# GAMETE CRYOPRESERVATION IN THE RECOVERY PROGRAM OF MEXICAN GRAY WOLF (*Canis lupus baileyi*): RESULTS OF REPRODUCTIVE SEASON OF 2018 IN MEXICO

Raúl Eduardo Piña-Aguilar[1] | Patricia Zúñiga-Sánchez[1] | Rosa María Díaz-Salazar[1] | Claudia González-Ortega[1] | Beatriz Santamaria-Jiménez[1] | Laura Elena Gómez-Montes[2] | Herbe Monrroy-Jacobo[3] | José de Jesús Barroso-Padilla[4] | Alvar Alonso Cruz-Tamayo[5] | Antonio Martín Gutiérrez-Gutiérrez[1]

[1] Instituto de Ciencias en Reproducción Humana (Instituto "Vida"), León, México

[2] Dirección Técnica y de Investigación de la Dirección General de Zoológicos y Vida Silvestre, Secretaria de Medio Ambiente Gobierno Ciudad de México, Ciudad de México, México

[3] Área de investigación y Conservación de Fauna, Comisión Estatal de Parques Naturales y de la Fauna (CEPANAF), Secretaria de Medio Ambiente Gobierno del Estado de México, Toluca, México

[4] Parque Zoológico de León, León, México

[5]Escuela Superior de Ciencias Agropecuarias, Universidad Autónoma de Campeche, Escárcega, Campeche

## Abstract

The Mexican gray wolf (*Canis lupus baileyi*) is a subspecies of gray wolf with unique morphologic, genetic and historical features. The Mexican gray wolf faced near-extinction during the 70s after decades of predator eradication actions. A binational *ex situ* management program between the United States and Mexico, relying primarily on zoos has enabled the slow recovery of the population. According tohe 2017 census, the population included approximately two hundred fifty animals in captivity and more than a hundred that have been returned to the wild. One of the components of the recovery program is cryobanking gonadal tissue: initially sperm, and more recently, ovaries and oocytes. During the reproductive season of 2018 our team cryopreserved sperm obtained by electrojaculation from five males maintained in three facilities (Zoológico San Juan de Aragón, Parque Zoológico de León and Zoológico El Ocotal) and vitrified oocytes and cryopreserved ovarian tissue obtained by ovariohysterectomy from a female maintained at Zoológico de Zacango. Improved methods were introduced, such as the use of a commercial dog sperm extender that enabled superior post-thaw survival and motility rates, the measurement of testosterone

## Relevancia

El desarrollo de tecnologías reproductivas ha permitido la criopreservación de gametos como parte de las acciones de conservación de los animales en cautiverio. El *Species Survival Plan* (SSP) del lobo mexicano es único entre los programas de manejo en zoológicos en hacer recomendaciones anuales sobre animales en quienes se criopreservará esperma, ovocitos y tejido ovárico en la época reproductiva. En este reporte detallamos nuestros resultados y abordaje con la criopreservación de gametos en México durante 2018.

**Revisado:** 18 de julio de 2019; **aceptado:** 12 de agosto de 2019; **publicado:** 15 de agosto de 2019. **Autor de correspondencia:** Raúl E. Piña-Aguilar, rpina.a@hotmail.com

**Cita:** Piña-Aguilar, R.E., P. Zúñiga-Sánchez, R.M. Díaz-Salazar, C. González-Ortega, B. Santamaria-Jiménez, L.E. Gómez-Montes, H. Monrroy-Jacobo, J.J. Barroso-Padilla, A.A. Cruz-Tamayo, A.M. Gutiérrez-Gutiérrez. 2019. Gamete cryopreservation in the recovery program of Mexican gray wolf (*Canis lupus baileyi*): Results of reproductive season of 2018 in Mexico. *Revista Mexicana de Mastozoología, nueva época*, 9(1):72-85. ISSN:2007-4484. www.revmexmastozoologia.unam.mx

and the use of ovary and oocyte cryopreservation techniques based on human procedures. In conclusion, we report the successful cryopreservation of Mexican gray wolf gametes based only in local resources and expertise. Our results will impact the long-standing efforts of the population management program to recover a flagship species of Mexican natural richness, the gray wolf.

**Key words:** Biobank, Oocyte vitrification, ovary cryopreservation, sperm freezing.

## Resumen

El lobo mexicano (*Canis lupus baileyi*) es una subespecie del lobo gris con características morfológicas, genéticas e históricas únicas. El lobo gris mexicano estuvo cerca de la extinción durante los años 70 después de décadas de acciones para la erradicación de depredadores. El programa binacional Estados Unidos-México de manejo *ex situ*, dependiente principalmente de los zoológicos, ha permitido una recuperación lenta. La población al censo de 2017 incluía aproximadamente doscientos cincuenta animales en cautiverio y más de una centena de regreso a la vida libre. Uno de los componentes del programa de recuperación es la criopreservación de tejido gonadal, inicialmente espermatozoides y más recientemente ovario y ovocitos. Durante la temporada reproductiva del 2018 nuestro equipo criopreservó espermatozoides obtenidos por electroeyaculación de cinco machos en tres instituciones (Zoológico San Juan de Aragón, Parque Zoológico de León y Zoológico El Ocotal), vitrificó ovocitos y criopreservó tejido ovárico después de una ovariohisterectomía en una hembra mantenida en el Zoológico de Zacango. Métodos mejorados fueron introducidos, como el uso de un diluyente comercial para perros con mejores resultados en las tasas de viabilidad y motilidad postcongelación, y el uso de técnicas de criopreservación para ovarios y ovocitos basadas en procedimientos para humanos. En conclusión, reportamos la criopreservación exitosa de gametos basados solamente en recursos y conocimiento local. Estos resultados impactarán los esfuerzos de larga duración que se han realizado en el programa de manejo de la población en la recuperación de una especie emblemática de la riqueza natural mexicana como el lobo gris mexicano.

**Palabras clave:** Biobanco, congelación de esperma, criopreservación de ovario, vitrificación de ovocitos.

## Introduction

The Mexican gray wolf (*Canis lupus baileyi*) is one of five subspecies of gray wolves in North America. Historically, this subspecies was distributed across northern and central Mexico and southern United States (US). (Heffelfinger *et al*., 2017). After facing extinction from hunting and poisoning, the last members of the Mexican gray wolf population were captured in Chihuahua and Durango, Mexico, from 1976 to 1980, and these individuals became the initial founders of an *ex situ* recovery program (U.S. Fish and Wildlife Service, 2017). The subspecies was included in the United States Endangered Species Act in 1976 and is currently listed as endangered; in Mexico it is listed as extinct by NOM-059-SEMARNAT-2010. The existing population of Mexican gray wolves today is descended from seven original founders comprising three different lineages: McBride, Ghost Ranch and the Aragon lineage has been genetically validated and incorporated more recently into the recovery program (Siminski, 2017). Interbreeding among the three lineages started in 1995 (U.S. Fish and Wildlife Service, 2017).

The *ex-situ* population management in facilities of Mexico and the US has sought to increase the number or individuals and eventually reintroduce animals to the wild. Reintroductions started in Arizona and New Mexico in 1998 with the establishment of the Mexican Wolf Experimental Population Area (Blue Free) of Arizona and New Mexico, and animals have been living stably in the wild since the initial introductions (Siminski, 2017). Reintroductions in Mexico started in 2011 and the first Mexican gray wolf pup was born in the wild in 2014 (Lara-Díaz *et al*., 2015). A small population currently lives in northern Mexico at Sierra Madre Occidental in the states of Sonora and Chihuahua. In 2017, at the time of publication of the breeding plan (July, 2017), the total number of wolves in the US and Mexico was 281: 138 males, 141 females and 2 unsexed individuals. 245 wolves were included in the management program and were distributed among 55 institutions, 19 of which were located in Mexico (Siminski and Spevak, 2017). The size of the population in management is lower than the goal of 300 individuals recommended by American Zoological Association (AZA) Canid Taxon Advisory Group. The main limiting resource in captive breeding is space in the participating institutions; as a result, during the 2018 reproductive season only 31 breeding pairs were recommended (Siminski and Spevak, 2017). As of the 2016 end-of-year census, it was estimated that

there are approximately 113 wolves in the wild in the US and 31 in Mexico (Siminski, 2017; U.S. Fish and Wildlife Service, 2017).

Assisted reproduction techniques are well-established for treatment of infertility and fertility preservation in humans and for intensive breeding in domestic animals. Some of these techniques have also been used effectively as part of conservation programs to maintain genetic diversity in wild populations. Maintaining genetic diversity is important because reproductive fitness is associated with the genetic health of a wild population. In the Mexican gray wolf, sperm quality has been reported to negatively correlate with level of inbreeding (Asa *et al*., 2007). Sperm cryopreservation is one of the most accessible assisted reproduction techniques and sperm cryobanking and artificial insemination have been used successfully to maintain genetic diversity in highly endangered species, such as the panda (*Ailuropoda melanoleuca*), black-footed ferret (*Mustela nigripes*) and scimitar oryx (*Oryx dammah*; Comizoli *et al*., 2018). In the case of black-footed ferret, a carnivore with original distribution in Mexico and US, that was reintroduced to the wild after being at the brink of extinction, cryopreserved semen and insemination has been crucial for the recovery of this species and are the part of the specific Special Survival Plan of the Association of Zoos and Aquariums (Howard *et al*., 2016).

In females, vitrification of Mexican gray wolf oocytes has been reported to maintain oocyte viability with a success rate greater than 50% (Boutelle *et al*., 2011). Crucially, the Mexican gray wolf recovery program has benefited from collaborations with human infertility clinics, especially in the case of female preservation, since the technique of oocyte vitrification is firmly established in human assisted reproduction (Silber *et al*., 2013). In this report, we detail the results of the application of assisted reproductive techniques for cryopreservation of gametes in the Mexican gray wolf by collaborating mexican institutions as part of the breeding and management plan for 2018.

## Material and Methods

### *Animals*

The 2018 gamete cryopreservation program was performed after a commission of the General Direction of Zoos and Wildlife, (Dirección General de Zoológicos y Vida Silvestre) of the Environment Ministry of Mexico City (SEDEMA, Secretaría Medio Ambiente Ciudad de México) and was approved and endorsed by the General Directorate for Wildlife (Dirección General de Vida Silvestre) of the Federal Ministry of Environment and Natural Resources (SEMARNAT, Secretaria Medio Ambiente y Recursos Naturales) by the "Oficio SGPA/DGVS/08333/17". Five males were selected for sperm cryopreservation: two males M982 (born 19/May/2005) and M1324 (born 26/April/2013) located at Zoológico San Juan de Aragón (SEDEMA, 2019) in Mexico City, two brothers: M1534 (born 30/April/2016) and M1535 (born 30/April/2016) in Parque Zoológico de León (Parque Zoológico de León, 2018) in León, Guanajuato and one male: M1379 (born 11/May/2014) in Zoológico El Ocotal (CEPANAF, 2018a) in Santiago Maxdá, State of Mexico. One female: F844 (29/April/2003), located at Parque Ecológico de Zacango (CEPANAF, 2018b), Toluca, State of Mexico was selected for the female gamete preservation procedure. Anesthesia was performed by a combination of physical restrain and administration of ketamine/xylacine under local protocols with induction maintained with isoflurane. Reproductive and cryopreservation procedures not performed in the field occurred at the Sciences in Human Reproduction Institute (RENIECYT, Registro Nacional de Instituciones y Empresas Científicas y Tecnológicas del CONACYT, 1702363), León, Guanajuato.

### *Testosterone measurement*

Blood samples were obtained by venipuncture of cephalic or saphenous veins and collected in serum tubes (BD, Vacutainer). Tubes were centrifuged and serum separated and maintained at -20°C until processing. Total testosterone was measured using a solid phase enzymatic competitive human assay (Total testosterone for Immulite 1000) with analytical sensitivity for human testosterone of 15 ng/dl. Samples were processed in a chemilumiscence immunoanalyzer (Immulite 1000, Siemens).

### *Sperm retrieval, evaluation and cryopreservation*

Before starting sperm collection, urine was removed by introducing a human neonatal feeding tube (5F) into the bladder. Next, sodium chloride solution 0.9% (w/v), approximately 100 ml, was instilled until bladder was completely clear. Electroejaculation was performed using an electroejaculation rectal probe of 2.5 cm of diameter and 16 cm long with 3 elec-

trodes (Model AC1, Beltron Instruments) using an ascending protocol delivering 4 seconds of stimuli and 4 seconds of rest in series of 3 stimuli up to 18V. Collection vessels were changed at first emission to keep fractions of ejaculate separate in case of urine contamination. Aliquots of samples were taken for vitality assessment by eosin/nigrosine staining and morphological assessment by Papanicolaou staining (WHO, 2010).

Ejaculate was centrifugated to remove seminal plasma and resuspended in CaniPlus Freeze® one-step (Minitube) with 10% (v/v) egg yolk or CaniPro ApX2 Freeze® (MOFA Global) two step extender with 20% (v/v) of egg yolk. Sperm was diluted when possible to a concentration of $20x10^6$/ml to $50x10^6$/ml and loaded in 0.25ml straws. Straws were equilibrated at 4°C for 4 hours, then were transferred to liquid nitrogen vapors for 20 min and finally plunged into liquid nitrogen. After evaluation, straws were transferred to cryostorage tanks at Zoológico de Chapultepec, which maintains guardianship of gametes for the Mexican gray wolf recovery program in Mexico.

### *Oocyte retrieval and vitrification*

Ovariohysterectomy involving a ventral technique with midline incision was used to retrieve the ovaries (Fossum, 2013). Ovaries were transported at room temperature in 25 mM HEPES-TCM199-Earle's salts medium (Gibco) containing 10% (v/v) fetal bovine serum (FBS; Gibco), 40 IU/ml heparin (Pisa), 100U-1 $\mu$g/ml penicillin-streptomycin (Sigma). Ovaries were processed by slicing (puncture of ovarian surfaces with an 11-blade) in a beaker containing HEPES-TCM199 +10% (v/v) FBS + heparin + penicillin/streptomycin medium. After processing both ovaries, medium containing cumulus-oocytes-complexes (COCs) was filtered through a 70 $\mu$M nylon mesh strainer (Fisher Scientific). Then the strainer was washed with medium to transfer COCs to a Petri dish. Vitrification of oocytes was performed using Kuwayama's method (Kuwayama, 2007) in a Cryotop® device (Kitazato). Three solutions were used: washing solution [HEPES-TCM199 (Gibco) with 20% (v/v) serum substitute supplement (SS; Irvine Scientific)], then equilibration solution [HEPES-TCM199 (Gibco), 20%(v/v) SS, 7.5% (v/v) dimethyl sulfoxide (DMSO) and 7.5% (v/v) ethylene-glycol] and finally vitrification solution [HEPES-TCM199 (Gibco), 20% (v/v) SS, 7.5% (v/v) dimethyl sulfoxide (DMSO), 7.5% (v/v) ethylene-glycol, 0.5 M sucrose]. COCs were transferred to washing solution for 3 minutes, then to equilibration solution for 3 minutes, then to a second well containing equilibration solution for 3 minutes, next to a third well containing equilibration solution by 10 minutes and finally vitrification solution for one minute, COCs in vitrification solution were loaded to a Cryotop®, plunged in liquid nitrogen and the Cryotop covered with its sheet.

### *Ovary tissue slow-freezing*

After processing ovaries for oocyte retrieval, ovaries were cut in small cubes of 2x1x1 mm, and the fragments were transferred to HEPES-TCM199 medium with 10% (v/v) FBS, 1.5 M DMSO and 0.1 M sucrose in cryovials for slow freezing using a controlled CL2200 cryobath (Freeze Control®, Cryologic). The initial cooling rate was 0.5 °C/min until -7 °C, then cryovials were seeded manually with frozen forceps and cooling continued at 0.5 °C/min up to -32 °C (Oktay *et al*., 2000). Finally, cryovials were transferred to liquid nitrogen for long-term storage.

### *Statistical analysis*

Sperm parameters are described using percentages and central tendency measurements.

## Results

### *Sperm freezing*

To optimize cryopreservation protocol of Mexican gray wolf sperm, we performed a preliminary experiment to test which of two commercial dog sperm extenders: CaniPlus Freeze® and CaniPro ApX2 Freeze® would result in superior post-thaw survival rates. We cryopreserved sperm samples from domestic dogs obtained by manual collection and electroejaculation with each of the two sperm extenders. Post-thaw sperm survival rates exceeded 30% in both conditions but was superior when using CaniPlus Freeze® (data not shown). Therefore, we decided to use this product as the main extender for sperm collections in the Mexican gray wolf.

Electroejaculation procedures for sperm collection were performed on March 14th, 2018 at Zoológico San Juan de Aragón, on March 15th, 2018 at Zoológico El Ocotal and March 20th at Parque Zoológico de León.

Results of hormone and sperm analysis in the five animals that underwent electroejaculation are shown in Tables 1 and 2. The levels of testosterone (Table 1) showed a positive tendency with sperm concentration, but more animals and species-specific validation of measurement kits are required to build a regression analysis and calculate statistical significance. Viability assessments by eosin-nigrosin staining of fresh and post-thaw sperm are shown in Figure 1A and 1B, respectively. Morphology evaluations by Papanicolau staining are shown in Figures 1C and 1D. Animal M1324 was found to have the highest quality sperm in terms of morphology and motility; sperm from the second fraction of ejaculate form this animal exhibited 63% progressive motility and 63% normal morphology. The two animals from Parque Zoológico de León M1534 and M1535 were one year and 10-month-old and they exhibited a low concentration of serum testosterone and smaller testicles (Table 1). These findings could potentially be explained by their juvenile age, or by inhibition of their testicular activity by the other higher rank males in the pack maintained at Parque Zoológico de León. These are also probable explanations of the lower sperm concentration and post-thaw survival in males M1534 and M1535 (Table 2).

The highest post-thaw sperm survival rate (Table 3) was obtained in third fraction of ejaculate from animal M1379, which exhibited 70% viability and 39% progressive motility, and in the second fraction of ejaculate from M1324, which exhibited 64% viability and 42% progressive motility. It was possible to compare the results of both semen extenders only in M1379 (Table 3). Viability (70% vs 48%) and progressive motility (39% vs 5%) were superior when using CaniPlus Freeze® compared with CaniPro ApX2 Freeze®. Inseminations in Mexican gray wol-

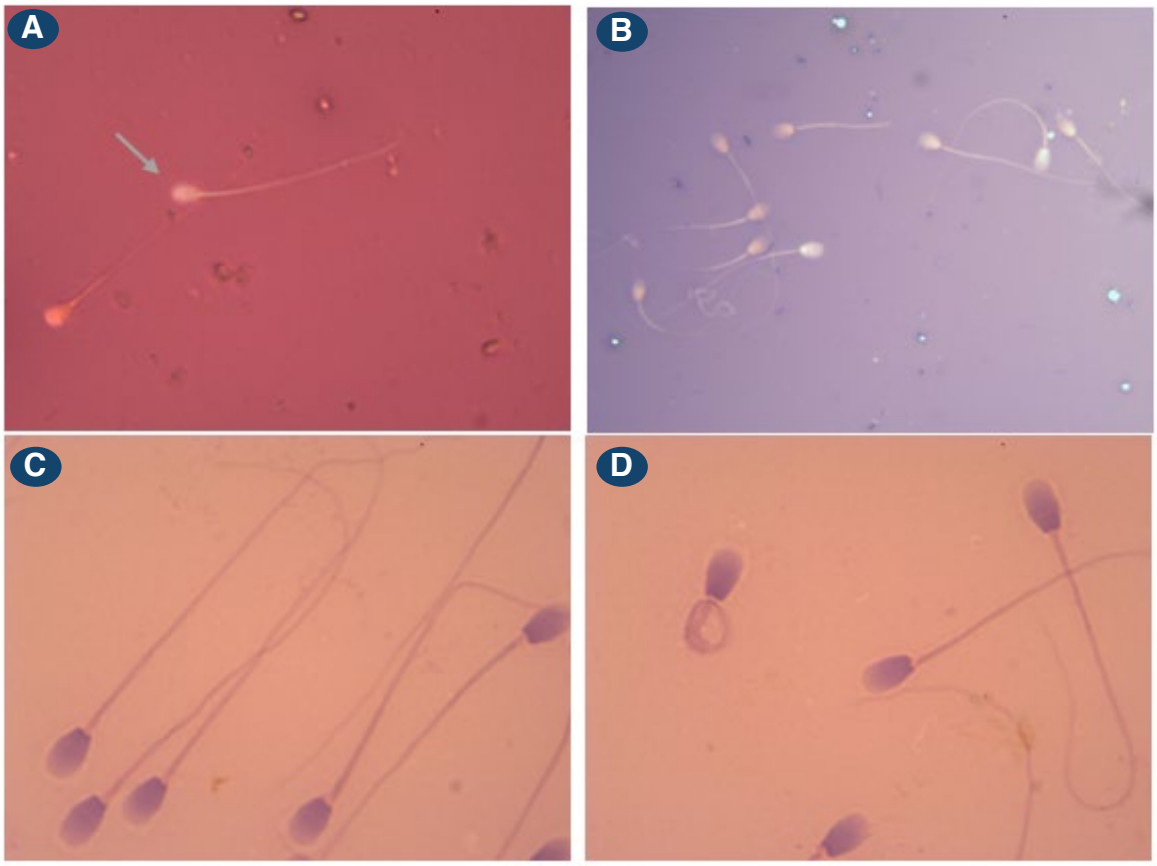


**Figure 1.** Analysis of sperm morphology in Mexican gray wolves. **A.** Eosin-nigrosin staining showing viability of sperm. Non-stained sperm are live (arrow) and red-dark sperm are dead, 400x optical x~1.5x digital zoom. **B.** Spermatozoa stained with eosin-nigrosin for viability in male M1534 post thaw, 400x. **C.** Morphology assessment by Papanicolau staining showing normal spermatozoa in male M1379 1000x. **D.** Spermatozoa with abnormal tail(left) in male M1534, 1000x.

Table 1. Age and weight of males, testosterone and testicular characteristics.

| Animal | Age | Testosterone concentration | Weight (kg) | Right testicle size (cm) | Left testicle size (cm) |
|---|---|---|---|---|---|
| M982 | 12y 9m | 180 ng/dl | 37 | 5x3.8 | 5x4 |
| M1324 | 4y 10m | 138 ng/dl | 28.5 | 4x3.5 | 4x3.7 |
| M1379 | 3y 9m | 978 ng/dl | 26.8 | 5x3.2 | 5x3.5 |
| M1534 | 1y 10m | 20 ng /dl | 29 | 4x2.5 | 4x2.4 |
| M1535 | 1y 10m | 54 ng/dl | 28 | 3x3.5 | 3x3.3 |

Table 2. Sperm analysis in the Mexican gray wolves included in the study.

| Animal | Age | Ejaculate fraction | Concentration (106/ml) | Viability (%) | Progressive motility (%) | Total motility (%) (pr+npr) | Normal morphology (%) | Head defects (%) | Middle piece defects (%) | Tail defects (%) | Teratozoospermia index |
|---|---|---|---|---|---|---|---|---|---|---|---|
| M982 | 12y 9m | 1 | 8 | 81 | 45 | 65 | 40 | 49 | 21 | 18 | 1.47 |
| | | 2 | 35 | 85 | 85 | 90 | 53 | 40 | 19 | 18 | 1.64 |
| M1324 | 4y 10m | 1 | 25 | 75 | 55 | 80 | 49 | 40 | 27 | 13 | 1.51 |
| | | 2 | 28 | 84 | 63 | 80 | 63 | 25 | 16 | 20 | 1..65 |
| M1379 | 3y 9m | 1 | 25 | 42 | 5 | 20 | 30 | 58 | 31 | 25 | 1.63 |
| | | 2 | 35 | 74 | 30 | 50 | 43 | 45 | 26 | 13 | 1.47 |
| | | 3 | 80 | 82 | 75 | 90 | 51 | 34 | 26 | 23 | 1.69 |
| M1534 | 1y 10m | 1 | 32 | 74 | 70 | 90 | 37 | 52 | 18 | 21 | 1.44 |
| | | 2 | 28 | 75 | 50 | 80 | 43 | 45 | 10 | 17 | 1.26 |
| M1535 | 1y 10m | 1 | 20 | 60 | 5 | 45 | 26 | 58 | 28 | 31 | 1.58 |
| | | 2 | 10 | 54 | 0 | 30 | 32 | 51 | 21 | 25 | 1.43 |

[1]PR: progressive, NPR: non-progressive

ves have been reported using 200 million spermatozoa. As we collected over 400 million spermatozoa from animal M1379 we consider that we were able to collect two doses of sperm ready for insemination from this animal. All ejaculates fractions from all animals were cryopreserved but only the samples from M1379 achieved the cryobanking requirements for reproductive purposes using insemination.

### *Ovary and oocyte cryopreservation*

Ovariohysterectomy of a female Mexican gray wolf, F844, was performed on March 22th, 2018 with the specific purpose of collecting ovaries. The animal was 14 years and 10 months-old at the time of surgery. Removed ovaries were transported to our laboratory in Leon for processing and cryopreservation. No visible antral follicles were found in the ovaries. Dissection of the ovaries with slicing method obtained six oocytes (Figure 2), no oocytes at germinal vesicle stage among them. Three oocytes appeared to be of good quality (top row in Figure 2) and all oocytes were vitrified using Cryotop®. After oocyte retrieval, the ovaries were cut in pieces for slow freezing (Figure 3). No post-thaw evaluation was performed, but we used our standard validated technique at several clinical for human ovarian tissue cryopreservation.

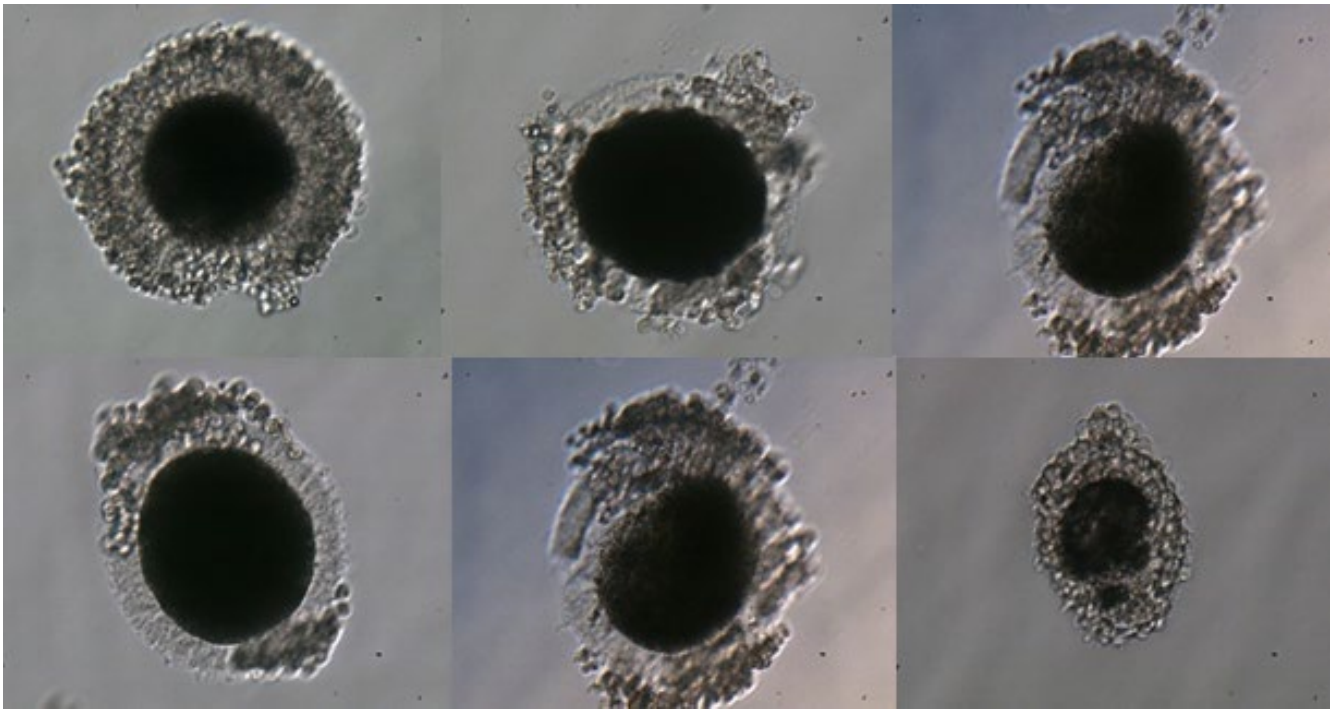

**Figure 2.** Oocytes obtained from Mexican gray wolf female F844. Mexican gray wolf oocytes surrounded by granulosa cells in cumulus-oocyte complexes.

## Discussion

The Species Survival Plan® for the Mexican gray wolf established a genome bank in the US in 1990, and male wolves are selected for sperm collection based on their representation in the gamete bank. As 2017, material from 125 males has been cryopreserved in the US (U.S. Fish and Wildlife Service, 2017). During the reproductive season of 2018, the Institute of Sciences in Human Reproduction

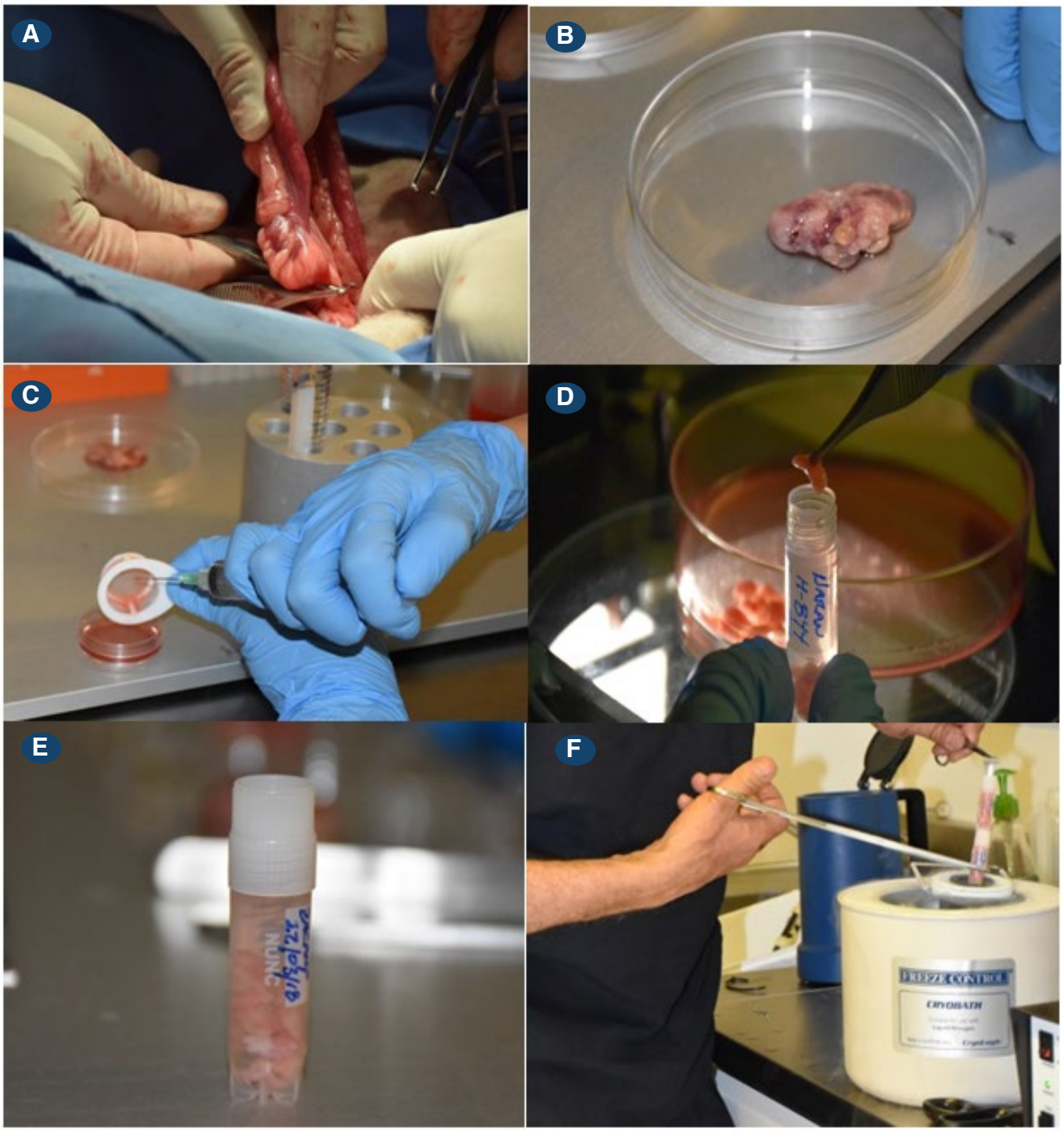


**Figure 3.** Cryopreservation of ovarian tissue from Mexican gray wolf female F844. **A.** Removal of ovary during surgery. **B.** Ovary without visible antral follicles. **C.** Washing of cell strainer to transfer COCs. **D.** Ovary tissue cube transferred to cryopreservation solution. **E.** Ovary pieces in cryovials ready for starting slow freezing protocol. **F.** Manual seeding in programmable freezer during slow freezing protocol.

(Instituto de Ciencias en Reproducción Humana), a Mexican human infertility clinic with a research lab, took part for the first time in the Mexican side of Mexican wolf gamete cryopreservation program. We introduced innovations based on our expertise in human, domestic and wild animal reproductive technology. The innovations included the measurements of serum testosterone along with sperm retrieval, the use commercial dog semen extender not used before in Mexican gray wolf gamete preservation and the use of a slow freezing technique for ovarian tissue.

### *Cryopreservation of male gametes*

Our results with the males show the difficulties in banking gametes for conservation: despite all spermatozoa we obtained were cryopreserved only two doses of sperm for insemination, as defined by current artificial inseminations protocols, were obtained (Table 3).

Our results were likely affected because sperm collection was performed at the end of March, at which time we would theoretically expect a lower

Table 3. Post-thaw analysis of seminal parameters in the Mexican gray wolves studied.

| Animal | Age | Ejaculate fraction | Extender | Viability (%) | Progressive motility (%) | Total motility (%) (pr+npr)[1] | Dosages[2] for insemination |
|---|---|---|---|---|---|---|---|
| M982 | 12y 9m | 1 | CaniPlus Freeze | 69 | 18 | 49 | 0 |
| | | 2 | CaniPlus Freeze | 72 | 41 | 65 | 0 |
| M1324 | 4y 10m | 1 | CaniPlus Freeze | 45 | 19 | 53 | 0 |
| | | 2 | CaniPlus Freeze | 64 | 42 | 63 | 0 |
| M1379 | 3y 9m | 1 | CaniPlus Freeze | 32 | 0 | 2 | 0 |
| | | 2 | CaniPlus Freeze | 66 | 4 | 29 | 0 |
| | | 3 | CaniPlus Freeze | 70 | 39 | 61 | 1 |
| | | 3 | CaniPro ApX2 Freeze | 48 | 5 | 35 | 1 |
| M1534 | 1y 10m | 1 | CaniPlus Freeze | 65 | 15 | 55 | 0 |
| | | 2 | CaniPlus Freeze | 60 | 5 | 54 | 0 |
| M1535 | 1y 10m | 1 | CaniPlus Freeze | 49 | 0 | 29 | 0 |
| | | 2 | CaniPlus Freeze | 48 | 0 | 21 | 0 |

[1]PR: progressive, NPR: non-progressive
[2] based in 200x106 spermatozoa per insemination

quality of sperm compared to that at the beginning of the reproductive season from January to February, when spermatogenesis is at its peak. Unfortunately, dates were out of our control due to time required for processing paperwork and logistic reasons. Nevertheless, we were able to obtain viable sperm in the five males. In other reports, i.e. Asa *et al*. (2007) analysis was limited to ejaculates obtained in January and February. However, no report has described serial collections in the Mexican gray wolf to understand the precise timing of spermatogenic activity. Collections should be optimized in relation to timing of reproductive season, age of the animals and potentially should be scheduled to allow for more than one collection per season as it is currently performed. Nevertheless, we have show that is possible to collect sperm for insemination even within a few weeks of the end of the reproductive season.

In cryobanking male gametes with the purpose of impacting conservation of an endangered mammal, it is important to clarify that the number of sperm straws is not a good indicator of the real potential of cryopreserved gametes. Straws are variable in volume (0.25 ml, 0.5 ml), the concentration of sperm in each straw (normally from $5x10^6$ to $100x10^6$), and the percentage of viable sperm. Therefore, it is important to use a better parameter that indicates the real potential of producing progeny. According to the present status of reproductive technology, artificial wolf insemination with frozen semen it is requires $200x10^6$ spermatozoa with at least 60% viability. In general, most animals collected in the US and Mexico did not produced the requirements for artificial insemination; the use of additives as Equex paste® (Zindl *et al*., 2006) or CaniPlus Freeze® extender used in the present report could potentially help to improve post-freezing viability in future seasons. Further research comparing different cryopreservation extenders is required.

We included sperm morphology analysis (Table 2) in this report that is certainly a less rigorous component of sperm analysis. In human medicine through the years, stricter criteria have been used and the threshold for normal sperm has decreased (currently it is 4%). In wild animals, no standardized methods and criteria for sperm analysis are available and morphology evaluation is biased by researcher subjectivity. Sperm morphology evaluation has been performed in the Mexican gray wolf using Spermac® stain (Zindl *et al*., 2006), but Asa *et al*. (2007) used eosin-nigrosin that is designed for vitality assessment and not for morphology. We used a standard Papanicolaou stain (Figures 2C and 2D) which is employed in human andrology labs (WHO, 2010).

Motility is also susceptible to high variability among observers. Computer Automated Sperm Analysis (CASA) systems provide more reproducible results but do not have widespread use in human andrology. Sperm motility assessment in fresh samples by CASA has been reported for gray wolf (Christensen *et al*., 2013). In the Mexican gray wolf, an improved sperm analysis may provide better conclusions regarding the fertility potential of sperm based on morphology and motility.

### *Oocyte and ovary cryopreservation*

A primary challenge in gamete cryopreservation efforts for the Mexican gray wolf is in preserving female gonadal tissue. Canids have a complex oocyte maturation system in which oocytes are ovulated while still immature, and as results the success of in vitro production to produce dog embryos is limited. A recent study reported the birth of pups from in vitro fertilized embryos but with oocytes matured in vivo (Nagashima *et al*., 2015). It will be important to test whether in vitro maturation of cryopreserved oocytes is also effective for the Mexican gray wolf, and our samples enables such studies.

Ovarian tissue cryopreservation has not been reported successful to produce offspring in wild animals (Comizzoli *et al*., 2018) and techniques for differentiation of stem cells into mature oocytes are at their infancy. Recently, researchers reported a limited in vitro growth of follicles from dog vitrified ovaries (Ackermann *et al*., 2017); however, it is not clear if this technique could provide normal ovarian function or at least mature oocytes. We decided to use our standard human protocol for ovarian cryopreservation using slow-freezing (Oktay *et al*., 2000). This protocol has proven to produce normal ovarian function and spontaneous pregnancies after autologous transplantation (Oktay and Oktem 2010). Presently, fertility preservation is only a reality in humans and laboratory rodents. In terms of conservation goals for Mexican gray wolf, cryopreservation of ovarian tissue should not provide a false hope that this cryopreserved tissue is useful for reproductive purposes in the near future. An active Mexican program of research wolf gonads oocyte and ovary cryobiology and transplantation using dog and generic gray wolf gonads is urgently needed to

establish a standard protocol for ovary cryobanking in Mexican gray wolf.

### *Implications of gamete cryopreservation for conservation*

A major aim of cryopreserving Mexican gray wolf gametes to increase or maintain genetic diversity within the population. Indeed maintaining genetic diversity has been a challenge in recovery efforts. The Mexican gray wolf recovery program currently decides yearly reproductive pairs based on low kinship of animals and logistical considerations; however, retained genetic diversity in captive population is only 83% (Siminski and Spevak, 2017). Fitak *et al*. (2018) recently reported SNP-microarray genotyping of 87 Mexican wolves that demonstrate a low representation of individuals from Aragon and Ghost Ranch lineages and a decline in genetic diversity after years of breeding among the three lineages. The combination of artificial insemination and the design of breeding pairs based on genomic information, methods that are well established in pure breed livestock associations, could help to maximize genetic diversity and increase representation of Aragon lineage, which is one of the long-term goals of recovery programs (U.S. Fish and Wildlife Service, 2017) and of specific interest for Mexico. For the wolves maintained in Mexico, the genotypes based on SNPs are not available. However, dog commercial SNP-microarrays are a cost-effective method to perform whole genome level genotyping of Mexican gray wolves. Genotyping the whole Mexican gray wolf population (in 2017, 87 animals) as well as any banked samples is an achievable goal and a desirable step for the future conservation and reintroduction planning activities for Mexican wildlife authorities.

Paternity testing is part of requirements to register domestic animals in Mexican pure breed associations of cattle, sheep and horses. This mandate is supported by Mexican federal government and works along with the National Livestock Registry (http://www.pgn.org.mx/). Pure breeder associations stopped using pedigrees for kinship assessment and paternity validity years ago and now all animals of particular breeds of productive species have been genotyped. These efforts are coordinated by the National Council for Livestock Genetic Resources (Consejo Nacional de Recursos Genéticos Pecuarios). Also, several cattle associations in Mexico are using genetic markers of interest for production and planning breeding based on genetic data. Consequently, genetic marker assisted reproduction is not a new concept in Mexico but has not been used in wildlife species. If used, it may aid in better population management of the endangered Mexican gray wolf.

Nowadays, perhaps only somatic nuclear transfer technology can return the genome of valuable individuals to the species management programs as previously discussed (Piña-Aguilar *et al*., 2009). Advanced technologies for conservation purposes should be considered as an alternative in Mexico (López-Saucedo *et al*., 2010). Domestic dog cloning was reported in 2005 using *in vivo* matured oocytes (Lee *et al*., 2006). The same technique has been successful for cloning gray wolves using domestic dog oocytes (Kim *et al*., 2007), including the use of cells obtained post-mortem for nuclear transfer (Oh *et al*., 2008). Using nuclear transfer with cryopreserved somatic cells (fibroblasts from skin, ovary or somatic cells from sperm) can bring back individuals of desired genomes who may have died many years ago or valuable animals who died after release in the wild. Potentially this can be a better approach considering the poor results with artificial insemination.

In other species, for which artificial insemination and intensive *ex-situ* management are in place (Howard *et al*., 2016), somatic nuclear transfer has been proposed as a tool to preserve the genetic pool present in deceased animals (Wisely *et al*., 2015). A discussion of the benefits and costs of conservation cloning, for the Mexican gray wolf (Piña-Aguilar *et al*., 2009) to increase or at least maintain genetic diversity should occur in the binational recovery program to include biobanking of somatic cells and not only gametes. If there is interest in conservation cloning, somatic cells should be banked in addition to gametes. An example is the plan to recover the Northern white rhino (*Ceratotherium simum cottoni*) using cryopreserved somatic cells in San Diego Zoo's Frozen Zoo (Tunstall *et al*., 2018). Mexico has already established a National Center of Genetics Resources (Centro Nacional de Recursos Genéticos, CNRG) in Tepatitlán, Jalisco with support of the federal government for the long-term preservation of germplasm of domestic species. A matching initiative for priority wildlife species would complement the existing efforts in Mexican gray wolf conservation and would enable long-term storage of somatic cells and gametes of this endangered species and provide a safer deposit than liquid nitrogen tanks in a specific zoo.

## Future actions

In our view, the Mexican component of the Mexican gray wolf recovery plan requires gamete and tissue retrieval of wolves during reproductive seasons before they are released to the wild. As the wild populations represent the main hope for a potential recovery of the species in Mexico, banking gametes and somatic cells from animals released to the wild can help decrease the burden of the loss of these valuable animals after release. Of the 41 individuals liberated in Mexico from 2012 to 2016, 18 (44%) died during the first year of poisoning, car accidents and other human interactions (U.S. Fish and Wildlife Service, 2017). Therefore, as establishing a stable growing population at Sierra Madre Oriental is difficult and not yet at the level of the US, banked reproductive and somatic cells of animals that are candidates for release are vital.

Starting a scheme of artificial insemination with fresh and frozen semen in mexican zoos is urgently needed. This will create the resources and develop species-specific expertise within the mexican community and facilitate the annual breeding plans without the need of move animals or disrupt social structures of packs already established at facilities. The research and results detailed in this report are the product of a longstanding collaboration between our Institute and several mexican zoos for the purpose of reproductive assessment and cryopreservation of gametes and embryos (López-Saucedo *et al*. 2013; Piña-Aguilar *et al*., 2016). Such collaborations enable animal conservationists access to advanced assisted reproduction techniques and have proven effective for other international groups as well (Comizzoli *et al*., 2018; Silber *et al*., 2013).

In conclusion, our team based in Mexico successfully cryopreserved gametes of Mexican gray wolf and innovated improved cryopreservation techniques that we recommend as standard operating procedures for the Mexican gray wolf *ex situ* recovery programs in Mexico and the US. In future reproductive seasons, an active integrative research and education program is required in Mexico to achieve the specific conservations goals of the mexican conservation community and stakeholders.

## Acknowledgements

We are thankful to all individuals in Mexico and US involved with the Recovery plan of Mexican gray wolf, including Mexican Wolf SSP. We thank to Dirección de Vida Silvestre de la Secretaria de Medio Ambiente y Recursos Naturales (SEMARNAT) for the opportunity to contribute to conservation of Mexican gray wolf in Mexico. Animal handling procedures and tissue recovery across the country required the support and contribution of many individuals (veterinary teams, animal keepers and students) at Zoologico San Juan de Aragón, Parques de la Comisión Estatal de Parques Naturales y de la Fauna (CEPANAF) and Parque Zoológico de León. We especially acknowledge the support of authorities in Zoos involved Claudia Lewy Sánchez-Aldana and Julieta Méndez Ruíz, Anna Sofía Manzur García-Maass and Rubén David Rocha-Lemus.

## References

Ackermann, C.L., C.S. Asa, R. Krisher, K. Bauman, S. Casey and M.D. Lopes. 2017. Evaluation of follicular growth and tissue viability in vitrified/warmed domestic dog ovaries after in vitro culture. *Reproduction in Domestic Animals*, 52:77-81.

Asa, C., P. Miller, M. Agnew, J.A. Rebolledo, S.L. Lindsey, M. Callahan and K. Bauman. 2007. Relationship of inbreeding with sperm quality and reproductive success in Mexican gray wolves. *Animal Conservation*,10:326-331.

Boutelle, S., K. Lenahan, R. Krisher, K.L. Bauman, C.S. Asa and S. Silber. 2011. Vitrification of oocytes from endangered Mexican gray wolves (*Canis lupus baileyi*). *Theriogenology*, 75:647-654.

CEPANAF. 2018a. Zoológico El Ocotal. Available at: http://cepanaf.edomex.gob.mx/zoologico_el_ocotal [Accessed on December 30, 2018].

CEPANAF. 2018b. Parque Ecológico de Zacango. Available at http://cepanaf.edomex.gob.mx/parque_ecologico_zacango [Accessed on December 30, 2018].

Christensen, B.W., C.S. Asa, C. Wang, K. Bauman, M.K. Agnew, S.P. Lorton and M. Callahan. 2013. Kinematic activity of gray wolf (*Canis lupus*) sperm in different extenders, added before or after centrifugation. *Theriogenology*, 79:953-960.

Comizzoli, P., E.E. Paulson, L.K and McGinnis LK. 2018. The mutual benefits of research in wild animal species and human-assisted reproduction.

*Journal of Assisted Reproduction and Genetics,* 35:551-560.

Fitak, R.R., S.E. Rinkevich and M. Culver. 2018. Genome-wide analysis of SNPs is consistent with no domestic dog ancestry in the endangered Mexican wolf (*Canis lupus baileyi*). *Journal of Heredity*, 109:372-383.

Fossum, T. 2013. *Small Animal Surgery.* 4th Edition. Elsevier Mosby: Missouri

Heffelfinger, J.R., Nowak, R.M. and D. Paetkau. 2017. Clarifying historical range to aid recovery of the Mexican wolf. *Journal and Wildlife Management*, 81:766-777.

Howard, J.G., C. Lynch, R.M. Santymire, P.E. Marinari and D.E., Wildt. 2016. Black-footed ferret gene restoration. *Animal Conservation*, 19:102-111.

Kim, M.K., G. Jang, H.J. Oh, F. Yuda, H.J., Kim, W.S. Hwang, M.S. Hossein, J.J. Kim, N.S. Shin, S.K. Kang, and B.C. Lee. 2007. Endangered wolves cloned from adult somatic cells. *Cloning Stem Cells*, 9:130-137.

Kuwayama, M. 2007. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology*, 67:73-80.

Lara-Díaz, N.E., C.A. López-González, H. Coronel-Arellano and J.L. Cruz-Romo. 2015. Nacidos libres: en el camino a la recuperación del lobo mexicano. CONABIO. *Biodiversitas*, 119:1-6.

Lee, B.C., M.K. Kim, G. Jang, H.J. Oh, F. Yuda, H.J. Kim, M.S. Hossein, J.J. Kim, S.K. Kang, G. Schatten and W.S. Hwang. 2006. Dogs cloned from adult somatic cells. *Nature*, 436:641.

López-Saucedo, J.L., J.P. Ramon-Ugalde and R.E. Piña-Aguilar. 2010. ¿Es posible la recuperación de especies silvestres extintas a través de la transferencia somática nuclear? *Revista Mexicana de Biodiversidad*, 81:587-589.

López-Saucedo, J., J.P. Ramón-Ugalde, J. Jesús, A.M. Gutiérrez-Gutiérrez, R. Fierro and R.E. Piña-Aguilar. 2013. Superovulation, *in vivo* embryo recovery and cryopreservation for Aoudad (*Ammotragus lervia*) females using osmotic pumps and vitrification: A preliminary experience and its implications for conservation. *Tropical Conservation Science*, 6:149-157.

Nagashima, J.B., S.R. Sylvester, J.L. Nelson, S.H. Cheong, C. Mukai, C. Lambo, J.A. Flanders, V.N. Meyers-Wallen, N. Songsasen and A.J. Travis. 2015. Live births from domestic dog (*Canis familiaris*) embryos produced by in vitro fertilization. *PLoS One*, 10:e0143930.

Oh, H.J., M.K. Kim, G. Jang, H.J. Kim, S.G. Hong, J.E. Park, K. Park, C. Park, S.H. Sohn, D.Y. Kim, N.S. Shin and B.C. Lee. 2008. Cloning endangered gray wolves (*Canis lupus*) from somatic cells collected postmortem. *Theriogenology*, 70:638-647.

Oktay, K., H. Newton and R.G. Gosden. 2000. Transplantation of cryopreserved human ovarian tissue results in follicle growth initiation in SCID mice. *Fertil Steril*, 73:599-603.

Oktay, K, and O. Oktem. 2010. Ovarian cryopreservation and transplantation for fertility preservation for medical indications: report of an ongoing experience. *Fertil Steril*, 93:762-768.

Parque Zoológico de León. 2018. Parque Zoológico de León: Available at: http://www.zooleon.org.mx. [Accesed on December 30, 2018].

Piña-Aguilar, R.E., J. Lopez-Saucedo, R. Sheffield, L.I. Ruiz-Galaz, J. de J. Barroso-Padilla and A. Gutiérrez-Gutiérrez. 2009. Revival of extinct species using nuclear transfer: hope for the mammoth, true for the Pyrenean ibex, but is it time for "conservation cloning"? *Cloning Stem Cells*, 11:341-6.

Piña-Aguilar, R.E., J. López-Saucedo, L.I. Ruiz-Galaz, J.J. Barroso-Padilla, M.C. Gallegos-Rivas, C. González-Ortega and A.M. Gutiérrez-Gutiérrez. 2016. A human reproductive approach to the study of infertility in chimpanzees: An experience at Leon's Zoological Park, Mexico. *Veterinary Research Forum*, 7:255-259.

SEDEMA. 2018. Zoológico de Aragón. Available at: http://data.sedema.cdmx.gob.mx/zoo_aragon/ [Accessed on December 30, 2018].

Silber, S.J., N. Barbey, K. Lenahan and D.Z. Silber. 2013. Applying clinically proven human techniques for contraception and fertility to endangered

species and zoo animals: a review. *Journal of Zoo and Wildlife Medicine*, 44:S111-122.

Siminski, D.P. 2017. *Mexican Wolf,* Canis lupus baileyi*, International Studbook, 2017*. The Living Desert, Palm Desert, California, USA.

Siminski, P. and E. Spevak. 2017. *Mexican wolf* (*Canis lupus baileyi*) *population analysis & breeding and transfer plan*. Association of Zoos and Aquariums. USA.

Tunstall, T., R. Kock, J. Vahala, M. Diekhans, I. Fiddes, J. Armnstrong, B. Paten, O.A. Ryder and C.C. Steiner. 2018. Evaluating recovery potential of the northern white rhinoceros from cryopreserved somatic cells. *Genome Research*, 28:780-788.

U.S. Fish and Wildlife Service. 2017. *Mexican Wolf Biological Report: Version 2*. Region 2, Albuquerque, New Mexico, USA.

Wisely, S.M., O.A. Ryder and R.M. Santymire, J.F. Engelhardt and B.J. Novak. 2015. A Road Map for 21st Century Genetic Restoration: Gene Pool Enrichment of the Black-Footed Ferret. *Journal of Heredity*, 106:581-592.

WHO (World Health Organization). 2010. *WHO laboratory manual for the examination and processing of human semen*. Fifth edition, Geneva, Switzerland.

Zindl, C., C.S. Asa and A.R. Günzel-Apel. 2006. Influence of cooling rates and addition of Equex pasta on cooled and frozen-thawed semen of generic gray (*Canis lupus*) and Mexican gray wolves (*C. l. baileyi*). *Theriogenology*, 66:1797-1802.