

## Effect of egg yolk on sperm cryopreservation of drone (*Apis mellifera*)

Efecto de la yema de huevo sobre la criopreservación espermática de zángano (*Apis mellifera*)

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### ABSTRACT

The aim of this study was to evaluate the effect of whole yolk (WEY) and centrifuged yolk (CEY) and its interaction with dimethylsulfoxide (DMSO) and glycerol, on the cryopreservation of drone semen. Six aliquots of semen were obtained, which were divided into four treatments: T1 (CEY+ glycerol), T2 (CEY + DMSO), T3 (WEY + glycerol) and T4 (WEY + DMSO). The diluted samples were evaluated after 10 minutes of incubation at 37 °C and after thawing. Motility was evaluated subjectively (scale 4 to 0), membrane integrity (Host test) and viability (IP/ SYBR-14). The results were analyzed with ANOVA. At the time of dilution, T1 was the highest percentage of motility, viability and integrity of the membrane presented (grade 4, 95.75% ± 1.20, 89.75% ± 0.42, respectively), after thawing the T2 was the highest percentage presented (grade 2, 59.10% ± 1.44, 84.35% ± 0.72, respectively). The results showed that T1 was the one that obtained the best sperm parameters at the time of dilution, while, after thawing, T2 was the diluent that best cryopreserved sperm samples from drone.

**Keywords:** Egg yolk, cryopreservation, semen, drone.

### RESUMEN

El objetivo del presente estudio fue evaluar el efecto de la yema de huevo entera (YHE) y centrifugada (YHC) y su interacción con dimetilsulfóxido (DMSO) y glicerol, sobre la criopreservación del semen de zángano. Se obtuvieron seis alícuotas de semen, las que se dividieron en cuatro tratamientos: T1 (YHC + glicerol), T2 (YHC + DMSO), T3 (YHE + glicerol) y T4 (YHE + DMSO). Las muestras diluidas se evaluaron después de 10 minutos de incubación a 37 °C y pos descongelación. La motilidad se evaluó subjetivamente (escala 4 a 0), la integridad de la membrana (test de Host) y la viabilidad (IP/SYBR-14). Los resultados se analizaron con un ANDEVA. Al momento de la dilución, el T1 fue el que mayor porcentaje de motilidad, viabilidad e integridad de la membrana presentó (grado 4, 95.75% ± 1.20, 89.75% ± 0.42, respectivamente), pos-descongelación el T2 fue el que mayor porcentaje presento (grado 2, 59.10% ± 1.44, 84.35% ± 0.72, respectivamente). Los resultados demostraron que el T1 fue el que obtuvo los mejores parámetros espermáticos al momento de la dilución, mientras que, pos descongelación el T2 fue el diluyente que mejor criopreservó las muestras espermáticas de zángano.

**Palabras clave:** Yema de huevo, criopreservación, semen, zángano.

## INTRODUCTION

The bee *Apis mellifera* is the main pollinator species used by man to increase crop productivity, and plays an important role in maintaining biodiversity. In recent years, the beekeeping sector has been seriously threatened by reported hive losses, raising concern about the genetic diversity of honey bee populations (Cobey *et al.*, 2012). It is believed that these losses are due to a multifactorial phenomenon, such as climate change, insecticides, pathogens, viral diseases, among others (Tentcheva *et al.*, 2004, van Angeldorp *et al.*, 2010, Guzmán-Novoa *et al.*, 2010, Neumann and Carreck, 2015).

In this sense, one of the alternatives to preserve the bees would be the cryopreservation of sperm cells of drones in liquid nitrogen (NL<sub>2</sub>), for later use in instrumental insemination techniques. In this way, long-term genetic material can be conserved and its availability can be used at any time of the year (Hopkins and Herr, 2010); nevertheless, the cryopreservation of sperm cells of drone presents low percentages of cryosurvival (Taylor *et al.*, 2009), negatively affecting the production of instrumentally inseminated queen offspring, achieving a low fertility rate of 47 %, with respect to the percentage with Fresh semen that is between 95-99 % (Hopkins and Herr, 2010). To minimize the negative effects that occur during and after cryopreservation, various solutions have been studied; among them, cryoprotectants such as glycerol, dimethylsulfoxide, ethylene glycol and dimethyl formamide; which prevent the formation of ice crystals and protect sperm (Nouri *et al.*, 2013, Medeiros *et al.*, 2002). However, among the cryoprotectants most used in freezing drone semen are Dimethylsulfoxide (DMSO) and glycerol. In the same way, infinity of components that are added to the freezing diluent have been evaluated; among them milk, glycine, glycerin, egg yolk, coconut water and soy lecithin. The purpose of these components is to preserve the functional characteristics of sperm cells, maintain the appropriate level of fertility and protect them from injuries caused by thermal shock (Stornelli *et al.*, 2005; Dadkhah *et al.*, 2016; Wegener and Bienefeld, 2012).

Egg yolk is currently one of the main components used in most semen cryopreservation diluents in almost all species (Cabrera *et al.*, 2005, Kulaksiz *et al.*, 2010), as it exerts a protective effect on sperm membranes undergoing cryopreservation processes (Fernández-Santos *et al.*, 2006; Briand-Amirat *et al.*, 2007). This protective effect is due to the contribution of low density lipoproteins (LBD) composed of triglycerides, phospholipids and cholesterol (Moussa *et al.*, 2002).

LBD prevents the formation of ice crystals and protects the integrity of the plasma membrane from cold shock (Hu *et al.*, 2010; Jian-Hong *et al.*, 2011). Likewise, it has been documented in different studies that the elimination of high density lipoproteins (LAD) improves semen quality, before and after freezing (Watson and Martin, 1975; Aboagla and Terada, 2004; Fernández-Santos *et al.*, 2006; Briand-Amirat *et al.*, 2007). Due to this, the centrifugation of the egg yolk has been used, with the purpose of eliminating some of its components and preserving mainly the LBD, which have allowed to improve the

freezing of the semen of several species (Fernández-Santos *et al.*, 2006; Briand-Amirat *et al.*, 2007; Pillet *et al.*, 2011; Nouri *et al.*, 2013). In drones, it has been documented that when the cryopreservation diluent contains whole egg yolk, the fertility of the inseminated queen is affected with a decrease in the number of eggs deposited in the cells (Hopkins *et al.*, 2011). It also represents a risk to the fertility of inseminated queen bees, since the egg yolk particles can clog the sperm oviducts and cause an infection (Hopkins *et al.*, 2012).

For all the above, the objective of the present study was to evaluate the effect of the whole and centrifuged egg yolk and its interactions with two types of cryoprotectants (DMSO and glycerol), on the cryopreservation of drone semen.

## MATERIAL AND METHODS

**Location and characteristics of the study area.** The semen collection was carried out at the National Institute of Forestry, Agricultural and Fisheries Research (INIFAP), located in the municipality of Mocochoá, Yucatán. Semen samples were evaluated in the laboratory of the Sheep Selection and Reproduction Center (CeSyRO) of the Technological Institute of Conkal.

**Capture and manipulation of drones.** The capture of the drones was carried out by means of exclusion grids, which were placed in front of the hole to avoid the entrance of the drones to the hive and thus facilitate their capture. These grilles were placed approximately before noon, because the departure of the drones for their tracking flights was ensured, so the capture was made at 5:00 p.m. on the same day, ensuring their return. The purpose of this technique is to obtain adult individuals approximately 16 days old (mature drone). Once the drones were captured, they were placed in 15x15x5 cm conveyor cages; which were filled to 35% capacity, so that the drones could move. Subsequently the drones were sheltered in orphan hives, in order to keep them fed throughout the night. The next day the drones were released in flight cages of 40x40 cm, for ten minutes, to encourage defecation and thereby reduce contamination of the samples; as well as to ensure the filling of their air sacs to obtain a better endothelial eversion (Taylor *et al.*, 2009).

**Semen Collection.** The technique described by Laidlaw (1977) was used, which involves manually forcing the partial and total erosion of the endophile, exerting pressure on the thorax and abdomen. Once the semen was detected in the endophylaus, it was collected by releasing a drop of saline solution over the exposed semen and with the help of a Harbo Schley syringe (ref. 104), and a stereoscopic magnifying glass model SMZ zoom Schley (ref. 2.00) and the semen was extracted. Semen was obtained in all drones, until a capillary tube with a capacity of 100 µl was filled, to be subsequently cryopreserved.

**Egg Yolk Processing.** To obtain the whole egg yolk, the egg was first sterilized with 70% alcohol, then the egg was opened carefully, pouring the yolk without breaking it into a shatter, to completely release the yolk from the egg white. To remove the membranes from the yolk, it was placed on a filter paper, making it roll to retain the remains and thus be able to extract the egg yolk with a syringe. In the case of the centrifuged yolk, the procedure was the same as that of the whole yolk, only 20 ml of distilled water was added to 20 ml of whole egg yolk and then centrifuged at 10,000 xg/20min, and finally, the supernatant was obtained from the yolk obtained by centrifugation (LBD), and the pellet that formed at the bottom of the tube was discarded (LAD).

**Thinners.** Four diluents were tested according to the method described by Harbo (1983). The diluents were prepared with minor modifications, so that T1 was constituted with: 25% glycerol, 25% CEY T2: 25% DMSO, 25% CEY, T3: 25% glycerol, 25% WEY and T4: 25% DMSO, 25% WEY. Each of the diluents were suspended with 50% Buffer solution, with 2.27g w/v Tris at 375mm; 0.369g p/v of citric acid at 124 mm, 7.28g p/v glucose at 41 mm and 0.025g p/v streptomycin, with a pH of 7.0.

**Sample Preparation.** Semen samples were diluted with their respective treatment at a 3: 2 concentration. Subsequently, an aliquot of diluted semen (20  $\mu$ l) was left for fresh evaluation and the rest was used for freezing.

**Evaluation of seminal quality in fresh and thawed.** The variables that were evaluated in the fresh semen were: the concentration, motility, viability and integrity of the sperm membrane. Upon defrosting, the variables evaluated were the same as fresh, except for sperm concentration that was not evaluated.

**Sperm concentration.** It was estimated by the technique described by Taylor et al., (2009), mixing 2.5  $\mu$ l of pure semen in 497.5  $\mu$ l of distilled water. The sperm count was observed with a Buker cell count chamber, and with a UOB UB203i phase contrast microscope at 400x.

**Sperm motility.** 5  $\mu$ l of fresh semen was deposited in an object holder on a 37 ° C thermal plate; it was subjectively estimated through visual observation, examining at least 5 fields of each sample in a MO-a 400x to 40x optical microscope. The assessment was estimated using the scale described by Locke and Peng (1993), where: 4 indicates more than 50% of sperm, presenting circular motion and progressive movement; 3 indicates presence of circular and progressive movement, and more than 50% with vibratory movement; 2 indicates more than 50% with vibratory motion; 1 indicates less than 50% with vibratory movement and 0 without movement.

**Sperm viability.** It was evaluated according to the technique described by Collins and Donoghue, (1999), with the fluorescence stains of propidium iodide and SYBR-14. 1  $\mu$ l of each fluorochrome was deposited in 100  $\mu$ l of each treatment diluted in saline (PBS), and allowed to incubate in the dark for 5 minutes. Subsequently, 5  $\mu$ l of each stained sample was deposited on an object holder and an object cover, to analyze the samples with a fluorescence microscope. Five fields were analyzed and 100 cells were counted, taking as stained sperm the green stains and the dead ones those that presented red.

**Integrity of the sperm membrane.** It was evaluated by the technique described by Nur *et al.* (2012), so 1.0  $\mu$ l of semen in 250  $\mu$ l of distilled water was added, then incubated at room temperature for 5 min. At the end of the time, 5  $\mu$ l of the sample was placed in an object holder and covers glass objects to count 100 sperm, among which there were coiled (E +) and unrolled (E-) tails.

**Cryopreservation and thawing of sperm samples.** All sperm samples of the treatments were packed in 0.25 ml straws and stored in a container with water at room temperature (37 °C). Then the container with the straws was placed in a refrigerator for 50 minutes, thus causing a gradual decrease in temperature at 5 °C/min. Subsequently the straws were placed at a distance of 4 cm from the surface of the liquid nitrogen for 20 minutes, for freezing and storage in NL2 (Taylor *et al.*, 2009). The sperm samples were thawed by immersion in water at 25 °C for 60 seconds (Taylor *et al.*, 2009).

**Statistical analysis.** Because of the variables showed parametricity, an ANDEVA was carried out and subsequently a Tukey test to determine the differences in means; The Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS, 2011) was used for this.

## RESULTS AND DISCUSSION

In diluted fresh semen, T1 was the one that best preserved motility, presenting 50% of circular mobile sperm and progressive movements (degree 4); then T2 and T3 followed, with circular and progressive movements, and more than 50% of vibratory movements (degree 3); and the T4 that was the one that presented less than 50% of vibratory movements (degree 1). Difference was observed between T4 and the other treatments ( $p < 0.05$ ) (table 1). These results are similar to those reported by Lensky and Schindler (1967) with fresh drone semen.

In the defrosted semen it was observed that T2 and T3 were the ones that obtained the greatest motility, with more than 50% of vibratory movements (degree 2); while T1 and T4 ( $p > 0.05$ ) were the ones with the lowest motility with less than 50% of movements (degree) (table 2). A significant reduction in motility was observed before and after the freezing process ( $p < 0.05$ ). These parameters evaluated are difficult to compare with other studies,

since they only describe the presence or absence of movement, and do not mention the type of movement (Hopkins and Herr, 2010; Taylor *et al.*, 2009).

Apparently the whole or centrifuged egg yolk and its interaction with glycerol or DMSO, if they act differently on the protection of drone sperm undergoing freezing; Therefore, it is necessary to find the best diluent that conserves or improves sperm motility after cryopreservation, since it plays an important role in the migration of sperm to the lateral sperm oviducts (Collins, 2000), and even It has been described that flagellar movement of sperm can increase the reproducibility of inseminated queens (Tofilski, 2014). Normally the production of working pups in queen bees inseminated with fresh semen is approximately 95 to 99%, while with defrosted semen less than 50% of worker bees are produced; observing a greater number of emerging drones (unfertilized eggs) (Hopkins *et al.*, 2012).

The sperm viability of the diluted fresh semen was higher in T1 ( $95.75 \pm 1.20$ ) compared to T3 ( $93.58\% \pm 0.57$ ), T2 ( $82.75 \pm 1.93$ ) and T4 ( $38.83 \pm 3.00$ ). No differences were found between T1 and T3 ( $p > 0.05$ ) (Figure 2). These results are similar to those reported by Nur *et al.* (2012), Collins and Pettis (2001) and Collins (2004) in fresh drone semen.

Upon defrosting, the viability was higher in T2 ( $59.10\% \pm 1.44$ ) compared to T3 ( $55.90\% \pm 0.98$ ), T1 ( $44.40\% \pm 0.66$ ) and T4 ( $33.65\% \pm 0.73$ ). No differences were found between T1 and T3 ( $p > 0.05$ ), nor between T2 and T3 ( $p > 0.05$ ) (table 2). A significant reduction in sperm viability was observed before and after the freezing process ( $p < 0.05$ ). These results are inferior to those reported after defrosting by Nur *et al.* (2012) with 87.2% and Taylor *et al.* (2009) with 68.3%.

The integrity of the sperm membrane in the diluted fresh semen samples was higher in T1 ( $89.75\% \pm 0.42$ ), compared to T3 ( $84.00\% \pm 1.08$ ), T2 ( $80.12 \pm 0.29$ ) and T4 ( $75.65 \pm 0.25$ ). Only T4 had differences with the other treatments ( $p < 0.05$ ) (table 1). The results are similar reported by Nur *et al.* (2012) with 92.2%.

Upon thawing, there was a greater integrity of the sperm membrane, in the samples where T2 was added ( $84.35\% \pm 0.72$ ); in comparison with T3 ( $83.25\% \pm 0.11$ ), T1 ( $82.75\% \pm 0.71$ ) and with T4 ( $72.50\% \pm 0.73$ ). No differences were found between T1, T2 and T3 ( $p > 0.05$ ) (figure 2). No differences were found in reducing the integrity of the sperm membrane, before and after the freezing process ( $p > 0.05$ ). It was observed that the tail membranes of the drone sperm are very resistant to the freeze-thaw process; This could be due to the fact that the cell membranes, the sperm of the drone, respond well to the regulation of osmotic changes during freezing and thawing, allowing the entry of cryoprotectants to the membranes and the expulsion of water (Karger *et al.*, 2016; Watson, 2000).

**Table 1. Effect of whole and centrifuged egg yolk on the motility, viability and endosmosis of fresh semen from drones**

Treatment	Motility (0 a 4)	Viability (%)	Host (%)
CEY + glycerol (T1)	4.00 ± 0.00 a	95.75 ± 1.20 a	89.75 ± 0.42 a
CEY + DMSO (T2)	2.33 ± 0.21 b	82.75 ± 1.93 b	80.12 ± 0.29 b
WEY + glycerol (T3)	2.67 ± 0.21 b	93.58 ± 0.57 a	84.00 ± 1.08 b
WEY + DMSO (T4)	2.00 ± 0.00 b	38.83 ± 3.00 c	75.65 ± 0.25 c

CEY: centrifuged egg yolk; WEY: Whole egg yolk; DMSO: dimethylsulfoxide a, b different literals per column indicate a significant statistical difference (p <0.05).

**Table 2. Effect of whole and centrifuged egg yolk on the motility, viability and endosmosis of frozen semen of drones**

Treatment	Motility (0 a 4)	Viability (%)	Host (%)
CEY + glycerol (T1)	1.36 ± 0.12 a	44.40 ± 0.66 a	82.75 ± 0.71 a
CEY + DMSO (T2)	1.83 ± 0.11 b	59.10 ± 1.44 a	84.35 ± 0.72 a
WEY + glycerol (T3)	2.13 ± 0.17 b	55.90 ± 0.98 a	83.25 ± 0.11 a
WEY + DMSO (T4)	1.06 ± 0.45 a	33.65 ± 0.73 b	72.50 ± 0.73 b

CEY: centrifuged egg yolk; WEY: Whole egg yolk; DMSO: dimethylsulfoxide a, b different literals per column indicate a significant statistical difference (p <0.05).

## CONCLUSION

Based on the results obtained in this study, it is concluded that the diluent based on CEY+glycerol (T1) is the most suitable for cryopreserve sperm samples of drone.

## ACKNOWLEDGMENT

To the National Council of Science and Technology (CONACYT), for the funding granted this research through the Basic Science project 164592.

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