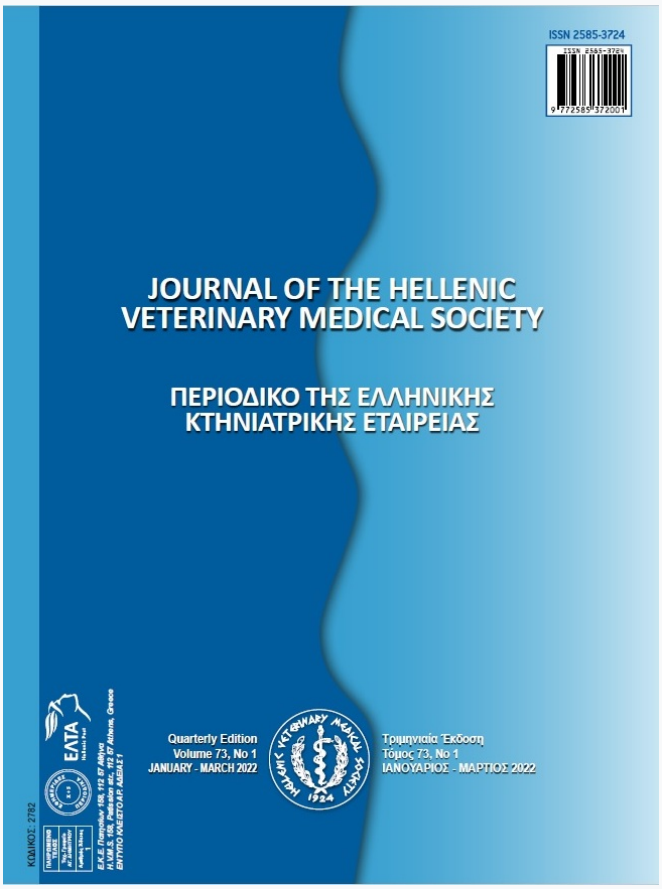

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Honey bee drone (*Apis mellifera*) sperm cryopreservation with rainbow trout seminal plasma supplemented extenders

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ABSTRACT: The current study aimed to investigate the effects of rainbow trout seminal plasma (RTSP) on frozen-thawed honey bee drone spermatozoa. Semen samples were collected from sexually mature drones and pooled. Then pooled semen was diluted with different concentrations of RTSP (2.5%/5% / 10%) and without RTSP (control) supplemented extenders. Motility, plasma membrane functionality, acrosome integrity, and mitochondrial function were negatively affected by the cryopreservation process but DNA integrity was not affected. Membrane lipid peroxidation status was also analyzed using the malondialdehyde (MDA) concentration at frozen-thawed. RTSP10 groups had a positive effect on sperm motility (64.00 ± 3.38%), plasma membrane functionality (72.07 ± 2.12%), acrosome integrity (86.20 ± 2.11%), and mitochondrial function (69.33 ± 2.94%) compared to the control group ($P< 0.05$). The study shows that RTSP supplemented extenders have beneficial effects on at frozen-thawed drone sperm parameters. The results of the present study demonstrated the advantage of using a 10% RTSP supplemented extender for drone sperm motility, plasma membrane functionality, and mitochondrial function.

Keywords: Drone semen, Rainbow trout, Seminal Plasma, Cryopreservation

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INTRODUCTION

The spermatozoon is one of the first living cells to be successfully cryopreserved (Benson et al., 2012). Cryopreservation of sperm without losing its fertilization feature contributes to the selection and protection of genelines with superior genetic characteristics (Nur et al., 2010). Achieving acceptable fertility results by freezing spermatozoa is under the influence of some important factors. These include freezing techniques, appropriate extenders, freezing and thawing process, species-specific spermatozoon physiology, and characteristics (Curry, 2000). Also, the freezing of sperm cells belonging to different species is privileged due to the different size, shape, and lipid components of the cells (Benson et al., 2012, Ustuner et al., 2016).Dronesemen has been successfully cryopreserved in recent years (Taylor et al., 2009, Hopkinset al., 2010, Wegener et al., 2014, Alcayet al., 2019b, Alcayet al., 2019a). However, the cryopreservation process may cause structural, biochemical, and functional damage in spermatozoa, leading to premature activation of spermatozoa against physiological stimulation in the female genital tract, and a decrease in sperm motility, vitality, plasma membrane integrity, acrosomal integrity, and fertility. These undesirable effects may cause an irreversible decrease in sperm parameters (Nur et al., 2010). Therefore, the success of the sperm cryopreservation process depends on the composition of the extenders which is a crucial factor to protect spermatozoon (Ustuner et al., 2016, Alcay et al., 2020).

Seminal plasma which contains biochemicals components regulates sperm function (Ciereszko et al., 2000, Glogowski et al., 2000). The seminal plasma has been used in the semen extenders for sperm cryopreservation of different species (Gunay et al., 2006, Ustuner et al., 2016, Alcay et al., 2020). Rainbow trout seminal plasma (RTSP) includes proteins (2.1 ± 0.3 mg mL), monosaccharides, aminoacids, free fatty acids, phospholipids, vitamins, and neutral lipids (Shaliutina-Kolešová et al., 2016, Ciereszko et al., 2000). Besides, RTSP supplemented extenders have been successfully used for ram and goat semen cryopreservation (Ustuner et al., 2016, Alcay et al., 2020). However, its effect on drone sperm cryopreservation has not been evaluated until now.

The cryopreservation of drone spermatozoa without losing its ability of fertilization contributes to the conservation of genelines. We hypothesized that RTSP supplementation in the semen extender could improve the frozen-thawed drone sperm viability and its longevity. Hence, the current study was designed to compare various concentrations of RTSP supplemented extenders for the cryopreservation of drone sperm using quality tests.

MATERIALS AND METHODS

This study was planned to research the efficiency of RTS supplementary extenders for cryopreservation of drone semen.To this end, extenders supplemented with various RTS concentrations (non-RTS, RTS 2.5%, RTSP 5%, and RTSP10%) were used to evaluate the frozen-thawed quality of drone spermatozoa.

Chemicals: The chemicals used in the study were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Extender preparation

According to the experimental design, various RTSP concentrations were added in certain proportions to each extender group.We prepared the experimental groups such as RTSP2, 5 (with 2.5% RTS), RTSP5 (with 5% RTSP), RTSP10 (with 10% RTSP), and control (non-RTS). Extenders contained Na Citrate (82.21 mmol), Catalase (1.59 mmol), KCI (5.34 mmol), NaHCO3 (24.87 mmol), Amoxicillin (0.82 mmol), and DMSO (10%). The pH value of the diluents prepared was scaled to be 8.1.

RTSP were obtained by the previously described method of Glogowski et al. (2000) and stored at −20 ºC for up to one monthbefore using for drone sperm cryopreservation.

Collection and dilution of semen

Healthy and strong honey bee colonies in Bursa Uludag University, Beekeeping Development-Application and Research Center were used for semen collection.

Sexually mature drones (16 days and older) were selected for semen collection and at least five colonies were used for this research. The pressure was applied to the thorax to induce ejaculation, and then the abdominal area was gently squeezed. Approximately 1 µl semen was collected from per drone using the Schley syringe under a stereomicroscope. Besides, to eliminate individual differences all semen was pooled. The volume of each pooled semen was portioned into four equal volumes, and a total of five pooled semen was used in the study. Each group of the extender was

individually diluted with control or RTS supplemented extenders to a final concentration of about 150x10^6 (spermatozoa / mL).

Semen freezing and thawing

The method of cryopreservation and thawing was based on (Alcay et al., 2019b).According to this method, equilibrated drone sperm was filled into 0.25 mL straws.After the filling process, straws were frozen at 3°C/min from +5°C to -8°C and at 15°C/min from -8°C to -120°C in liquid nitrogen vapor using the Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France).Then the sperm-filled straws were immersed in liquid nitrogen and then stored in a liquid nitrogen container.

Semen evaluation

In the evaluation of frozen-thawed semen, plasma membrane integrity, acrosome integrity, sperm motility, and DNA integrity parameters were examined. A hypoosmotic swelling test (HOST) was used for plasma membrane functionality. FITC-Pisum sativum agglutinin (PSA-FITC) was used for acrosome integrity. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) wasused to assess DNA integrity. Evaluations were made by the same person during the study.

Motility

Drone semen motility assessment was performed using a phase-contrast microscope at 400x magnification (Olympus BX51-TF - OlympusOptical Co., Ltd., Japan) with the slide heated to 37 ° C.

Membrane functionality

For the assessment of the plasma membrane integrity, the hypoosmotic swelling test method was performed according to Alcayet al. (2019b). Following this method, the membrane integrity of the drone sperm was evaluated by observing the frizzled tails.

Acrosome Integrity

For this evaluation, a sample of 10 µl spermatozoa was added in 100 mL of PBS and centrifuged for 5 minutes. The sperm pellet obtained after centrifugation was resuspended with 100 mL PBS. Afterwards, smeared-slides were prepared and dried. After the drying process is over, the smears were left in acetone fixation at 4 °C for 15 minutes in a glass chalet (vertical, Hellendahl type).After fixation, smears were stained with FITC PSA solution for 1 hour at 37 ° C in a light-proof sample kit. After the staining process was completed, at least 200 drone spermatozoa emitting fluorescent light were evaluated under a fluorescent attachment microscope (Alcay et al., 2019a).

Mitochondrial Activity

Double fluorescent stains, PI, and Rhodamine (R123), was used to examine the mitochondrial integrity. For this analysis, Fraseret al. (2002) method was used. The results are expressed as a percentage (%).

DNA fragmentation

DNA fragmentation was evaluated by the TUNEL technique using In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications (Wegener et al., 2014).

Malondialdehyde (MDA) concentrations

To assess the MDA concentrations the method of Sharafi et al. (2015) was performed. Briefly, 0.25 ml of diluted semen sample was treated with 0.25 ml of cold 20% (w/v) trichloroaceticacid to precipitate the protein. During the centrifugation, the precipitated protein was pelleted and the supernatant was incubated with (w/v) thiobarbituric acid for 10 minutes in a 100° C boiling water bath. After the incubation in the hot water bath, the sample was allowed to cool. Absorbance was determined using the Spectrophotometer (Mannheim Boehringer Photometer 4010). MDA concentrations were expressed as nmol/ml.

Statistical analysis

All of the data obtained in the study were analyzed using SPSS (SPSS 23.0 for Windows; SPSS, Chicago, IL, USA) and the results were represented as mean ± standard deviation. ShapiroWilk test was used as a normality test. Semen parameters were analyzed using one-way ANOVA followed by Tukey.

RESULTS

In the study, the percentages of motility, plasma membrane functional integrity, acrosome integrity, mitochondrial function, and DNA fragmentation rates of pooled semensamples were 87.00±4.47, 91.40±1.14, 94.00±1.58, 92.20±2.28, and 0.20±0.45 respectively. Spermatozoa quality was negatively affected by the cryopreservation process compared with the fresh pooled semen ($P < 0.05$). The table 1 shows the effects of different concentrations of RTSP on drone sperm parameters at frozen-thawed.

Table 1. The mean of studied sperm post-thawing parameters on different extender groups

Variable	RTSP Concentrations (%)			
	0	2.5	5	10
Motility (%)	52.00±3.68[a]	53.3±2.44[a]	59.67±4.42[b]	64.00±3.38[c]
HOST (%)	62.93±3.06[a]	65.73±3.55[b]	68.53±1.92[c]	72.07±2.12[d]
Acrosome integrity (%)	82.73±2.43[a]	83.00±3.25[a]	84.60±2.85[ab]	86.20±2.11[b]
Mitochondrial function (%)	59.73±2.71[a]	64.53±2.70[b]	66.27±2.63[b]	69.33±2.94[c]
DNA fragmentation (%)	0.71±0.99	0.53±0.74	0.60±0.74	0.53±0.74
MDA level (nmol/ml)	2.78±0.83	2.44±0.73	2.22±0.66	2.33±0.50

Data is presented in Mean± S.D.
Different letters within the same rows show significant differences among the groups ($p<0.05$).

The sperm motility was higher in RTSP5 and RTSP10, compared to control group ($P<0.05$). Also, the highest percentage of motility rates were obtained from the RTSP10 group ($P < 0.05$).The percentages of plasma membrane functional integrity were higher in RTSP compared to the control group ($p<0.05$). Besides, the best membrane integrity rate was obtained in the RTSP10 group ($P < 0.05$).

The better acrosome integrity was obtained in the RTSP10 group compared to the control and RTSP2.5 groups ($P<0.05$). The percentage of acrosome integrity wasnot found significantly different between RTSP5 and RTSP10 groups ($P > 0.05$).A better mitochondrial function rate was obtained in the RTSP groups compared with the control group ($p<0.05$). No significant differences were observed in sperm DNA damage between all groups ($p>0.05$). As shown in Table 1, it was found that the MDA levels were not significantly different between all groups ($P>0.05$).

DISCUSSION

Sperm freezing ensures that genetic material is preserved for a long time. However, it is known that the freezing-thawing process has negative effects on the fertilization ability of spermatozoa. These undesirable effects decrease motility, viability, plasma membrane, and acrosome integrities of spermatozoa (Nur et al., 2010, Alcay et al., 2019).In the study, we evaluated the effect of exogenous addition of RTSP in extenders on drone sperm quality at frozen-thawed.

Motility is one of the main sperm evaluation parameters and has an important effect on oocyte penetration (Yániz et al., 2020). In this study, we have shown that the presence of RTSP concentrations in cryopreservation media increased drone sperm motility compared to the control group at frozen-thawed ($P<0.05$). The motility values of drone spermatozoa cryopreserved with various semen extenders ranged between 25% - 62% (Alcay et al., 2015, Alcay et al., 2019b, Alcay et al., 2019a, Wegener et al; 2014, Wegener et al., 2012). Our study shows that frozen-thawed sperm motility rates in high dose RTSP groups (RTSP5 and RTSP10) were in good agreement with the findings of these studies. In our study, although RTSP supplementation caused a clear increase in motility, the RTSP2.5 group had not sufficient effect to make a statistical difference compared with the control group. When the RTSP doses were compared among each other, increasing doses of RTSP caused a gradual increase in motility.

The functional integrity of the plasma membrane that is essential for spermatozoon metabolism plays a crucial role in the oocyte fusion of spermatozoon (Yániz et al., 2013). However, cold shock and lipid peroxidation have negatively affect membrane permeability and integrity during cryopreservation (El-Kon et al., 2011). Therefore, it is crucial to keep integrity during the cryopreservation process to avoid cellular damage. HOST is the optimized test for detecting the subtle changes of spermatozoon membrane functionality (Maxwell et al., 1993, Nur et al., 2010). In the study, the plasma membrane functional integrity values in the RTSP10 group were higher than in the other groups ($P < 0.05$). The HOST values are in agreement with the earlier researches (Alcay et al., 2015, Alcay et al., 2019a, Alcay et al., 2019b).

The defected acrosome is one of the adverse effects of the sperm cryopreservation process (Ustuner et al., 2016, Nur et al., 2010). During oviposition, the queen releases few spermatozoa from the spermatheca, and then the acrosome reaction releases lytic enzymes that aid in the penetration of the vitelline membrane to fertilize the egg. Therefore, acrosome integrity is crucial for the fertility of frozen-thawed spermatozoa. In the study, there was no statistical difference among RTSP groups (RTSP2.5 and RTSP5) and the control group

for acrosome integrity rates. RTSP10 group preserved acrosome integrity better thanRTSP2.5 and control groups. These results are in agreement with the previous research (Alcay et al., 2019a, Alcayet al., 2019b, Nur et al., 2020).

Sperm needs the energy to carry out its functions and it can mostly obtain ATP through the glycolytic and oxidative phosphorylation pathways (Storey et al., 2008, Moscatelle et al., 2017). Mitochondria play an essential role in regulating sperm function (Yániz et al., 2020). Therefore, it is important to evaluate the mitochondrial function for sperm quality. In the study, mitochondrial function was better preserved in RTSP groups compared to the control group at frozen-thawed ($P<0.05$). Also, high dose RTSP preserved mitochondrial function compared to the other groups. Similar results were obtained in previous research (Ciereszko et al., 2017)

Protecting the integrity of DNA during freezing also has great importance not to disrupt the early development of the embryo (Nur et al., 2010). In this study, it was observed that drone semen was resistant to freezing-thawing process. No significant differences of DNA fragmentation were noticed between all groups ($P>0.05$). Similar result was obtained in previous research (Wegener et al., 2014). Oxidative damage may be evaluated by MDA levels which is a key product of polyunsaturatedfatty acid's peroxidation in thecells. In our study, no statistical differences were obtained for MDA levels in all groups ($P>0.05$). Catalase which was used in the main extenders for all groups might have been a positive effect against oxidative damage.

Considering all sperm parameters; the RTSP10 group was the optimum for drone semen preservation. Future studies might be focusing on RTSP supplementation to improve the cryopreservation values and to evaluate reproductive success (viable offspring) when used to fertilize the queens.

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CONFLICT OF INTEREST

None declared.

REFERENCES

Alcay, S., Cakmak, S., Cakmak, I., Mulkpinar, E., Gokce, E., Ustuner, B., Sen, H., Nur, Z. (2019a). Successful cryopreservation of honey bee drone spermatozoa with royal jelly supplemented extenders. *Cryobiology,* 87, 28-31. https://doi.org/10.1016/j.cryobiol.2019.03.005.

Alcay, S., Ustuner, B., Aktar, A., Mulkpinar, E., Duman, M., Akkasoglu, M., Cetinkaya, M., (2020). Goat semen cryopreservation with rainbow trout seminal plasma supplemented lecithin-based extenders. *Andrologia,* Vol. 52. https://doi.org/10.1111/and.13555.

Alcay, S., Çakmak, S., Çakmak, İ., Mülkpınar, E., Toker, M. B., Üstüner, B., Şen, H., Nur, Z. (2019b). Drone Semen Cryopreservation with Protein Supplemented TL-Hepes Based Extender.*Kafkas Univ Vet Fak Derg.,* 25 (4), 553-557. DOI: 10.9775/kvfd.2018.21311.

Alcay, S., Üstüner, B., Çakmak, İ., Çakmak, S., Nur, Z. (2015). Effects of various cryoprotective agents on post-thaw drone semen quality. *Kafkas Univ. Vet. Fak. Derg.,* 21 (1), 31-35. DOI: 10.9775/kvfd.2014.11515

Benson, J. D., Woods, E. J., Walters, E. M., Critser, J. K. (2012). The cryobiology of spermatozoa. *Theriogenology,* 78, 1682-1699. https://doi.org/10.1016/j.theriogenology.2012.06.007

Ciereszko, A., Wilde, J., Dietrich, G. J., Siuda, M., Bak, B., Judycka, S., Karol, H. (2017). Sperm parameters of honeybee drones exposed to imidacloprid. Apidologie, 48, 211-222. https://doi.org/10.1007/s13592-016-0466-2.

Ciereszko, A., Glogowski, J., Dabrowski, K. (2000). Biochemical characteristics of seminal plasma and spermatozoa of freshwater fishes. T.R. Tiersch, P.M. Mazik (Eds.), Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, Louisiana, 20-48.

Curry, M. R. (2000). Cryopreservation of semen from domestic livestock. *Reviews of Reproduction*, 5, 46-52. https://doi:10.1530/ror.0.0050046.

El-Kon I., (2011). Testing usability of bovine serum albumin (BSA) for preservation of Egyptian Buffalo Semen. Environ. Sc., 11, 495-502.

Fraser, L., Lecewicz, M., & Strzezek, J. (2002). Fluorometric assessments of viability and mitochondrial status of boar spermatozoa following liquid storage. *Polish Journal of Veterinary Sciences*, 5, 85-92.

Glogowski, J., Kwaoenik, M., Piros, B., Dabrowski, K., Goryczko, K., Dobosz, S., & Ciereszko, A. (2000). Characterization of rainbow trout milt collected with a catheter: Semen parameters and cryopreservation success. *Aquaculture Research,* 31, 289-296. https://doi.org/10.1046/j.1365-2109.2000.00400.x

Gunay, U., Dogan, I., Nur, Z., Manolov, I., Sagırkaya, H., Soylu, M.K., Kaptan, C., Akpınar, L. (2006). Influence of bull seminal plasma on post-thaw ram semen parameters and fertility. *Bull. Vet. Inst. Pulawy*, 50, 503-507.

Hopkins, B. K., Herr, C. (2010). Factors affecting the successful cryopreservation of honey bee (Apis mellifera) spermatozoa. *Apidologie,* 41, 548-556.https://doi.org/10.1051/apido/20010006.

Maxwell, W. M., Salamon, S. (1993). Liquid storage of ram semen: a review. Reprod Fertil Dev., 5 (6), 613-638. https://doi.org/10.1071/RD9930613

Moscatelli, N., Spagnolo, B., Pisanello, M., Lemma, E. D., De Vittorio, M., Zara, V., Pisanello, F., Ferramosca, A. (2017). Single-cell-based evaluation of sperm progressive motility via fluorescent assessment of mitochondria membrane potential. Sci. Rep., 7, 1-10. https://doi.org/10.1038/s41598-017-18123-1.

Nur, Z., Cakmak, S., Cakmak, I., Onder, N. T., Gokce, E., Ustuner, B., Alcay, S., Toker, M. B., Soylu, M. K. (2020). Effect of trehalose supplementation on post-thaw sperm quality of honey bee drones. Journal of Animal and Feed Research, 10 (5), 191-196. https://dx.doi.org/10.51227/ojafr.2020.27

Nur, Z., Zik, B., Ustuner, B., Sagirkaya, H., & Ozguden, C. G. (2010). Effects of different cryoprotective agents on ram sperm morphology and DNA integrity. Theriogenology, 73 (9), 1267-1275. https://doi.org/10.1016/j.theriogenology.2009.12.007

Shaliutina-Kolešová, A., Kotas, P., Štěrba, J., Rodina, M., Dzyuba, B.,

Cosson, J., Linhart, O. (2016). Protein profile of seminal plasma and functionality of spermatozoa during the reproductive season in the common carp (Cyprinus carpio) and rainbow trout (Oncorhynchus mykiss).*Mol Reprod Dev.,* 83 (11), 968-982. https://doi.org/10.1002/mrd.22737

Sharafi, M., Zhandi, M., Shahverdi, A., Shakeri, M. (2015). Beneficial effects of nitric oxide induced mild oxidative stress on post-thawed bull semen quality. International Journal Of Fertility & Sterility, 9, 230-237. 10.22074/IJFS.2015.4244.

Storey, B.T. (2008). Mammalian sperm metabolism: Oxygen and sugar, friend and foe. Int. J. Dev. Biol., 52, 427-437. https://doi.org/10.1387/ijdb.072522bs.

Taylor, M.A., Novoa, E. G., Morfin, N., Buhr. M. M. (2009). Improving viability of cryopreserved honey bee (Apis mellifera L.) sperm with selected diluents, cryoprotectants, and semen dilution ratios. *Theriogenology,* 72, 149-159. https://doi: 10.1016/j.theriogenology.2009.02.012.

Ustuner, B., Alcay, S., Toker, M. B., Nur, Z., Gokce, E., Sonat, F.A, . Gul, Z., Duman, M., Ceniz, C., Uslu, A., Sagirkaya, H., Soylu, M.K. (2016). Effect of rainbow trout (Oncorhynchus mykiss) seminal plasma on the post-thaw quality of ram semen cryopreserved in a soybean lecithin-based or egg yolk-based extender. *Anim Reprod Sci,* 164, 97-104. https://doi.org/10.1016/j.anireprosci.2015.11.017

Wegener, J., May, T., Kamp, G., Bienefeld, K. A succesfull new approach to honeybee semen cryopreservation. (2014). Cryobiology, 69 (2), 236-242. https://doi:10.1016/j.cryobiol.2014.07.011.

Wegener, J., May, T., Knollmann U., Kamp G., Müller K., Bienefeld K. (2012). In vivo validation of in vitro quality tests for cryopreserved honey bee semen. Cryobiology, 65 (2), 126-131. https://doi.org/10.1016/j.cryobiol.2012.04.010.

Yániz, J. L., Silvestre, M. A., Santolaria, Pilar. (2020). Sperm Quality Assessment in Honey Bee Drones.Biology, 9, 174. https://doi.org/10.3390/biology9070174.

Yániz, J.L., Palacín, I., Vicente-Fiel, S., Gosálvez, J., López-Fernández, C., Santolaria, P. (2013). Comparison of membrane-permeant fluorescent probes for sperm viability assessment in the ram. Reprod. Domest. Anim., 48, 598-603. https://doi.org/10.1111/rda.12132.