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In vitro embryo production in the Iberian lynx (*Lynx pardinus*) using oocytes recovered post-mortem and cryopreserved spermatozoa

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ABSTRACT

The Iberian lynx (*Lynx pardinus*), once considered the most endangered felid in the world, has become a remarkable example of conservation success thanks to intensive *ex situ* and *in situ* management efforts. However, the long-term viability of the species remains under threat. The implementation of assisted reproductive technologies (ARTs) would contribute to ensure the species viability, but the use of ARTs is limited in this species and there are still important gaps in the knowledge of its physiology. Here, we report the first *in vitro* fertilization (IVF) and early embryo development in the Iberian lynx using oocytes retrieved post-mortem and cryopreserved spermatozoa. Oocytes from six females were subjected to *in vitro* maturation using two sets of conditions. In the first series, oocytes incubated *in vitro* for 24 h, exhibited a very low proportion (0–37 %) reaching metaphase II (MII). Attempted IVF with cryopreserved spermatozoa resulted in no fertilization. In a second series, oocytes were incubated *in vitro* for 28 h; grade I-II oocytes exhibited 40 % maturation rate (MII), whereas grade III oocytes showed 28 % maturation rate. Matured oocytes from one female were co-incubated with cryopreserved sperm for 18 h resulting in cleavage rates of 39.3 % (grade I–II) and 7.1 % (grade III) and the opportunity to vitrify one blastocyst and six morulae. These results demonstrate the feasibility of using ARTs in this species, validating domestic cat protocols for Iberian lynx gametes, and providing a foundation for further refinement of IVF protocols, embryo cryopreservation, and ultimately, embryo transfer strategies for use in conservation programs.

1. Introduction

The rapid extinction of animal species driven by human activity and the effects of climate change represent one of the most pressing challenges faced by the scientific community in the 21st century [1]. Despite the scale of this threat, significant progress has been made in the preservation of certain species through the implementation of coordinated *in situ* and *ex situ* conservation programs [2,3]. According to the IUCN Red List, nearly half of all wild felids are currently classified as either endangered or vulnerable [4]. The Iberian lynx (*Lynx pardinus*) serves as a notable example of the effectiveness of such conservation strategies. Once considered the most endangered felid species in the world, the Iberian lynx was reclassified as "vulnerable" in 2024 [4], following more than two decades of intensive conservation efforts.

Thanks to the establishment of *ex situ* conservation programs, assisted reproductive technologies (ARTs) have begun to be applied over the past few decades to support and enhance conservation efforts in wild felids. These technologies include artificial insemination (AI), *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) for in *vitro* embryo production (IVP), embryo transfer (ET), and the cryopreservation of gametes or somatic cells for the creation of genetic resource banks [3]. The implementation of the latter has led to significant achievements in wild felid reproduction, such as successful laparoscopic and transvaginal AI in clouded leopards, African lions or Siberian tigers [5–7], the birth of offspring following IVP and subsequent ET in cheetahs, tigers, caracals, servals, African wildcats, fishing cats, black-footed cats, sand cats,

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ocelots and oncillas [8–16], and even the production of viable descendants through intra or interspecific transfer of somatic cell nuclear transfer (SCNT) embryos in caracals, African wildcats or sand cats [17–20]. These accomplishments have been closely linked to the development of ARTs in the domestic cat, as a model for its wild counterparts. This species has allowed the availability of samples, which has offered the opportunity to explore and begin the refinement of these techniques in felids, prior to the adaptation to endangered and valuable individuals [21–23].

Until now, conservation strategies for the Iberian lynx have relied heavily on *ex situ* breeding programs [24], which offer important advantages such as enabling close monitoring of individual animals, facilitating research on reproductive biology and behavior, and allowing the controlled breeding of individuals for reintroduction into the wild [25]. One of the critical challenges in managing these programs, particularly in small and genetically constrained populations, is the limited number of founders available for breeding. This restriction has been therefore linked to a certain level of inbreeding [26,27] and underscores the need for rigorous genetic management to preserve and, whenever possible, enhance genetic diversity within the *ex situ* population [28,29].

In this context, a key conservation strategy implemented for the Iberian lynx has been the establishment of a genetic resource bank in 2003 [30]. This biobank was designed to preserve a wide range of cryopreserved materials (including somatic cells, gonadal tissue, and frozen semen) with the goal of supporting future applications of ARTs in the species by reintroducing lost genetic material [30,31]. In parallel, ongoing research efforts have focused on developing and refining protocols for IVF, including the generation of hybrid embryos by fertilizing domestic cat oocytes with cryopreserved Iberian lynx spermatozoa, as a test of their fertilizing capacity [32]. Furthermore, work has focused on cryopreservation of oocytes and embryos, specifically adapted to the reproductive physiology of the Iberian lynx [24,32].

Although these achievements represent major milestones, the use of ARTs in wild felids remains limited and anecdotal for most species. This is largely due to the aforementioned factors, in combination with their unique anatomical and physiological characteristics, which present specific challenges for the application of these techniques. Therefore, the development of standardized protocols for the implementation of ARTs is essential to broaden their applicability and effectiveness in the conservation of endangered felid species [33,34].

The objective of the present study was to evaluate the effectiveness of IVM and IVF techniques, originally developed for the domestic cat, in the vulnerable Iberian lynx. These protocols are essential tools for future attempts of genetic recovery and management of the species. The use of assisted reproduction will significantly improve the species long-term survival, benefiting from the cryopreserved genetic material available in the genetic resource bank, and the one that becomes available in the future. This study was designed to first examine conditions for IVM, taking advantage of oportunistic access to ovaries from animals that die in the wild as a result of road kills or other accidents [30]. Due to such oportunistic access, it is not possible to plan ahead or control the timing of ovary retrieval from cadavers. In addition to the unpredictability of accidents, animals that die in the wild have to go through a protocol with a mandatory intervention of authorities and forensic centers [24]. In some cases, ovaries can be retrieved from females that are castrated or sacrificed in the captive breeding centers, allowing for more precise timing. An additional factor to bear in mind is the fact that Iberian lynx females experience an extended period of corpus luteum persistence after the breeding season [22,24] which would influence follicular physiology. All these conditions result in a low number of samples available at different times of the year with an inevitable impact on the IVM protocols. On the other hand, storage of cryopreserved semen from males of proven fertility in the species genetic resource bank [30,32] ensures a readily available source of spermatozoa for IVF studies. Previous studies have indicated that such stored semen can retain high quality and fertility potential [32]. Thus, it is possible to expect less variation due to sperm quality and a more consistent IVF success in case the conditions for IVM are adequate. Overall, two experimental series of IVM with ovaries retrieved at different times of the year served to assess *in vitro* conditions for oocyte maturation. This was complemented by attempts to fertilize and incubate presumptive zygotes to explore development potential.

2. Material and methods

2.1. Animals

The females included in this study were free-ranging ($n = 5$) or captive-born ($n = 1$) Iberian lynxes. Three of them died as a result of road kills in Andalusia or Castilla-La Mancha, Spain, two died while under quarantine and the last one was ovariectomized as a result of recurrent uterine infections (Table 1). The ovaries were collected and transported under refrigeration for a maximum period of 28 h to the National Museum of Natural Sciences in Madrid, and were processed there or at the Reproduction Laboratory of the Complutense University of Madrid for collection of oocytes. Three males of proven fertility were used for semen collection.

All animal manipulations were conducted in compliance with Spanish Animal Protection legislation (RD 53/2013), which conforms with the European Union Directive 2010/63/UE.

2.2. Reagents and media preparation

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain). Media were prepared, filtered through 0.22 μm membrane filters and aliquoted into sterile 15 mL tubes. Tubes were tightly sealed and stored at 4 °C until use, for a period of 2 weeks.

2.3. Oocyte collection and in vitro maturation

Two series of oocyte collection and maturation were carried out. In the first series (females #1 – #4), ovaries were obtained in the autumn and were transported to the laboratory at 4 ºC in physiological saline solution containing 0.1 mg/mL each of penicillin and streptomycin. The ovaries were sectioned longitudinally with a scalpel blade and sliced using two 21-gauge needles to release cumulus-oocyte complexes (COCs) from follicles under a dissecting microscope (Nikon SMZ 645, Tokyo, Japan). COCs were retrieved in a Petri dish containing medium TCM-199, supplemented with 15 mM HEPES, 15 mM $NaHCO_3$, 0.36 mM sodium pyruvate, 1 mM glutamine, 2.2 mM calcium lactate, 0.4 % (w/v) bovine serum albumin (BSA; A-8412; Sigma) and penicillin-

Table 1
Iberian lynx females from which oocytes were recovered.

Female ID	Age (months)	Season of death	Cause of death	Interval between ovary retrieval and oocyte collection (hours)
1	20	Early Autumn	neumonia	20
2	30	Early Autumn	road kill	12
3	18	Early Autumn	road kill	28
4	31	Early Autumn	electrocution	16
5	108	Mid Winter	(ovariectomy) *	18
6	12	Early Spring	road kill	26

* This female had several episodes of pyometra.

streptomycin (0.1 mg/mL each) [32,35,36]. A mouth pipette was used for handling COCs. Oocytes were morphologically evaluated and classified based on cumulus cell layers and cytoplasmic characteristics, following established criteria [37]. A total of 47 oocyte-cumulus complexes were incubated in four-well dishes (Nunc®, Roskilde, Denmark) with 500 μL of TCM-199 supplemented with 25 mM $NaHCO_3$, 0.36 mM sodium pyruvate, 2 mM glutamine, 2.2 mM calcium lactate, 1.12 mM cysteine, 0.4 % (w/v) BSA (FractionV; A-9418; Sigma), 25 ng/mL epidermal growth factor (EGF [38];), ovine FSH/LH (10 μg/mL each), 1 μg/mL estradiol, penicillin and streptomycin (0.1 mg/mL each). Oocytes were cultured at 38.5 ºC under 5 % CO_2/air and maximum humidity for 24 h [36,39,40]. At the end of incubations, oocytes from females #1 and #2 were fixed and stained to assess maturation stages, whereas those from females #3 and #4 were coincubated with spermatozoa.

In the second series (females #5 – #6), ovaries were available in mid-winter and early spring and were transported to the laboratory at 4 °C in sterile physiological saline solution for either *in vitro* maturation (female #5) or *in vitro* embryo production (female #6). COCs were retrieved under a stereomicroscope (Nikon SMZ 745) by slicing the ovary with a scalpel blade in a Petri dish containing HEPES-buffered TCM-199 medium (Sigma-Aldrich), maintained at 38.5 °C. All collected COCs were morphologically evaluated and classified based on cumulus cell layers and cytoplasmic characteristics [37], as performed in series 1. A total of 65 oocytes were recovered from female #5 and 85 oocytes from female #6, of which 58 and 69, respectively, were selected for IVM and distributed into two groups according to their morphological quality: grade I–II ($n = 33$ and $n = 37$, respectively) and grade III ($n = 25$ and $n = 32$, respectively). From the oocytes collected of female #5, 7 oocytes were discarded due to signs of degeneration, while 16 oocytes were discarded from those collected from female #6 for the same reasons. In this series, the oocytes were incubated for 28 h (instead of 24 h), under the same conditions as in series 1, and the same IVM medium, with the exception of FSH/LH, estradiol and antibiotic supplementation. We found no differences in previous IVM experiments using cat oocytes when the latter supplements were added (unpublished data).

Following maturation, all oocytes from female #1, #2 and #5 were denuded using 0.1 % hyaluronidase and incubation times varying between 30 s and 10 min; manual pipetting was applied as needed until all cumulus cells were fully removed. Oocytes were then fixed in 4 % paraformaldehyde (PFA), and washed in phosphate-buffered saline (PBS). Chromatin configuration in some oocytes was assessed by staining with 1 μg/mL Hoechst 33342. Oocytes were washed and mounted in a medium composed of 50 % PBS, 50 % glycerol, and 0.0025 μg/mL Hoechst 33342 between a glass slide and a coverslip. Fluorescence microscopy was used to evaluate nuclear maturation, and oocytes were classified according to meiotic stage as germinal vesicle, metaphase I, metaphase II, or with an extruded polar body. Oocytes were regarded as mature when a metaphase II or an extruded polar body was observed.

2.4. Semen collection, evaluation, storage and preparation for IVF

Semen collection was carried out under anesthesia as described [32,35]. Electroejaculation was performed with sets of 10 pulses at incremental voltages (2 – 5 V), divided into four sequential series: the first series at 2, 3, and 4 V; the second at 3, 4, and 5 V; and the third and fourth series at 4 and 5 V. Semen was collected in sterile, pre-warmed 25-mL polypropylene containers (Lab Center, Madrid, Spain). Semen was assessed for volume, pH (Medi-Test Combi 9 strips; Macherey-Nagel, Düren, Germany) and sperm motility (using a pre-warmed slide and a coverslip under phase contrast microscopy), recording the percentage of motile spermatozoa and the quality of movement in a scale from 0 to 5 (0 = no movement; 5 = rapid, linear forward progression). Acrosome integrity and morphology was assessed in sperm fixed with 4 % PFA and stained with Coomassie Brilliant Blue [41].

Semen was diluted 1:1 (v/v) with Ham's F-10 medium containing HEPES (Irvine Scientific, Santa Ana, CA, USA), L-glutamine, pyruvate, gentamicin sulfate, penicillin G, streptomycin, and neomycin, and 5 % heat-inactivated fetal bovine serum (FBS; Gibco Invitrogen, Barcelona, Spain) [32,35]. Diluted semen was centrifuged 8 min at 500 × g, the pellet gently resuspended in TEST extender (4.83 % TES, 1.15 % Tris, 0.4 % glucose penicillin G, streptomycin, 20 % egg yolk, and 4 % glycerol) and the sample loaded into 30 μL straws. Refrigeration was performed using a controlled cooling process for 2 h (from 20 ºC to 5ºC, at −0.125ºC/min) in a programmable dry block temperature system (Thermostat 35780, Eppendorf, Hamburg, Germany) equipped with a dry thermoblock chamber (CombiBox, Eppendorf) [32]. After cooling, straws were frozen using a two-step protocol [42], placing them first 7.5 cm above the surface of liquid nitrogen for 1 min, then at 2.5 cm above liquid nitrogen for 1 min, and finally plunging them in liquid nitrogen.

In the first series, cryopreserved semen was thawed by immersing straws in a 37 °C water bath for 30 s. Thawed spermatozoa were transferred to 1.5 mL tubes and diluted (1:3 v/v) slowly by the addition of a modified Tyrode's solution as used for IVF ([36]; see below). In the second series, spermatozoa were thawed for 50 s at 37 ºC and then selected by density gradient centrifugation using a BoviPure® Bottom Layer (BoviPure®, Nidacon, Sweden) [43]. The resulting pellet was carefully resuspended in 60 μL of IVF medium previously equilibrated under incubation conditions. Aliquots were taken for assessments of sperm motility, acrosome integrity and mitochondrial potential (evaluated using JC-1 fluorescent probe).

Upon semen collection, sperm motility was, on average, 70–90 %, with a score of 3.0 – 3.5, 40 – 60 % acrosome integrity, and 20 – 60 % morphologically normal sperm. Upon thawing, in diluted sperm (series I), motility was 35 – 50 % and acrosome integrity was 40 %, whereas in density-gradient selected sperm, progressive motility was 65 % and acrosome integrity was 58 %. Assessments of mitochondrial membrane potential showed 12 % of spermatozoa with high potential, 42 % with medium potential, and 46 % with low potential.

2.5. In vitro fertilization

Prior to IVF, oocyte maturation from females #3, #4 and #6 was assessed by observation of cumulus cell expansion before fertilization. Subsequently, oocytes from females #3 and #4 were coincubated with frozen–thawed Iberian lynx spermatozoa. Oocytes (10 – 20 per drop) were placed in 50 – 100 μL drops of Tyrode's solution supplemented with 15 mM $NaHCO_3$, 0.36 mM pyruvate, 2.2 mM calcium lactate, 1 mM glutamine, 0.1 mg/mL penicillin, 0.1 mg/mL streptomycin and 0.6 % (w/v) BSA (fatty acid free; catalogue no. 1265479; Calbiochem, Madrid, Spain [36,44]). Oocytes were coincubated, under mineral oil, with 0.5 – 1.5 × 10^5 motile spermatozoa/mL in an atmosphere of 5 % CO_2/air at 38.5 ºC for 18 – 20 h.

Matured oocytes of female #6 were transferred to a laboratory-prepared medium (FERT-TALP media, supplemented with 25 mM $NaHCO_3$, 22 mM sodium lactate, 1 mM sodium pyruvate, 46 mg/mL fatty acid free BSA, and 10 μg/mL heparin), previously shown to sustain IVF and early embryo development in the domestic cat [43,45,46]. Oocytes were co-incubated with frozen-thawed and density-gradient selected spermatozoa (1 × 10^6 spermatozoa/mL) for 18 h at 38.5 ºC under 5 % CO_2/air, with maximum humidity, in 60 μL droplets under mineral oil. Oocytes were cultured in two plates, in three separate drops, placing grade I–II oocytes ($n = 12$, n = 12, and $n = 13$) and grade III oocytes ($n = 11$, $n = 11$, and $n = 10$) in different droplets.

2.6. In vitro culture

Presumptive zygotes from oocytes of females #3 and #4 ($n = 10$ and $n = 19$, respectively) were placed in four-well dishes with 500 μL Tyrode's solution containing 1 % (v/v) minimum essential medium

(MEM) non-essential amino acids, 0.3 % (w/v) BSA (fatty acid free), 15 mM $NaHCO_3$, 0.36 mM pyruvate, 2.2 mM calcium lactate, 1 mM glutamine, 0.1 mg/mL penicillin and 0.1 mg/mL streptomycin [32,35,36,44]. Incubation was carried out at 38.5 ºC under 5 % CO_2/air at maximum humidity. Cleavage was evaluated 44 – 48 h later.

Presumptive zygotes from oocytes of female #6 ($n = 47$) were group-cultured *in vitro* for 10 days post-insemination (dpi) [46]. Groups based on the original oocyte grading were maintained. Incubation was carried out at 38.5 °C under 5 % CO_2, 5 % O_2, 90 % N_2, with maximum humidity. For the first four days of culture post-insemination (1 – 4 dpi), zygotes were maintained in synthetic oviductal fluid (SOF) supplemented with 3 mg/mL BSA. On day 4 post-insemination, the culture medium was replaced with SOF supplemented with 10 % FBS. Cleavage rates were recorded at 4 dpi, and morula and blastocyst formation were monitored daily from 6 to 10 dpi. Diameters (including the zona pellucida) of morula- and blastocyst-stage embryos and thickness of the zona were measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

2.7. Embryo vitrification

Day 6 blastocyst ($n = 1$) and morulae ($n = 6$) were cryopreserved using the Kitazato protocol and the Cryotop® open system (Kitazato Corporation, Tokyo, Japan). Briefly, embryos were initially placed in Equilibration Solution (ES) for 12 min, then transferred to Vitrification Solution (VS) for two consecutive 30-s exposures. Subsequently, they were carefully loaded onto the Cryotop® sheet with minimal medium volume and directly plunged into liquid nitrogen [47,48]. Vitrification was performed individually for both blastocysts and morulae, with one embryo deposited per Cryotop®. Samples were subsequently stored in liquid nitrogen.

3. Results

3.1. Experimental Series I - in vitro oocyte maturation

Incubation of oocytes recovered from female #1 ($n = 2$) resulted in no maturation since the oocytes were degenerated when examined after 24 h of culture. Oocytes recovered from female #2 ($n = 16$) exhibited 37.5 % maturation, reaching MII. The remaining oocytes degenerated during incubation (Table 2).

Table 2
Experimental series I - *In vitro* maturation and *in vitro* fertilization of Iberian lynx oocytes. Ovaries were received at the laboratory 12–28 h after necropsy. Oocytes were retrieved from ovaries upon arrival. The majority of oocytes were grade III and they were incubated for 24 h. For two females, *in vitro* fertilization was attempted using cryopreserved spermatozoa with incubation during 18–20 h and subsequent incubation for additional 48 h. Oocytes from females #1 and #2 were evaluated at the end of the maturation period by examining for presence of MII. Oocytes from females #3 and #4 were retrospectively assessed as MII or degenerated after IVF and additional incubation.

Female ID	No. of oocytes	No. degenerated after IVM (%)	No. reaching MII (%)	No. fertilized or cleaved (%)
1*	2	2 (100)	0 (0)	ND
2	16	10 (62.5)	6 (37.5)	ND
3	10	ND	ND	0 (0)(1)
4	19	ND	ND	0 (0)(2)

ND, not done/not determined.
(1) 1 MII oocyte and 9 degenerated oocytes after 18–20 h of incubation for IVF and additionl 48 h incubations.
(2) 1 MII oocyte and 18 degenerated oocytes after 18–20 h of incubation for IVF and additionl 48 h incubations.
* Only one ovary was available for oocyte retrieval.

3.2. Experimental Series II - in vitro oocyte maturation

Maturation rates for oocytes retrieved from female #5 were 40 % for grade I–II oocytes and 28.5 % for grade III oocytes. Following IVM, oocytes were classified according to nuclear status as degenerated, immature (germinal vesicle [GV] or metaphase I [MI]), or mature (metaphase II [MII] and/or exhibiting polar body [PB] extrusion) (Figs. 1, 2, Table 3).

3.3. In vitro fertilization, cleavage, morula and blastocyst rate

In vitro matured oocytes collected from females #3 and #4, co-incubated with cryopreserved spermatozoa, did not show evidence of fertilization or cleavage. After 48 h in culture a single oocyte from each female was in MII stage, unfertilized in both cases, and all the remaining ones were degenerated (Table 2).

Cleavage rates for oocytes retrieved from female #6 were 39.3 % in the grade I–II group and 7.1 % in the grade III group. Morula rates were 30.3 % and 7.1 %, respectively, while blastocyst relative to cleaved embryos was 7.6 % and 0 %, respectively (Fig. 3**A,B,** Table 4).

For confirmation of early blastocyst development, embryo measurements were compared. A morula on 4 dpi measured 163 μm in its maximum diameter and had a 29 μm thick zona pellucida. When it developed to early blastocyst on 6dpi, its maximum diameter was 167 μm and the zona was 17 μm thick, confirming expansion of the cells (Fig. 3**A,B**).

3.4. Embryo cryopreservation

Morulae and early blastocyst cryopreservation was carried out, and visualization of media absorption occurred during the process. Future studies are needed to evaluate the viability of these embryos.

4. Discussion

To the best of our knowledge, this is the first report describing IVM, IVF and early-stage *in vitro* embryo development in the Iberian lynx. This achievement is especially significant given the species' endangered status and its dramatic population recovery over the past two decades. From a population of less than 100 individuals in 2002, the number of Iberian lynxes surpassed 2000 by 2024 [49]. This recovery has been largely attributed to the implementation of an *ex situ* breeding program, which has played a pivotal role in establishing biological resource banks and facilitating the creation of new populations through the reintroduction of controlled captive-bred individuals into their natural habitats [24,30,31].

Nevertheless, the recovery of the Iberian lynx from a critically low number of founders still presents significant genetic challenges. A reduced gene pool has led to an increased prevalence of inherited disorders, such as juvenile idiopathic epilepsy, which may have become more frequent due to inbreeding [29,50]. In this context, the application of IVM-IVF and subsequent ET could serve as a key tool to counteract the negative effects of limited genetic diversity. These approaches would not only enable the use of cryopreserved semen from genetically valuable individuals that are no longer alive, but also promote genetic exchange between *ex situ* populations without requiring the physical movement of animals. This would facilitate more flexible and controlled genetic management, ultimately contributing to the health, resilience, and long-term sustainability of the species [51].

The IVM studies show favorable results in the second experimental series when compared to the first one. Different factors may explain these different outcomes. Firstly, the source of the samples between the two series needs to be considered, since oocytes from the first were retrieved outside the reproductive season and those matured in the second series were retrieved during the breeding period. Season has been highlighted in felids as a contributing factor for oocyte retrieval

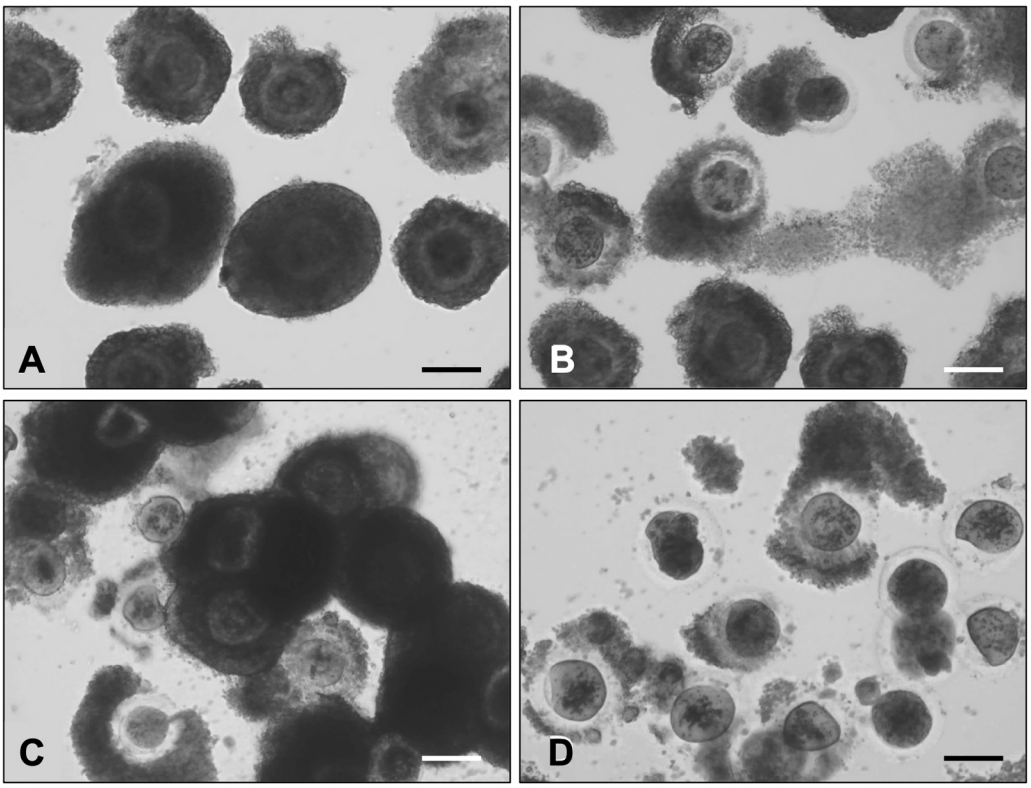

Fig. 1. Iberian lynx (female #5) oocytes retrieved by ovarian slicing prior to *in vitro* maturation and after completion of the maturation process. (A,B) After slicing. (C,D) After *in vitro* maturation. (A) Grade I–II after slicing, (B) Grade III after slicing. (C) Grade I–II after 28 h of *in vitro* maturation. (D) Grade III oocytes after 28 h of *in vitro* maturation. Scale bars = 100 μm.

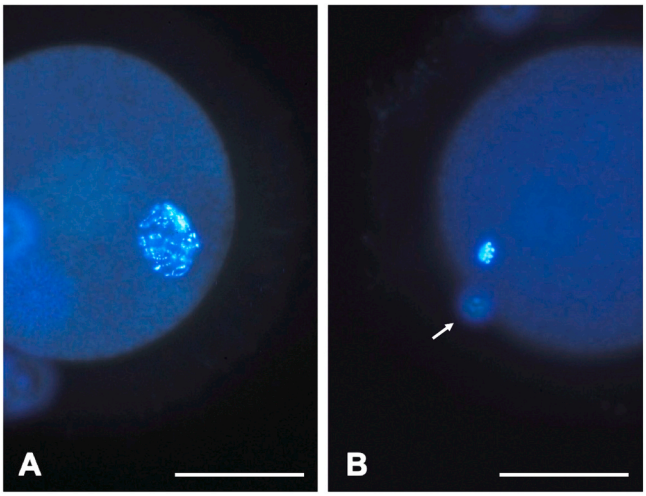

Fig. 2. Iberian lynx oocytes after *in vitro* maturation showing (A) germinal vesicle (immature oocyte), and (B) mature oocyte with an extruded polar body (arrow). Scale bars = 50 μm.

and blastocyst production [52], being a plausible cause for these differences. The methods applied in each series were practically identical except for the length of incubation. The outcomes, therefore, favor IVM for 28 h in contrast to 24 h, following prior findings that support that feline oocytes may not be fully competent after 24 h of IVM; as well as comparable IVP rates when maturing the retrieved oocytes for 26–28 h prior to IVF [46,53,54].

The results obtained with the IVM-IVF procedures performed in this study are promising, demonstrating the feasibility of applying advanced ARTs developed in the domestic cat to the Iberian lynx. Although the observed maturation and cleavage rates were lower than those typically reported in the domestic cat, approximately 60 % for maturation, 60 % for cleavage, and around 20 % blastocyst development from cleaved zygotes depending on the author [20,55,56], the present outcome still represents a significant milestone in feline conservation biology.

It is important to note that, in several instances, ovaries were recovered from road-killed females, with no information available regarding the stage of the estrous cycle. Furthermore, ovaries were transported to the laboratory under refrigeration during varying times (range 12 – 28 h). In the domestic cat, a 24 h-transport at around 4 °C has been shown to have minimal impact on the maturation capacity and blastocyst production [57,58]. There is still no information for the Iberian lynx on the effect of time and temperature of transportation of

Table 3
Experimental series II - *In vitro* maturation of Iberian lynx oocytes. Oocytes were recovered from female #5. IVM was performed using conditions similar to those used in Series I, with the exception that the incubation time was 28 h instead of 24 h. See Materials and methods for details. Results show nuclear maturation status of grade I–II or grade III oocytes after *in vitro* maturation.

Oocyte quality	No. oocytes	No. oocytes degenerated (%)	No. in GV	No. GVBD	No. MI	No. MII/PB	% Maturation rate
Grade I-II	33	8 (24.2)	3	4	8	10	40.0
Grade III	25	11 (44.0)	2	4	4	4	28.5

Abbreviations: GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II; PB, polar body extruded.

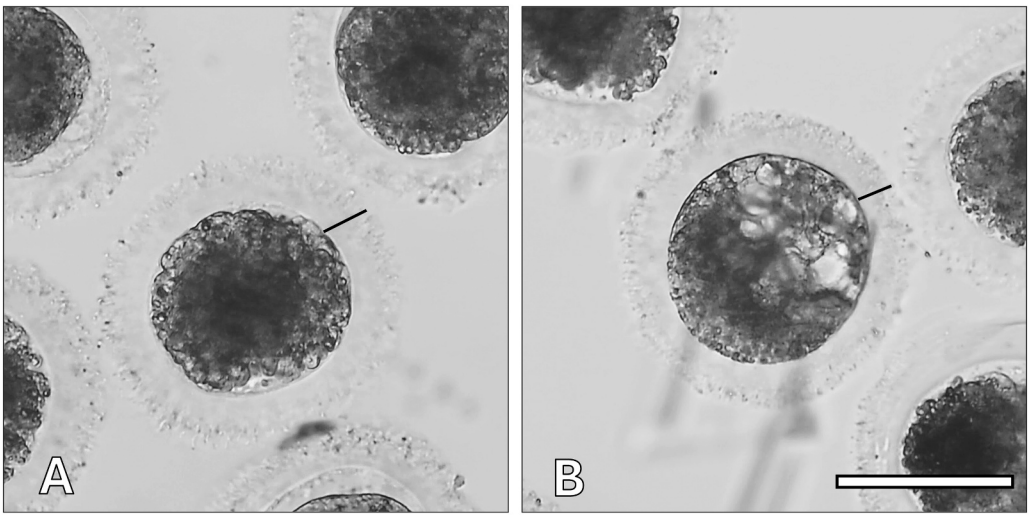

Fig. 3. Embryo development stages after *in vitro* fertilization. (A) Morula on day 6. Maximum diameter (including zona pellucida): 163 μm. Thickness of zona: 29 μm. (B) Grade II early blastocyst on day 6. Maximum diameter (including zona pellucida): 167 μm. Thickness of zona: 17 μm. Scale bar = 100 μm.

Table 4
In vitro maturation (IVM) and fertilization (IVF) of Iberian lynx oocytes collected from female #6. Results show number of oocytes selected for IVM, presumptive zygotes incubated after IVF, cleavage rate, morula rate and blastocyst rate. *In vitro* embryo production was performed by maturing *in vitro* selected oocytes for 28 h, and subsequently coincubating them with frozen-thawed sperm using conventional IVF. *In vitro* culture of selected presumptive zygotes was done during 10 days, with a change in culture medium on day 4 post insemination, at which time cleavage was also recorded. Morula and blastocyst development was evaluated from day 6 to day 10 post insemination.

Oocyte quality	Oocytes selected for IVM	Presumptive zygotes	Cleaved/ zygotes (%)	Morulae (%)	Blastocyst (%)	Blastocyst/ cleaved (%)
Grade I-II	37	33		10 (30.3)	1 (3)	1/13 (7.6)
		13 (39.3)				
Grade III	32	14		1 (7.1)	0	0
		1 (7.1)				

ovaries on embryo production, so these variables could still contribute to the low developmental rates observed in this study. This is futher confounded by the fact that the exact period of time elapsed between the animal's death and ovary retrieval was unknown. This is a common limitation for wildlife where response time depends entirely on when the carcass is discovered, reported and handled. In such scenarios, rapid action is often constrained by logistics and external notification, making standardization particularly challenging. In any case, the development of an early blastocyst in this context is encouraging, as it supports the feasibility of utilizing ovaries retrieved post-mortem or following ovariohysterectomy due to disease for *in vitro* embryo production in this endangered species.

This outcome also supports the validity of the IVM-IVF protocol previously developed by us [43] as suitable for the *in vitro* production of Iberian lynx embryos. The results suggest not only that the incubation conditions - including media composition and supplementation - are appropriate for this species, but also that the quality of the previously cryopreserved semen was adequate to enable fertilization and early embryonic development. Notably, the semen used in this study was obtained from males of proven fertility, as they had sired multiple litters through natural mating, further confirming the viability of spermatozoa after cryopreservation.

Having access to cryopreserved embryos offers a valuable opportunity to investigate and establish a suitable ET protocol for this species. To date, no such procedures have been performed in the Iberian lynx, and key parameters, such as the optimal hormonal stimulation regime, the most suitable embryonic stage for transfer, and the most effective transfer technique, remain unknown. However, as ET has already been successfully applied in other wild felids [8,12,14,17], it provides a solid foundation to develop and adapt similar strategies for the Iberian lynx.

Despite the success represented by this achievement, it is crucial to develop further studies to better understand the physiology of these animals in order to adapt our protocols for greater efficacy. Moreover, although the IVP protocol proved effective in this case, further research using the domestic cat as a model is still required to identify the optimal culture media and supplementation to enhance developmental rates.

5. Conclusion

This study presents the first report of IVM and IVF resulting in early embryo development in the Iberian lynx, representing a significant advancement in the application of ARTs for wild felid conservation. The use of post-mortem oocytes and cryopreserved semen from genetically valuable males demonstrates the potential of *in vitro* generated embryos to enhance genetic management in this genetically challenged species. Although developmental rates remain lower than those reported in domestic cats, the ability to generate and cryopreserve embryos opens new avenues for future ET protocols. Further research is required to optimize culture conditions and identify the most effective strategies for ET, ideally supported by continued studies in domestic felids as translational models. These findings make a significant contribution to the conservation toolbox for the Iberian lynx and underscore the importance of integrating ARTs into comprehensive conservation strategies.

CRediT authorship contribution statement

Andrea Priego-González: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis. **Ana Munoz-Maceda:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Raquel González:**

Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Juan Antonio Rielo:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Natalia Gañán:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Ana Sanchez-Rodriguez:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Pilar Villar-Moya:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Ana Cantos Rubert:** Writing – review & editing, Methodology, Investigation. **María Jesús Sánchez-Calabuig:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Eduardo R.S. Roldan:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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