scientific reports

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Heat stress affects the functionality of the ovine cumulus-oocyte complex and subsequent in vitro embryo production

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Global population growth requires an increase in the production of food, particularly meat, with an expected increase in sheep farming. However, climate change challenges livestock management, with heat stress negatively impacting reproductive performance. In vitro embryo production (IVP) in sheep farming is promising, although optimizing embryo quality and efficiency remains challenging. Heat stress impairs oocyte developmental competence, affecting IVP outcomes. This study investigated the effects of season on oocyte quality and embryo production given seasonal variations in the temperature and temperature–humidity index (THI) and in vitro-induced heat stress. In the first experiment, ovaries were collected in four seasons (winter, spring, summer and autumn), with differences in THI, and in the second experiment, ovaries were exposed to 30 °C (control), 38.5 °C, 40 °C and 41 °C. The results indicated that elevated summer temperatures significantly compromised oocyte and cumulus cell viability, DNA integrity, mitochondrial distribution, and blastocyst quality. These detrimental effects persisted into autumn, likely due to a carry-over effect from summer heat stress. Furthermore, in vitro exposure to temperatures at or above 38.5 °C led to marked decreases in oocyte quality and blastocyst rates. Understanding these effects is essential for developing strategies to mitigate heat stress and enhance reproductive outcomes in sheep.

Keywords Ovine, High temperature, Oocyte quality, Embryo quality, Temperature–humidity index

Driven by growth in developing countries, the world population is projected to reach 8.5 and 9.7 billion people by 2030 and 2050, respectively, thereby requiring an approximately 70% increase in food production globally[1]. Annual meat production, for example, would have to grow by more than 200 million tons[1]. Particularly in Europe, the prevalence of sheep meat is also expected to increase due to the diversification of meat diets and changes in population structure[2]. To meet these demands and reduce environmental impacts, advances in animal agriculture through reproductive biotechnologies will be necessary to provide increasingly efficient and productive livestock and adapt to climate change and global warming[3]. In this context, the application of advanced assisted reproductive technologies, such as in vitro embryo production (IVP), in the farming sheep industry is highly promising and offers several advantages, as it allows the production of embryos from oocytes recovered from unstimulated ovaries, nonfertile females, and prepubertal, pregnant, senile, and even dead or slaughtered animals[4]. In addition, IVP facilitates the preservation of valuable biospecimens and provides secure transportation of biological material. Nevertheless, despite substantial improvements, the efficiency and quality of IVP-derived embryos are still among the main challenges of livestock farming[5,6].

Globally, new research into how climatic conditions strongly affect reproduction in domesticated farm animals is gaining interest throughout the agricultural sector[7]. From the standpoint of economic efficiency, the reproductive ability of domestic food animals such as sheep is the most critical trait that may be compromised by climate stressors[8]. Previously, fundamental research into environmental stress during summer and its influence on sheep has provided evidence that heat stress can negatively influence oocyte developmental competence and result in embryonic loss[9]. Heat stress may lead to changes in oocyte membrane properties, such as decreased phospholipid polyunsaturated fatty acid contents[10], which are essential for gamete fertility[11]. Furthermore, it

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may alter the transcript levels of genes involved in oogenesis, folliculogenesis, and embryonic development[12] and impair nuclear and cytoplasmic maturation events such as the translocation of cortical granules and cytoskeletal rearrangement[13], which can eventually lead to apoptosis[14]. In addition, heat stress may affect the spatial distribution of mitochondria within the oocyte, possibly through alterations in the cytoskeleton[15], as well as the proportion of highly polarized mitochondria and the expression of developmentally important genes[16,17].

Moreover, in summer, it is not unusual for sheep IVP systems to endure some periodic reductions in embryo yield[18]. In fact, most laboratories cease their activity during the hottest months. Previously, variability in ovine IVP output throughout the year was attributed to an effect of season related to photoperiod[19]. However, among the several variables that influence seasonal patterns of blastocyst production is also environmental temperature[20]. Consequently, understanding the effects of high temperatures that prevail throughout the warm season on oocyte quality may be a prerequisite for the successful implementation of IVP[21].

Therefore, in the present study, the first objective was to evaluate the effects of the season (periods with differences in ambient temperature and humidity) in which the sheep ovaries are harvested (winter, spring, summer, and autumn) on oocyte quality and in vitro embryo production. Additionally, we examined the impact of heat stress through elevated ovarian temperature in an in vitro model to better understand the physiological mechanisms of oocyte damage. Such research may ultimately help develop novel strategies to mitigate the impact of summer heat stress on oocyte quality and protect the integrity of the female germline in small ruminants.

Materials and methods

All chemicals were acquired from Merck Life Sciences (Madrid, Spain) unless otherwise stated.

Experimental design

Figure 1 shows the experimental design. In Experiment 1, to study the effects of season on oocyte quality and developmental competence, adult sheep ovaries were collected twice a month from an abattoir located in southeastern Spain (e.g., Murcia; latitude: 37° 59' 13.34" N; longitude: −1° 07' 48.14" W) from surrounding areas during winter (December–February), spring (March–May), summer (June–August), and autumn (September–November) and transported to the laboratory within 3 h at 30 °C in physiological saline (8.9 gr/L NaCl) supplemented with penicillin (0.1 g/L). Immediately after arrival, the ovaries were processed. To study maturation rates and oocyte quality parameters, 980 cumulus–oocyte complexes (COCs) were matured in vitro and further examined. The remaining 2115 COCs were subjected to in vitro fertilization (IVF) and in vitro culture (IVC) to evaluate their fertilization potential, embryo development, and blastocyst quality.

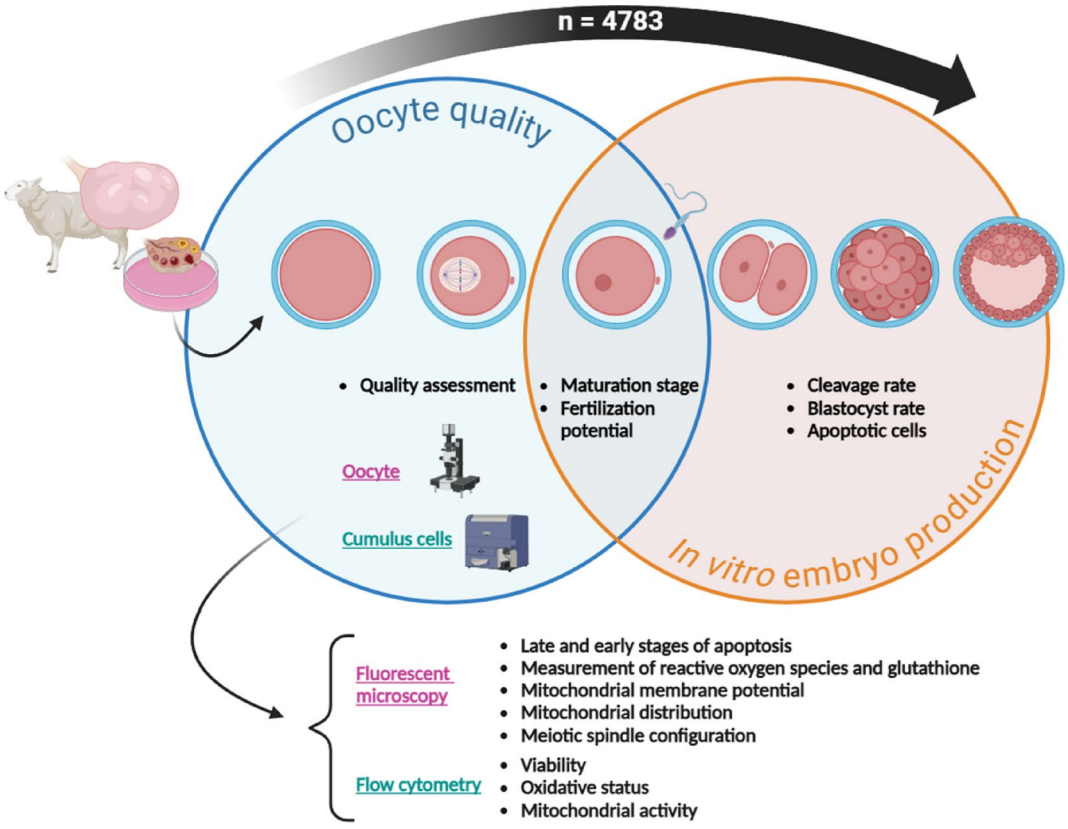

Fig. 1. Schematic representation of the experimental design. Illustration created using BioRender (https://biorender.com).

In Experiment 2, to investigate the effects of induced heat stress on oocyte quality and developmental competence, an *in vitro model* was developed. For this purpose, adult sheep slaughterhouse ovaries were collected postmortem from the same abattoir used in Experiment 1, receiving sheep from surrounding areas during late autumn and winter. Afterward, the ovaries were transported in physiological saline (8.9 g/L NaCl) supplemented with penicillin (0.1 g/L) at 30 °C, 38.5 °C, 40–41 °C and stored for 3 h before processing. The different temperatures were maintained with incubators connected to the transport vehicle. The quality and developmental potential of 1135 oocytes were subsequently evaluated in the laboratory after in vitro maturation (IVM), and 553 COCs were subjected to in vitro fertilization (IVF) and in vitro culture (IVC) to evaluate their fertilization potential, embryo development, and blastocyst quality.

In both experiments, all the oocyte characteristics described in this manuscript were evaluated at the same time for each replicate or collection date; therefore, the number of oocytes available for each specific analysis was inherently limited.

All experiments involving the collection and evaluation of COCs, in vitro maturation and fertilization of oocytes, and the assessment of fertilized oocytes, embryos and cumulus cells were conducted following the procedures previously described by our working group[22–24].

Climate data and calculation of the temperature–humidity index for heat stress assessment

The daily observed meteorological data from 2019 to – 2020 (time of ovarian collection) for each ovary collection season were obtained from the State Meteorological Agency of Spain for the location of Puerto Tocinos (Murcia). Daily values for maximum temperature measurements and relative humidity (RH hereafter) were used to determine the mean values for each variable per season. As previously described by Carabaño et al.[25], the temperature-humidity index (THI) was used to assess the potential for heat stress in sheep at our latitude. The THI was formulated specifically for ruminant species[26] and was examined for seasonal patterns of variability. The THI formula used is shown below, with temperatures in degrees Celsius and RH expressed as a percentage:

THI ruminant = $(1.8\ T_{max} + 32) - ((0.55 - 0.0055\ RH)\ (1.8\ T_{max} - 26.8))$.

The level of heat stress was considered as follows: normal ≤ 74; moderate 75–78; severe 79–83; and very severe (emergency) ≥ 84 [27].

Oocyte collection and in vitro maturation

Immature COCs were retrieved from the follicles using a scalpel blade in 2 mL of collection medium (TCM199 medium supplemented with 2.38 mg/mL HEPES, 2 µL/mL heparin, and 4 µL/mL gentamicin). Immediately, COCs with clear or moderately granular ooplasm surrounded by at least three layers of packed cumulus cells were selected and homogeneously distributed in selection medium (TCM199 medium supplemented with 2.38 mg/mL HEPES and 4 µL/mL gentamicin). The COCs were subsequently washed with TCM199 and 4 µL/mL gentamicin. The COCs were then homogeneously distributed in 4-well plates with 500 µL of maturation medium: TCM199 and 4 µL/mL gentamicin, 100 µM cysteamine, 10 µg/mL follicle-stimulating hormone (FSH), 10 µg/mL luteinizing hormone (LH), and 10% fetal calf serum (FCS). The maturation medium was covered with mineral oil (Nidacon, Gothenburg, Sweden), and the COCs were incubated for 24 h at 38.5 °C, 5% CO_2, and maximal humidity.

In vitro fertilization

Groups of approximately 40–45 mature oocytes were placed in four-well dishes containing 500 µL of fertilization medium: synthetic oviductal fluid (SOF)[28] supplemented with 10% estrous sheep serum (ESS). Oocytes were subjected to IVF using the frozen semen of two rams from the germplasm bank of the "Biology of Reproduction Group" of the Universidad de Castilla-La Mancha (UCLM), which is authorized for the collection and storage of sheep semen (ES008007). Thawed spermatozoa were separated using a Percoll© density gradient (45%/90%) and capacitated for 15 min at 38.5 °C and 5% CO_2 in fertilization medium. Then, the spermatozoa (1×10^6/mL) and oocytes were coincubated at 38.5 °C in 5% CO_2 with maximal humidity.

In vitro embryo culture

At 18 h post insemination (hpi), the putative zygotes were washed by repeated pipetting and transferred to 25 µL drops (approximately one embryo per µL) of culture medium (SOF supplemented with 3 mg/mL bovine serum albumin), covered with mineral oil and cultured until Day 8 post insemination (dpi) at 38.5 °C in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2 in air.

Evaluation of fertilization and embryo production rates

After IVF, the oocytes were fixed in 0.5% glutaraldehyde (v/v) for 15 min at room temperature and stored at 4 °C until analysis. To examine sperm penetration, the cells were stained with Hoechst 33,342 (5 µg/mL) for 20 min at room temperature, washed in phosphate-buffered saline (PBS) supplemented with 0.1% PVA (w/v; PBS-PVA), and then analyzed with 20× augmentation by fluorescence microscopy (Eclipse 80i, Nikon Instruments Europe, Amsterdam, Netherlands). Oocytes containing both female and male pronuclei (regardless of the stage of decondensation) relative to the total number of oocytes that matured were considered fertilized and were classified as normal (2PN) according to the numbers of swollen sperm heads and pronuclei in the cytoplasm.

The cleavage and blastocyst rates were checked at 48 hpi and at 6, 7, and 8 dpi. All expanded blastocysts were fixed in 0.5% glutaraldehyde (v/v) and stored at 4 °C for TUNEL analysis and cell number evaluation.

Determination of the nuclear maturation stage

After maturation, the oocytes were washed in PBS-PVA, denuded from cumulus cells by gentle pipetting, fixed in 0.5% glutaraldehyde (v/v) for 15 min and stored at 4 °C. The day of the analysis, the oocytes were placed

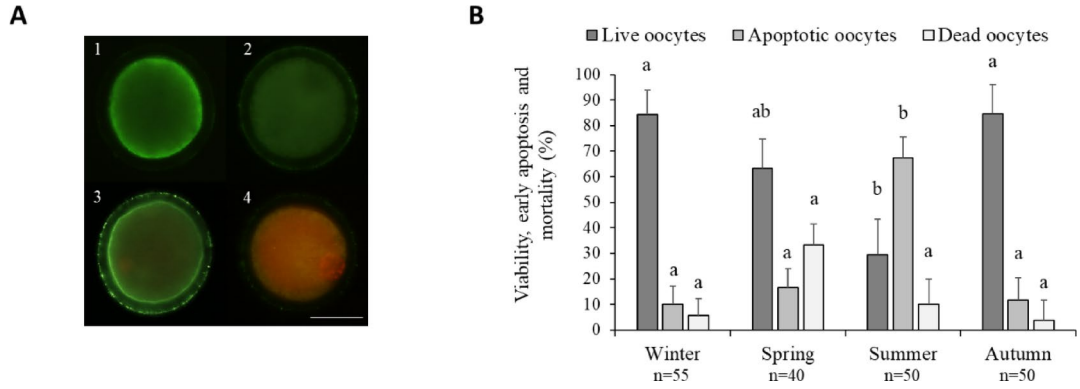

Fig. 2. (**A**) Representative images of early apoptosis detection in sheep oocytes after maturation. 1: Early apoptotic oocytes: Annexin V positive, represented by a green signal across the oocyte membrane. 2: Viable oocytes: no green Annexin V or red PI signal. 3–4: Dead cells: PI-positive signal. Scale bar = 50 µM. (**B**) Effects of season on the viability and early apoptosis rates of sheep oocytes after maturation. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between seasons.

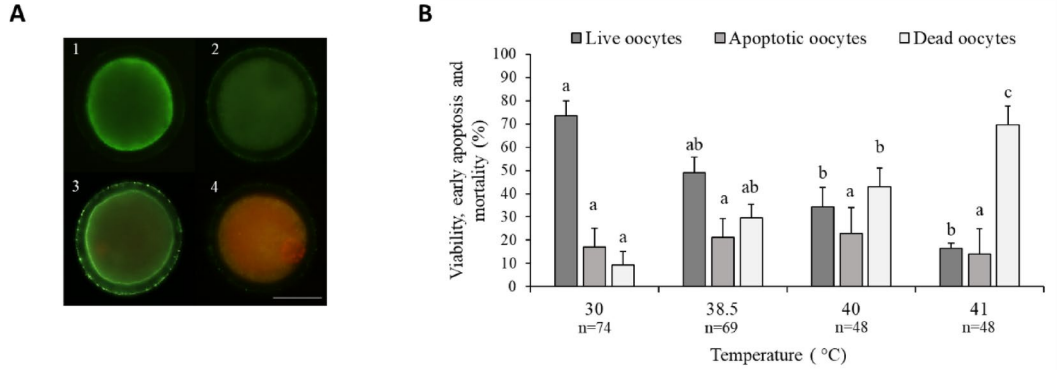

Fig. 3. (**A**) Representative images of early apoptosis detection in sheep oocytes after maturation. 1: Early apoptotic oocytes: Annexin V positive, represented by a green signal across the oocyte membrane. 2: Viable oocytes: no green Annexin V or red PI signal. 3–4: Dead cells: PI-positive signal. Scale bar = 50 µM. (**B**) Effects of temperature on the viability and early apoptosis rates of sheep oocytes after maturation. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between temperatures.

on a glass slide with a 1 µL drop of SlowFade™ and 5 µg/mL Hoechst 33,342 under a coverslip. After 20 min at room temperature, chromatin configurations were analyzed using fluorescence microscopy (Eclipse 80i, Nikon Instruments Europe, Amsterdam, The Netherlands) at 40× magnification with an excitation (Ex) filter of 365/28 nm, a dichroic mirror (DM) of 405 nm and an emission (Em) filter of 445/50 nm. Oocytes showing a germinal vesicle (GV) chromatin configuration were considered immature, and those showing a metaphase plate and a polar body were categorized as mature metaphase II (MII) oocytes.

Viability, early apoptosis, and mortality assessment

Following the manufacturers' protocol, early apoptosis was assessed using Annexin V staining (Invitrogen©, Thermo Fisher Scientific, Barcelona, Spain). After maturation, denuded oocytes were placed in 100 µL of Annexin V binding buffer droplets containing 5 µL of fluorescein isothiocyanate (FITC)-conjugated Annexin V and 1 µL of propidium iodide (PI; 100 µg/mL) and incubated at 37 °C on a heated plate in the dark for 15 min. After washing three times in PBS-PVA, the oocytes were mounted on slides under coverslips. The oocytes were analyzed using fluorescence microscopy (Eclipse 80i, Nikon Instruments Europe, Amsterdam, The Netherlands) at 20× magnification with an Ex filter of 475/30 nm, a DM 405 nm, and an Em filter of 530/40 nm. Oocyte status was classified into the following categories: viable (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), and dead (Annexin V-/PI + and Annexin V+/PI+), and the percentage of oocytes in each category was calculated. Representative images of different categories are shown in Figs. 2A and 3A.

Measurement of reactive oxygen species (ROS) and reduced glutathione (GSH)

After maturation, the oocytes were incubated in 10 µM CM-H_2DCFDA (Thermo Fisher Scientific, Barcelona, Spain) and 10 µM Cell Tracker Blue (Thermo Fisher Scientific, Barcelona, Spain) for 30 min at 37 °C in the dark to detect intracellular reactive oxygen species (ROS) and reduced glutathione (GSH), respectively. The oocytes

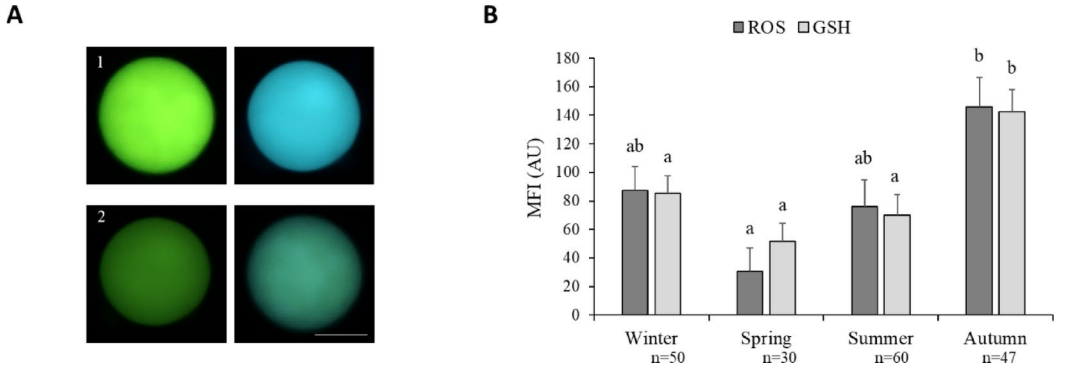

Fig. 4. (**A**) Representative images of reactive oxygen species (ROS) and glutathione (GSH) levels in sheep oocytes after maturation. 1: High ROS and GSH intensity. 2: Low ROS and GSH intensity. Scale bar = 50 µM. (**B**) Effects of season on ROS and GSH levels (mean fluorescence intensity (MFI)) in oocytes after maturation. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between seasons.

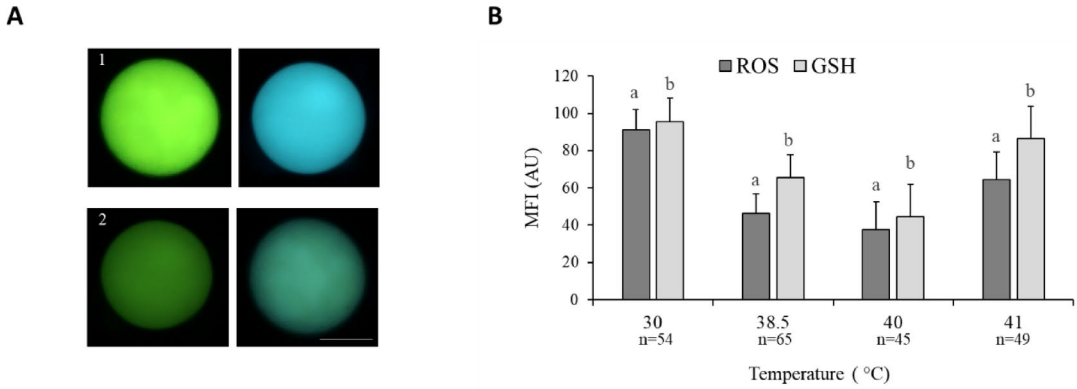

Fig. 5. (**A**) Representative images of reactive oxygen species (ROS) and glutathione (GSH) levels in sheep oocytes after maturation. 1: High ROS and GSH intensity. 2: Low ROS and GSH intensity. Scale bar = 50 µM. (**B**) Effects of temperature on ROS and GSH levels (mean fluorescence intensity (MFI)) in oocytes after maturation. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between temperatures.

were then washed three times in PBS-PVA and placed on slides under coverslips for evaluation. The fluorescence intensities of Cell Tracker Blue and CM-H_2DCFDA were assessed via fluorescence microscopy (Eclipse 80i, Nikon Instruments Europe, Amsterdam, the Netherlands) at 20× magnification with an Ex filter of 365/28 nm, a DM of 405 nm, an Em filter of 445/50 nm, an Ex filter of 475/30 nm, a DM of 405 nm and an Em filter of 530/40 nm. The mean fluorescence intensity (MFI) of each fluorochrome was quantified using ImageJ (version 1.45s; National Institutes of Health, Bethesda, USA) by measuring the mean pixel intensity within the selected region of interest in 16-bit images and is expressed in arbitrary units (AUs) in Fig. 4A. To account for autofluorescence, the MFI of the region of interest was corrected by subtracting the MFI of four background regions within the same image. This correction minimizes background interference and ensures reliable quantitative comparisons between samples. Representative images of ROS and GSH levels in sheep oocytes are shown in Figs. 4A and 5A.

Mitochondrial membrane potential analysis

The mitochondrial membrane potential was assessed by incubating oocytes after maturation for 30 min at 37°C in 0.5 µM JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Thermo Fisher Scientific, Barcelona, Spain), followed by two 5-min washes and mounting on a glass slide with a coverslip. JC-1 accumulates in mitochondria and reflects the membrane potential across the inner mitochondrial membrane. This dye exhibits two distinct emission peaks: red fluorescence from J-aggregates, indicative of hyperpolarized mitochondria (high membrane potential), and green fluorescence from JC-1 monomers, associated with depolarized mitochondria (low membrane potential). The relative mitochondrial membrane potential was determined as the ratio of green to red MFI[29,30] using ImageJ 1.45s software (National Institutes of Health, Bethesda, USA). A fluorescence microscope (Eclipse 80i, Nikon Instruments Europe, Amsterdam, The Netherlands) at 20× magnification with an Ex filter of 475/30 nm, DM: 405 nm, and Em filter of 530/40 nm was used for this evaluation. MFI quantification and normalization were performed as previously described for fluorescence intensity evaluations, ensuring consistency in data processing and minimizing background

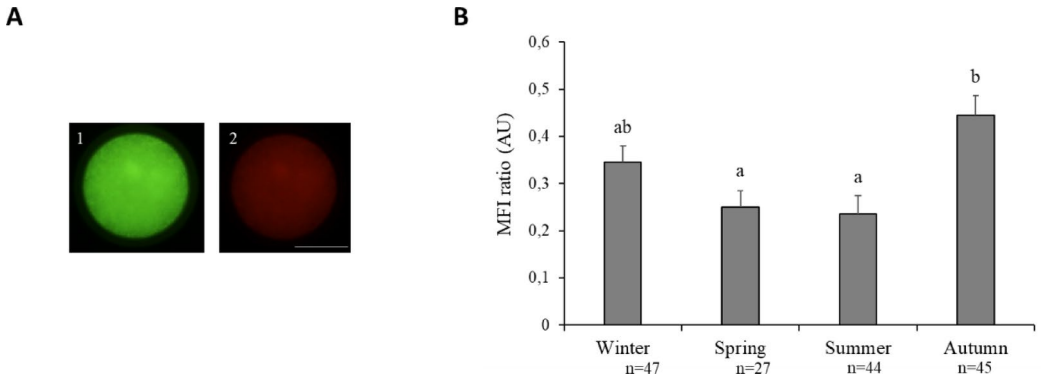

Fig. 6. (**A**) Representative images of JC-1-stained mitochondria in sheep oocytes after maturation. 1: Mitochondria with low membrane potential (green fluorescence). 2: Mitochondria with high membrane potential (red fluorescence). Scale bar = 50 µM. (**B**) Effect of season on the mitochondrial membrane potential: red (high membrane potential)/green (low membrane potential) fluorescence ratio (JC-1 staining). The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between seasons.

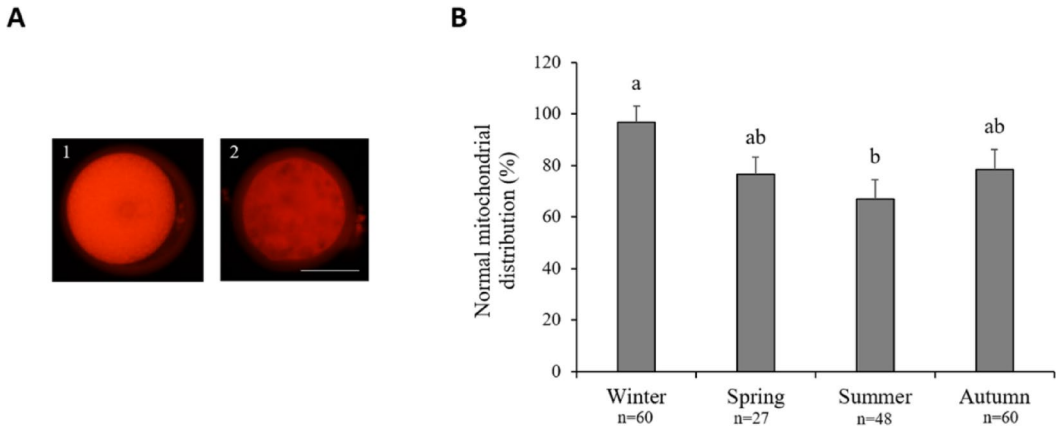

Fig. 7. (**A**) Mitochondrial distribution classification in oocytes after maturation. 1: Normal distribution. 2: Abnormal mitochondrial distribution. Scale bar = 50 µm. (**B**) Effect of season on normal mitochondrial distribution in oocytes after maturation. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between seasons.

interference. The fluorescence intensity ratio in Fig. 6A is expressed in arbitrary units (AUs). Representative images of JC-1-stained mitochondria in sheep oocytes are shown in Fig. 6A.

Assessment of mitochondrial distribution

To determine the mitochondrial distribution patterns, the oocytes were subjected to double staining with MitoTracker© Red CMXRos (Thermo Fisher, Barcelona, Spain), a mitochondria-specific probe, and Hoechst 33,342 to stain the chromosomes. Following IVM, the oocytes were incubated for 20 min in PBS-PVA plus 100 nM MitoTracker© Red CMXRos at 37 °C in the dark. The oocytes were washed three times under agitation for 5 h and then placed on glass slides under a coverslip. Oocytes were examined using fluorescence microscopy (Eclipse 80i, Nikon Instruments Europe, Amsterdam, The Netherlands) at 20× magnification. Hoechst 33,342 fluorescence was detected using an Ex filter at 365/28 nm, a DM filter at 405 nm, and an Em filter at 445/50 nm. MitoTracker® Red CMXRos fluorescence was detected with an Ex filter of 560/20 nm, a DM filter of 585 nm, and an Em filter of 630/40 nm. Mitochondrial distribution was classified into two categories: abnormal mitochondrial distribution in the cytoplasm and normal distribution (Figs. 7A and 8A).

DNA fragmentation assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was used to detect DNA fragmentation in oocytes after maturation and in expanded blastocysts. Samples were fixed in 4% glutaraldehyde for 15 min and permeabilized in 0.5% Triton X-100 for 1 h at room temperature. An In Situ Cell Death Detection Kit (Merck Life Sciences, Madrid, Spain) was subsequently used to detect DNA strand breaks following the manufacturers' instructions. Briefly, oocytes and blastocysts were placed in 30 µL drops of TUNEL reagent with deoxyuridine 5-trisphosphate (dUTP)-conjugated isothiocyanate fluorescein and incubated for 1 h at 37 °C.

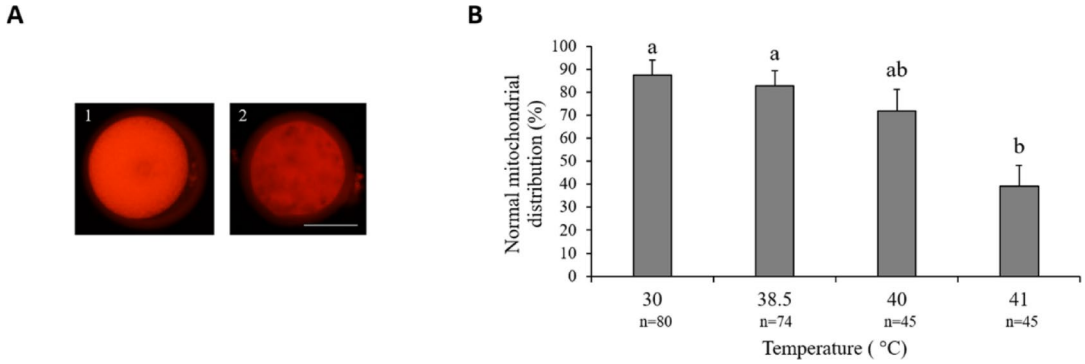

Fig. 8. (**A**) Mitochondrial distribution classification in oocytes after maturation. 1: Normal distribution. 2: Abnormal mitochondrial distribution. Scale bar = 50 µm. (**B**) Effects of temperature on normal mitochondrial distribution in oocytes after maturation. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between temperatures.

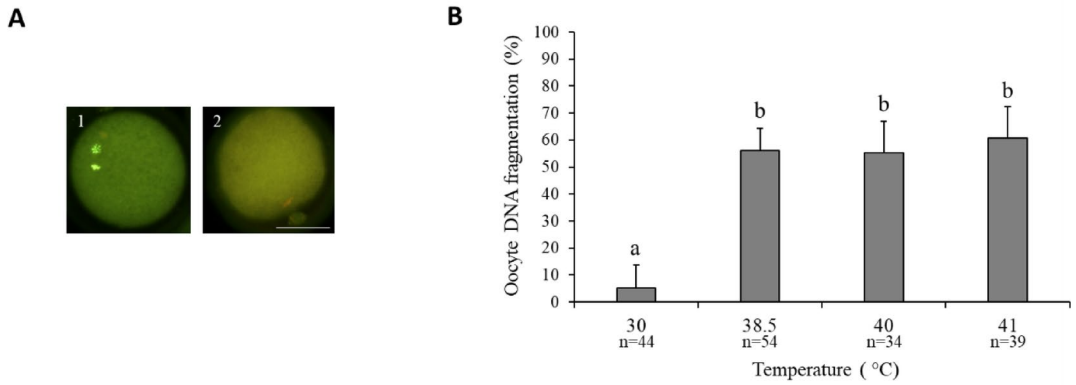

Fig. 9. (**A**) TUNEL staining of oocytes after maturation. 1: TUNEL-positive oocytes (green/yellow nuclei). 2: TUNEL-negative oocytes (red nucleus). Scale bar = 50 µm. (**B**) Effects of temperature on DNA fragmentation in oocytes after maturation. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between temperatures.

The positive control was preincubated with DNAse (0.2 U/µL) for 1 h at 37 °C, and the negative control was incubated in the absence of the deoxynucleotidyl transferase enzyme. After that, the samples were washed three times in PBS-PVA and placed on slides in a 1 µL drop of SlowFade™ with 5 µg/mL Hoechst 33,342 under a coverslip. The samples were evaluated using fluorescence microscopy (Eclipse 80i, Nikon Instruments Europe, Amsterdam, The Netherlands) at 20× magnification. For TUNEL evaluation, an Ex filter of 365/28 nm, a DM of 405 nm, and an Em filter of 445/50 nm were used. For Hoechst 33,342, an Ex filter of 475/30 nm, a DM of 405 nm, and an Em filter of 530/40 nm were applied. Oocytes (Fig. 9A) and blastomeres (Fig. 10A) with DNA damage, e.g., with fragmented nuclei, were classified as TUNEL positive, and those without damage were classified as TUNEL negative.

Assessment of meiotic spindle configuration

Mature oocytes were denuded and fixed with methanol (1:1) in PBS for 20 min at room temperature and stored in PBS-PVA at 4 °C until use. The oocytes were subsequently incubated in a permeabilizing solution (0.5% Triton-X-100 in PBS-PVA) for 30 min at room temperature, washed twice in PBS-PVA, and then blocked with 2% FCS in PBS for 45 min. Microtubules were detected using an anti-α-tubulin primary antibody (1:300; Cat. no. # 62204; Invitrogen©; Thermo Fisher Scientific, Barcelona, Spain) at 4 °C overnight. After rinsing twice with 2% FCS in PBS for 5 min per wash, the samples were incubated for 1 h at room temperature with the FITC–labeled secondary antibody (1:300; Cat. no. # F0257; Sigma Aldrich, Madrid, Spain) at room temperature for 30 min. Oocytes were mounted on a glass slide in a 1 µL drop of SlowFade with 5 µg/mL Hoechst 33,342 under a coverslip to visualize chromosomes. Oocytes were assessed using fluorescence microscopy (Eclipse 80i, Nikon Instruments Europe, Amsterdam, The Netherlands) at 40× magnification. For DNA identification, an Ex filter of 365/28 nm, a DM of 405 nm, and an Em filter of 445/50 nm were used. For meiotic spindle evaluation, an Ex filter of 475/30 nm, a DM of 405 nm, and an Em filter of 530/40 nm were applied. Oocytes with a classical symmetric barrel-shaped spindle with chromosomes aligned regularly in a compact group along the equatorial

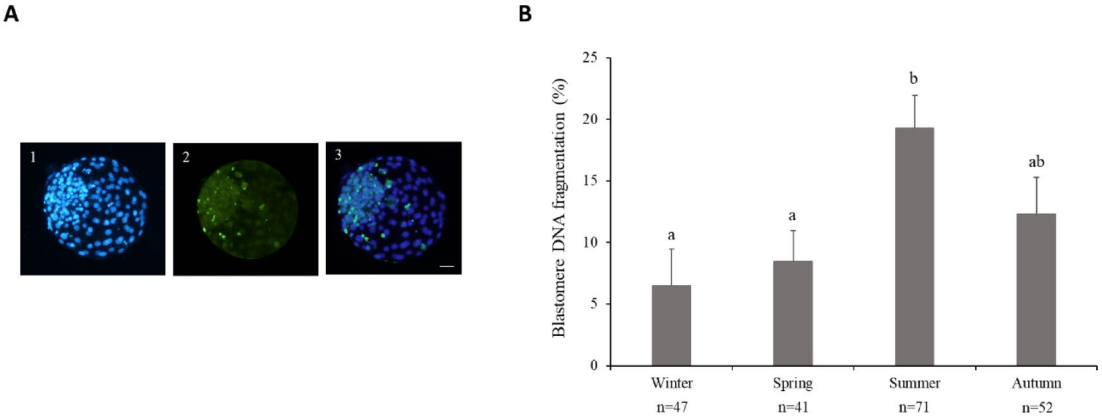

Fig. 10. (**A**) TUNEL staining of blastocysts. 1: Hoechst 3342 staining. 2: Blastocyst with TUNEL-positive cells in green. 3: Merge. Scale bar = 30 µm. (**B**) Effects of season on DNA fragmentation in sheep blastocysts. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between seasons.

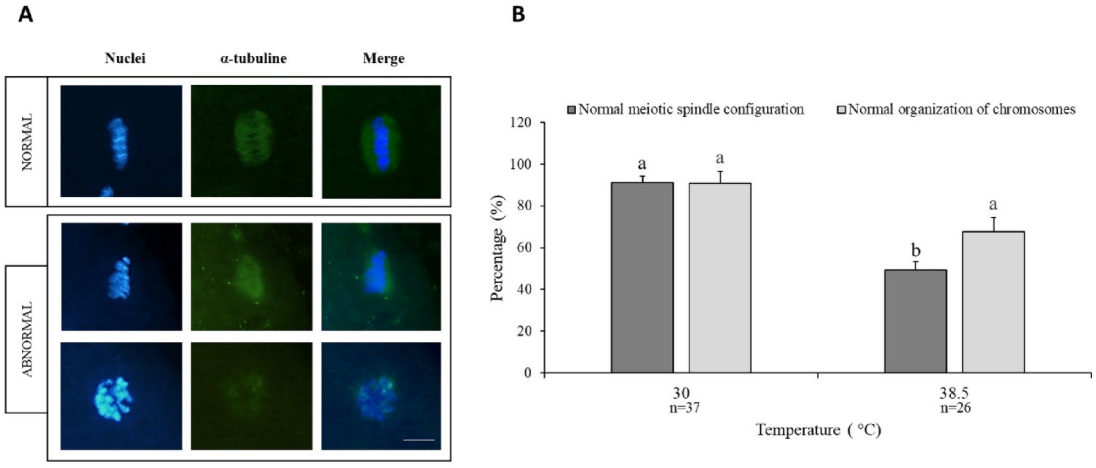

Fig. 11. (**A**) Representative images of spindle (green) morphology and chromatin (blue) alignment in ovine oocytes following immunostaining. Notably, the normal morphology shows symmetrical barrel-shaped meiotic spindles with chromatin aligned regularly along the equatorial plane of the spindle, whereas the abnormal morphology illustrates a reduced spindle, an absent spindle or asymmetrical spindles and chromatin. Scale bar = 50 µm. (**B**) Effects of in vitro heat stress during ovary storage on spindle and chromosomal organization after oocyte maturation in sheep. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between temperatures for each parameter.

plane were considered normal. In contrast, oocytes with spindles that were disorganized, clumped, dispersed, or missing (entirely or partially) with aberrations in chromatin arrangement, clumping, or dispersal from the spindle center were considered abnormal (Fig. 11A).

Flow cytometry analysis of cumulus cells

Cumulus cells were collected from mature COCs and examined using a FlowSight® Imaging Flow Cytometer (Amnis, Merck-Millipore, Germany) as previously described [28]. Briefly, samples were stained with 10 µM YO-PRO-1 (Thermo Fisher Scientific, Barcelona, Spain) and 0.5 µM PI (Thermo Fisher Scientific, Barcelona, Spain) to study viability, apoptosis, and mortality. Viable cells were recorded as YO-PRO-1-/PI-, whereas YO-PRO-1+/PI- cells were deemed apoptotic. The cells stained with PI were considered dead. For mitochondrial activity, the cells were incubated with 200 mM MitoTracker™ Deep Red (Thermo Fisher Scientific, Barcelona, Spain) for 20 min at 38.5 °C in the dark and then stained with 10 µM YO-PRO-1 and 0.5 µM PI. Viable cells with active mitochondria were considered MitoTracker+/YO-PRO-1. To study the intracellular levels of ROS and GSH in viable cells, the samples were incubated with 10 µM CellTracker™ Blue (Thermo Fisher Scientific, Barcelona, Spain) and 10 µM CM-H_2DCFDA (Thermo Fisher Scientific, Barcelona, Spain) for 30 min at 38.5 °C, followed by 0.5 µM PI staining. The AMNIS® flow cytometer was equipped with three lasers with excitation wavelengths of

Station	Viable cells (%)	Apoptotic cells (%)	Dead cells (%)	Active mitochondria (%)	ROS (MFI (AU))	GSH (MFI (AU))
Winter	77,65 ± 3.90 ab	2.77 ± 0.49	19.28 ± 3.64 ab	34.56 ± 5.41	1467.95 ± 578.40	6594.69 ± 3185.61
Spring	83.36 ± 2.87 a	2.69 ± 0.45	13.47 ± 3.37 a	43.96 ± 5.41	2792.63 ± 578.40	12946.67 ± 3185.61
Summer	67.32 ± 4.28 b	2.82 ± 0.54	29.34 ± 3.99 b	42.36 ± 5.41	765.79 ± 633.61	2708.51 ± 3489.66
Autumn	74.83 ± 1.98 ab	1.98 ± 0.49	22.88 ± 3.64 ab	41.20 ± 4.94	1968.84 ± 578.40	8030.63 ± 3185.61

Table 1. Effects of season on cumulus cell quality parameters in sheep. The data are expressed as the means ± SEMs. [a, b] Different letters in the column indicate significant differences ($p \leq 0.05$) between seasons.

Temperature (°C)	Viable cells (%)	Apoptotic cells (%)	Dead cells (%)	Active mitochondria (%)	ROS (MFI (AU))	GSH (MFI (AU))
30	73.414 ± 3.760 a	24.014 ± 3.762 a	2.300 ± 0.511	37.767 ± 2.327 a	1592.163 ± 180.420	6730.419 ± 624.351
38.5	49.257 ± 3.760 bc	47.786 ± 3.762 bc	2.414 ± 0.511	17.300 ± 2.327 b	1118.009 ± 180.420	5096.497 ± 624.351
40	50.136 ± 5.480 bc	48.012 ± 5.483 bc	1.857 ± 0.745	16.983 ± 3.680 b	889.332 ± 263.005	5908.562 ± 910.140
41	56.486 ± 5.480 ac	41.437 ± 5.483 ac	1.870 ± 0.745	22.383 ± 3.680 b	1034.637 ± 263.005	7717.344 ± 910.140

Table 2. Effects of in vitro heat stress during ovary storage on cumulus cell quality parameters in sheep. The data are expressed as the means ± SEMs. [a, b] Different letters in columns indicate significant differences ($p \leq 0.05$) between temperatures.

405 (10 mW), 488 (30 mW) and 785 nm (10 mW) and a 10× microscope objective. The brightfield and emission wavelengths of specific markers were acquired with the following channels: two brightfield channels (channels 1 and 9 (547/57 and 577/35, respectively)); (i) side scatter emission (SSC; channel 6 (772/55)); (ii) CellTracker™ Blue at 451 nm (detected by channel 7 (457/45; DAPI channel); (iii) YO-PRO-1 and CM-H2DCFDA at 521 nm (detected by channel 2 (532/55; FITC channel)); (iv) PI at 620 (detected by channel 4 (610/30; phycoerythrin (PE) channel); and (v) MitoTracker Deep Red at 662 nm (detected by channel 11 (702/86; allophycocyanin (APC) channel)). The percentages and MFIs (AUs) of several markers under study in viable cells were recorded using IDEAS® software (AMNIS EMD Millipore). The cell population was gated according to the aspect ratio and area parameters, and out-of-focus cells, debris, and cell clumps were also excluded from the analysis. A minimum of 1,000 cumulus cells were examined. The percentages of viable, apoptotic, and dead cells, as well as those with active mitochondria, and the MFIs (AUs) of ROS and GSH are shown in Tables 1 and 2. Representative images of the dot-plots in the different experimental conditions are shown in supplementary figures S1 to S6.

Statistical analysis

Statistical analyses were performed using IBM SPSS 24.0 (IBM Corp.; Armonk, NY, USA) software. The data were tested for a normal distribution (Kolmogorov–Smirnov and Shapiro–Wilk tests) and homogeneity of variance (Levene test). First, the maximum temperature, maximum relative humidity and THI values were analyzed by factorial ANOVA followed by the Bonferroni *post hoc* correction considering season (winter, spring, summer, or autumn) as the fixed effect. In Experiment 1, oocyte viability, early apoptosis, mortality, oxidative status, the mitochondrial membrane potential and distribution, DNA fragmentation, maturation, fertilization, embryo development rates, blastocyst quality and cumulus cell activity were analyzed by factorial ANOVA followed by the Bonferroni *post hoc* correction. For that purpose, season was considered the fixed effect. In Experiment 2, oocyte viability, early apoptosis, mortality, oxidative status, mitochondrial membrane potential and distribution, DNA fragmentation, maturation rates, meiotic spindle and chromosome organization, fertilization rates, embryo production and cumulus cell parameters were also analyzed by factorial ANOVA followed by the Bonferroni *post hoc* correction. For that, the ovary storage temperature (30 °C, 38.5 °C, 40 °C and 41 °C) and the replicates were considered fixed effects. Differences with probabilities of $p \leq 0.05$ were considered significant, and the results are presented as the means ± SEMs.

Results

Temperature–humidity indices as indicators of the heat stress of Climatic conditions

The ranges of the maximum temperature, maximum relative humidity and THI values for each season between 2019 and 2020 are listed in Table 3. The THI was significantly greater ($p < 0.05$) in summer than in winter and spring, although there was no difference from autumn.

Experiment 1

Effects of season on oocyte and cumulus cell quality parameters

After IVM, the collection of COCs during summer resulted in reduced ($p < 0.05$) oocyte viability (29.37 ± 14.07%) compared with collections during winter and autumn (84.44 ± 9.38 and 84.58 ± 11.49%, respectively) and a greater percentage ($p < 0.05$) of apoptotic oocytes (67.50 ± 8.14%) than in the other seasons (winter = 10.00 ± 7.18%; spring = 16.67 ± 7.18%; and autumn = 11.67 ± 8.79%; Fig. 2B). The percentage of dead oocytes was similar among the different seasons ($p > 0.05$).

To determine the effect of season on ovine oocyte oxidative status, the levels of intracellular ROS and GSH in the corresponding oocytes after maturation were determined. The level of ROS (MFI) was significantly greater ($p < 0.05$) in the oocytes collected during autumn (145.97 ± 20.44) than in those collected during spring

Season	Tmax (°C)	Max relative humidity (%)	THI
Winter	19.95 ± 1.39 a	79.22 ± 2.89	66.62 ± 2.26 a
Spring	23.31 ± 1.55 a	81.49 ± 3.23	72.38 ± 2.53 a
Summer	29.64 ± 1.66 b	84.01 ± 3.46	82.99 ± 2.70 b
Autumn	23.38 ± 1.79 ab	85.35 ± 3.73	72.55 ± 2.92 ab

Table 3. Climate and temperature–humidity index (THI) values between 2019 and 2020 in Murcia (Spain). The data are expressed as the means ± SEMs. [a, b] Different letters indicate significant differences ($p \leq 0.05$) between seasons. Tmax: maximum temperature.

Station	Total oocyte (n)	Maturation MII (%)	Fertilization 2 PN (%)	Cleavage (%)	Total blastocysts (%)	Blastocysts/cleaved (%)
Winter	686	77,63 ± 5,29	39,32 ± 6,90	58,64 ± 4,29	23,12 ± 2,31 a	37,63 ± 1,93 a
Spring	779	83,75 ± 5,29	50,55 ± 7,39	61,65 ± 4,77	22,94 ± 2,57 ab	36,44 ± 2,14 a
Summer	470	76,35 ± 6,11	42,17 ± 8,86	66,27 ± 5,71	11,00 ± 3,07 c	17,42 ± 2,56 b
Autumn	420	68,09 ± 6,11	36,64 ± 7,64	66,44 ± 4,98	13,15 ± 2,68 bc	18,69 ± 2,23 b

Table 4. Effects of season on oocyte meiotic status, fertilization potential and embryo development in sheep. The data are expressed as the means ± SEMs. [a, b, c] Different letters in the column indicate significant differences ($p \leq 0.05$) between seasons.

(30.38 ± 16.69). Additionally, GSH (MFI) was significantly greater ($p < 0.05$) in autumn (142.76 ± 15.45) than in the other seasons (winter = 85.17 ± 12.62; spring = 51.55 ± 12.62; and summer = 70.01 ± 14.31; Fig. 4B).

Both the mitochondrial membrane potential (fluorescence intensity ratio) and the normal distribution of mitochondria throughout the oocyte were significantly lower ($p < 0.05$) in summer (0.23 ± 0.04 and 67.14 ± 7.36%, respectively) than in autumn (0.44 ± 0.04) in the former and in winter (96.67 ± 6.49%) in the latter (Figs. 6B and 7B), although there were no differences from the other seasons.

The percentage of oocytes with fragmented DNA did not differ ($p > 0.05$) between seasons (winter = 11.42 ± 7.19%; spring = 1.22 ± 7.19%; summer = 25.92 ± 8.15%; and autumn = 0 ± 8.80%).

As shown in Table 1, the cumulus cell live/death status was significantly ($p < 0.05$) lower in summer than in spring, as a lower number of viable cells and a greater number of dead cells were found between these two seasons, although differences were not found between summer and winter and autumn. We also assessed cumulus cell apoptosis, the number of active mitochondria, and the intracellular ROS and GSH contents. For all the parameters, cumulus cells presented similar values ($p > 0.05$) throughout the year (Table 1).

Effect of season on the subsequent maturation and developmental competence of sheep oocytes

The results did not reveal significant differences ($p > 0.05$) in the maturation or fertilization rates (Table 4). Although the oocytes collected throughout the four seasons had similar cleavage rates ($p > 0.05$), the percentage of blastocysts from the initial number of oocytes was significantly greater ($p < 0.05$) in winter than in summer and autumn (Table 4). In addition, the number of blastocysts from the cleaved embryos at 48 hpi was also significantly greater ($p < 0.05$) during winter and spring than during summer and autumn (Table 4).

In vitro-generated blastocysts produced during the summer are of reduced quality

Although the numbers of in vitro-produced blastocysts were similar ($p > 0.05$) among all seasons (winter = 122.02 ± 8.69%; spring = 127.11 ± 7.34%; summer = 117.61 ± 7.93%; and autumn = 104.11 ± 8.69%), the percentage of blastomeres showing DNA fragmentation was greater during summer (19.25 ± 2.71%) than during winter (6.49 ± 2.97%) and spring (8.47 ± 2.51%; Fig. 10B), although there was no difference from autumn.

Experiment 2

Increasing the ovary storage temperature in an in vitro ovary storage model impairs the quality of sheep oocytes and cumulus cells

As shown in Fig. 3B, the results revealed that storage of slaughterhouse sheep ovaries for 3 h at 30 °C produced greater ($p < 0.05$) oocyte viability values (73.44 ± 6.48%) than storage at 40 °C (34.23 ± 8.29%) or 41 °C (16.48 ± 2.29%; Fig. 3B). Additionally, the lowest ($p < 0.05$) percentages of dead oocytes were observed at 30 °C (9.37 ± 5.84%) and 38.5 °C (29.68 ± 5.84%), in contrast to 41 °C (69.55 ± 8.05%; Fig. 3B).

There was no difference ($p > 0.05$) in the ROS levels (MFI) between the ovary storage temperatures (30 °C = 91.21 ± 10.70; 38.5 °C = 46.20 ± 10.70; 40 °C = 37.67 ± 14.74 and 41 °C = 64.48 ± 14.74; Fig. 5B). Moreover, the values for the GSH content (MFI) were similar between the temperatures (30 °C = 95.64 ± 12.41; 38.5 °C = 65.33 ± 12.41; 40 °C = 44.68 ± 17.10 and 41 °C = 86.57 ± 17.10; Fig. 5B).

The normal distribution of mitochondria was greater at 30 °C and 38.5 °C (87.5 ± 6.43% and 82.68 ± 6.43%, respectively) than at 41 °C (39.02 ± 8.87%; Fig. 8B).

The number of oocytes with fragmented DNA, as measured by the TUNEL assay, was significantly lower ($p < 0.05$) after ovary storage at 30 °C (5.21 ± 7.83%) than after storage at other temperatures (38.5 °C = 56.06 ± 7.83%; 40 °C = 55.44 ± 10.79% and 41 °C = 60.84 ± 10.79%; Fig. 9B).

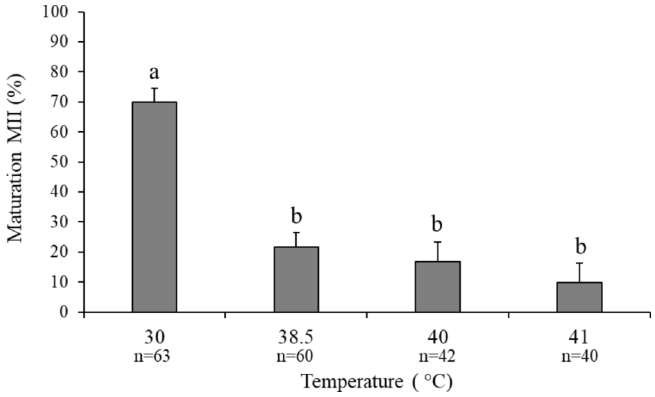

Fig. 12. Effects of in vitro heat stress during ovary storage on oocyte nuclear maturation. The results are expressed as the means ± SEMs. [a, b] Different letters indicate differences between temperatures for each parameter.

Temperature (°C)	Total oocyte (n)	Fertilization 2 PN (%)	Cleavage (%)	Total blastocysts (%)	Blastocysts/cleaved (%)	Total cell number (%)	Blastocyst DNA fragmentation (%)
30	273	37.04 ± 11.20	54.78 ± 6.28 a	17.54 ± 2.54 a	34.51 ± 5.90 a	120.54 ± 6.42	6.23 ± 1.32 a
38,5	229	39.48 ± 11.20	22.50 ± 6.28 b	4.25 ± 2.54 b	8.74 ± 5.90 b	91.00 ± 7.86	19.72 ± 1.62 b

Table 5. Effects of in vitro heat stress during ovary storage on fertilization potential, embryo competence and blastocyst quality. The data are expressed as the means ± SEMs. [a, b] Different letters in columns indicate significant differences ($p \leq 0.05$) between temperatures.

As shown in Table 2, the percentage of viable cumulus cells was significantly greater ($p < 0.05$) at 30 °C than at 38.5 °C and 40 °C, although there was no difference in relation to 41 °C. The number of active mitochondria in cumulus cells was significantly greater ($p < 0.05$) at 30 °C than at 38.5 °C, 40 °C and 41 °C. However, the other parameters (intracellular ROS and GSH contents) presented similar values ($p > 0.05$) across the different temperatures.

Stages of nuclear maturation, meiotic spindles, and chromosome organization with increasing ovary storage temperature

The influence of storage temperature on oocyte nuclear maturation, cytoskeletal integrity, and chromosome organization after maturation was assessed. The percentage of oocytes reaching the MII phase was greater ($p < 0.05$) in the 30 °C group (69.82 ± 4.97%) than in the other groups (38.5 °C = 21.73 ± 4.67%; 40 °C = 16.87 ± 6.43%; and 41 °C = 9.82 ± 6.43%; Fig. 12). Given the significantly compromised maturation rates at 40 °C and 41 °C, the evaluation of meiotic spindle configuration was limited to the 30 °C and 38.5 °C groups. This limitation was necessary, as the low maturation rates at elevated temperatures made reliable assessment of spindle integrity unfeasible. As expected, most of the 30 °C group oocytes contained a typical MII spindle (91.24 ± 3.09%; Fig. 11B). However, in the 38.5 °C group, a reduced ($p < 0.05$) number of oocytes (49.41 ± 3.79%) presented a normal meiotic spindle configuration. Regardless, the proportion of oocytes with a normal organization of chromosomes was not significantly different ($p > 0.05$) between the temperature groups (30 °C = 90.96 ± 5.61%; 38.5 °C = 67.53 ± 6.88%; Fig. 11B).

Increasing the ovary storage temperature reduces in vitro embryo production rates and blastocyst quality in sheep

Although no differences were observed in the fertilization rates ($p > 0.05$), the results reported in Table 5 show that the number of oocytes collected from ovaries stored at 30 °C was higher ($p < 0.05$) than that collected at 38.5 °C (only these 2 temperatures were studied because of the extremely low number of mature oocytes at 40 °C and 41 °C). Moreover, the number of expanded blastocysts from the total number of oocytes in culture and the number of cleaved embryos at 48 hpi followed the same trend and were significantly greater at 30 °C than at 38.5 °C (Table 5). Although the total number of blastocyst cells was similar ($p > 0.05$) among the temperatures, compared with 30 °C, 38.5 °C increased ($p < 0.05$) the percentage of blastomere DNA fragmentation.

Discussion

The ever-growing global population is driving a notable increase in global food production[31]. Annual meat production, particularly sheep meat demand in Europe, is expected to increase due to dietary diversification and demographic changes[2], highlighting the growing importance of sheep and goat farming in the future[32]. When increasing livestock production and productivity are key to meeting the escalating demand for animal protein globally, the climate is changing faster than predicted, posing a significant challenge to the long-term viability of livestock production systems[33].

Mammals are able to maintain body temperatures higher than the environment temperature (35–39 °C) through a balance of body heat production and loss[34]; however, global warming can affect the proper functioning of their metabolism. Changes in ambient temperature over recent years, especially the increase in the global average surface temperature of approximately 0.6 °C during the 20th century, can upset the balance[35]. Temperature clearly affects mammalian gamete function, which has shown high sensitivity to heat stress[36] in both in vivo and in vitro systems[37]. Extreme high-temperature conditions can increase body temperature and adversely impact mammalian biological functions, leading to impaired production and reproductive traits[38,39]. Most reproductive processes, including gametogenesis, fertilization or embryonic development, can be influenced by extremely high environmental temperatures that directly impact reproductive performance[33,40,41]. Although sheep are well adapted to different environmental conditions, including high environmental temperatures[32], heat stress significantly impairs reproduction and poses a risk to the efficiency of meat production[41].

To meet increasing global demands and minimize environmental consequences, advances in animal agriculture via reproductive technologies will be essential. These technologies aim to increase livestock efficiency and productivity while adjusting to climate change and global warming[3]. Currently, assisted reproduction techniques are widely used in livestock, although IVP has rarely been explored because of its poor performance[42].

The collection of developmentally competent oocytes is a drawback that may limit the applicability of IVP. Several studies have demonstrated that heat stress adversely impacts the fertility process, especially oogenesis, oocyte function, maturation, fertilization, and blastocyst development[43]. The molecular study of oocyte damage triggered by heat stress could help to avoid this type of injury. The present work compares, for the first time, whether the season-dependent effects of heat stress (winter, spring, summer, and autumn) are replicated in an in vitro model, which is crucial for a deeper understanding of the physiological mechanisms underlying oocyte damage. Thus, we evaluated the evolution of oocyte competence, fertility and blastocyst rates throughout the seasons and the effects of high in vitro temperatures on oocyte quality and competence for in vitro embryo production.

Different authors have shown that the quality of the oocyte, and consequently the production of embryos, is affected by seasonality in small ruminants. Souza-Fabjan et al.[44] reported that blastocyst production in goats was greater during fall and lower in spring. Moreover, Serra et al.[45] demonstrated a seasonal effect in sheep with better oocyte quality results in spring than in autumn, although this study was carried out in prepubertal animals, where the endocrine profile is quite different from that of adults[46]. In our case, the data generally revealed poorer oocyte quality in the summer months. We hypothesize that this fact was due to the high temperatures registered in our country at that time of the year. Table 3 shows that the level of heat stress reached during summer was considered to be severe (79–83) for ruminant species, whereas the other seasons presented values considered normal (≤ 74). We believe that the low quality shown during summer is not due to a photoperiod seasonal effect, as some of the parameters analyzed presented better results during spring, which is a nonoptimal reproductive season for sheep species at our latitude. Therefore, what should have been expected during spring considering the photoperiod is a worse quality at this time of the year.

Apoptosis is a form of programmed cell death that plays crucial roles in maintaining the homeostasis of various biological processes[47] and in the disruption of the normal function of oocytes under thermal stress[48]. In this study, Annexin-V staining was used as an early marker of apoptosis, and alterations in the oocyte phospholipid membrane preceding the late stages of apoptosis were detected by TUNEL staining. Early apoptosis, assessed using the V-FITC assay, is more common in oocytes subjected to heat stress, suggesting that high summer temperatures can induce oocyte apoptosis[49]. In fact, our results reported by Annexin-V staining revealed that the percentage of viable oocytes decreased during summer, whereas the apoptotic oocyte rate increased. Similarly, Ahmadi[50] reported a significant interaction effect between season and thermal stress on apoptosis in sheep, where the reduced developmental competence observed in heat-stressed oocytes was partially linked to changes in their plasma membrane. Similarly, the percentage of viable oocytes decreased at higher in vitro temperatures (40 °C and 41 °C) than at 30 °C. Our results are in agreement with previous studies performed in bovines, where oocytes subjected to heat stress before 12 h of maturation[49], or even in the short term at the early stage of maturation, resulted in increased Annexin-V binding and oocytes that underwent early apoptosis compared with those in the control group[51].

Heat stress has been shown to activate apoptotic cascades, inducing alterations in the oocyte phospholipid membrane[16] and ultimately promoting detrimental effects on oocyte developmental competence[50]. However, in our study, the stress caused by the temperature of 30 °C was not enough to damage oocyte DNA, although in vitro temperatures greater than 38.5 °C dramatically damaged the oocyte DNA. The different effects observed between ambient heat stress and in vitro heat stress may be caused by the differences in the responses of both models to high temperatures. Under physiological conditions, if stress is not excessive, an organism's defense system will address potential injuries[52]. Thus, during the summer months, early apoptosis greatly increases, unlike the percentage of dead cells or DNA damage. Early apoptosis can be reversed by the body's defense system through the synthesis of antiapoptotic proteins or antioxidants[52]. Indeed, some studies have shown that heat shock induces early apoptotic events upstream of DNA fragmentation[51]. This in vivo adaptational phenomenon was previously noted in bovine[53] and sheep[50] oocytes and even spermatozoa[54]. The development of heat tolerance could be associated with the expression of heat shock proteins, such as heat shock protein-70, which protects oocytes from apoptotic stimuli that can harm DNA[55]. Thus, it would also be of interest to develop future experiments to determine whether heat stress significantly impacts the expression levels of specific genes in both in vitro and in vivo models. Despite the results observed during the seasons, after exposure of the oocytes to temperatures of 38.5 °C or above, the oocyte may not be able to counteract this damage, as it may not have an active defense system, resulting in irreversible damage that leads to an increased percentage of dead cells and DNA damage. Our findings are in line with other works in which ovaries exposed to severe in vitro heat stress

(high storage temperature and/or long-term storage) increased the number of oocytes with fragmented DNA nuclei[56].

With respect to the oxidative balance, our results revealed that GSH production was greater in autumn than in the other seasons, whereas ROS production was greater in spring, as previously reported[45]. The balance between ROS production and antioxidant capacity also affects the developmental competence of oocytes. Under physiological conditions, ROS regulate specific cellular functions, although high levels lead to various forms of cellular damage to DNA, proteins, and lipids and ultimately affect oocyte quality and viability[57]. Although an excess of ROS may lead to a harmful effect on oocytes, the presence of ROS derived from mitochondrial respiration is necessary for certain cell signaling pathways involved in folliculogenesis, oocyte maturation, embryogenesis and implantation[58]. At our latitude, the reproductive season in small ruminants begins from the end of summer to the beginning of winter. This fact suggests that during this period, greater cellular activity is needed, the mitochondria are more active, and more ROS are generated. Additionally, our results are in line with other studies performed on sheep at similar latitudes, where oocytes collected during autumn also showed significantly greater ROS production than those collected during other seasons did[45]. Among the main antioxidants that protect the oocyte against oxidative damage, GSH plays a key role in determining the degree of cytoplasmic maturity and the quality of the oocyte after in vitro maturation[59]. The regulation of the intracellular redox potential in the oocyte is a crucial determinant of fertility and embryo development[60,61]. Thus, the high concentrations of GSH observed during autumn, compared with those observed during the other seasons, could have increased to achieve an oxidative balance and physiologically counteract the high production of ROS. Similarly, other works have reported that animals may respond to temperature challenges by upregulating the expression levels of antioxidant enzymes[62]. Moreover, variation in antioxidant levels was suggested not only as a defense mechanism to counteract oxidative damage but also as a consequence of environmental conditions[63]. In vitro heat stress did not affect the production of ROS or GSH. This may be due to the high percentage of dead oocytes that we found at temperatures greater than 38.5 °C, where the physiological system that determines the oxidation state of a cell could not be activated. Furthermore, we hypothesize that live oocytes may be unable to display the mechanisms that lead to the production of antioxidant enzymes due to the damage produced; hence, this effect could not be visualized[64].

With respect to mitochondrial parameters, dysfunction in one or more aspects of mitochondrial biology results in reduced oocyte developmental competence. We observed that both mitochondrial activity and the homogeneous distribution of mitochondria were affected throughout the seasons. Moreover, this study revealed not only heat stress-induced alterations in mitochondrial distribution[57] but also a trend in which homogeneous mitochondrial distribution aligns with the mitochondrial membrane potential during summer. Thus, mitochondrial activity was greater during the autumn season than in the spring and summer seasons, and the percentage of oocytes with a normal distribution of mitochondria was lower in the summer than in the winter. These results agree with other studies where the mitochondrial distribution differed between the cold and warm seasons[57,65]. In particular, our findings are in line with those of previous studies indicating that mitochondria exhibit a less uniform distribution during the summer[66] or after severe in vitro heat exposure (41 °C)[49]. This reduced uniformity could be due to an alteration of the cytoskeletal proteins caused by heat, as has been previously demonstrated by Gendelman and Roth (2012). Extremely high temperatures have been shown to denature cytoskeletal proteins, leading to their abnormal distribution in the oocyte[12], leading mitochondria to relocate to the cell periphery instead of maintaining a homogeneous distribution throughout the cytoplasm[67]. This alteration of cytoskeletal elements can even lead to disruption of nuclear maturation and disassembly of the meiotic spindle[14].

Meiotic spindle microtubules are susceptible to temperature changes, which can ultimately cause chromosomal imbalance and cell death[67,14,68]. The microtubular network in the oocyte is critical for meiotic spindle formation, chromosome segregation, fertilization, and embryonic development[45]. Microtubules and their posttranslational modifications, especially tyrosination and acetylation in sheep oocytes[69], play crucial roles in oocyte maturation and fertilization because of their influence on meiotic spindle assembly and chromosome movement. A balance between tyrosination (more abundant in dynamic microtubules) and acetylation (characterizing stability) is essential for the normal function of the meiotic spindle, which requires both dynamism and stability. Nevertheless, temperature has been shown to disrupt this balance, potentially affecting oocyte developmental competence[45]. Several studies have demonstrated that in vitro heat stress has detrimental effects on various aspects of cytoplasmic and nuclear oocyte maturation[70], including cytoskeletal rearrangement[14], meiotic spindle formation[14,67] and early embryonic mitotic failure[71]. In the present study, we also observed that the meiotic spindle was affected by a short exposure for 3 h at 38.5 °C, resulting in a lower percentage of oocytes with a normal spindle configuration. This parameter was clearly associated with the low maturation rates at high temperatures (from 38.5 °C). However, oocyte maturation was not affected by the temperatures recorded throughout the seasons. Unfortunately, the spindle configuration could not be assessed in this experiment; therefore, no data are available on this parameter. Our findings are consistent with other studies conducted on bovine oocytes, which reported no significant seasonal effects on nuclear maturation[53,72] and a normal uniform alignment of the chromosomes on the spindle[53].

There were no differences in the cleavage rate between seasons, although the percentage of blastocysts was lower during the summer and autumn. The fact that the number of oocytes obtained in the summer season did not differ from that collected during the rest of the year in terms of nuclear maturation and the cleavage rate could indicate that immature oocytes can be fertilized and undergo cleavage without developing to the blastocyst stage[41]. In addition, the number of apoptotic cells was greater in the embryos obtained during those months than in those obtained during winter and autumn. Similar results were reported by Gendelman and Roth (2012). Furthermore, genetic studies suggest that heat stress during the summer months impedes embryo development, likely because altered expression under these conditions is associated with a reduced developmental capacity

of oocytes and embryos[73]. Moreover, storing the ovaries for 3 h at temperatures above 38.5 °C produced a lower percentage of cleavage and blastocysts than did storing them at 30 °C, which is in line with previous studies[49]. This decrease is likely a consequence of hyperthermia. Importantly, these blastocysts were obtained from oocytes subjected to heat stress, which was previously reported to affect their quality and developmental competence in sheep[41]. Compared with those of their unexposed counterparts, the nuclear maturation and spindle configuration of oocytes exposed to in vitro heat stress were significantly impaired. This impairment could result in irreparable damage to the percentage of oocytes that progress past the cleavage stage, leading to a subsequent decline in embryo developmental potential[49]. Similarly, the number of apoptotic cells was greater for blastocysts from ovaries stored at 38.5 °C. These results agree with those obtained by Gendelman and Roth (2012a, b)[12,66].

Cumulus cells play critical roles in the maturation of the nucleus and cytoplasm in the oocyte, as they allow the interchange of molecules with the oocytes[74]. In addition, they support energy production in COCs and protect against oxidative stress-induced apoptosis. CCs are also important for the thermal protection of oocytes, as they provide extracellular thermoprotective molecules through gap junctions[75,76]. In addition, CCs also produce regulatory molecules that activate thermoprotective mechanisms within the oocyte[77]. Nevertheless, heat stress has been reported to reduce the effectiveness of gap junction communication between cumulus cells and oocytes[78]. As expected, we reported a lower percentage of viable CCs during the summer season as well as a higher percentage of dead cells than during the other seasons. Nevertheless, the percentage of apoptotic cells, the ROS/GSH ratio and mitochondrial activity were not affected. Conversely, a lower percentage of viable bacteria was observed at 38.5 °C, supporting the results of the oocyte apoptosis assay. Similarly, different studies have shown that the incidence of apoptosis in cumulus cells is negatively correlated with the developmental competence of oocytes and subsequent embryonic development after fertilization[79–81]. Nevertheless, other works have shown that oocytes are more sensitive to heat stress than cumulus cells are[75] and that long-term exposure to heat stress conditions may overwhelm the protection capacity of cumulus cells[82]. Moreover, although we have not studied morphological defects, cumulus morphology can also be affected by in vitro heat stress during ovary storage[83] as well as during summer rather than in winter[50].

To the best of our knowledge, no studies have simultaneously evaluated the effects of collection season on oocyte quality and IVP in sheep while also monitoring the impact of varying ovarian storage temperatures on oocyte quality and competence. The results revealed that extreme environmental temperatures during summer significantly decreased oocyte and cumulus cell viability and the normal distribution of mitochondria. Furthermore, ovaries collected during summer presented a lower percentage of blastocysts, and summer-produced blastocysts exhibited a significantly greater degree of DNA fragmentation compared with winter-produced blastocysts. This harmful effect was not limited to the summer season, as it continued until autumn, indicating a carry-over effect caused by heat stress during the summer. Similarly, the severity of the damage caused during in vitro heat stress was related to the highest temperatures selected, where temperatures over 38.5 °C caused major injuries to the oocyte quality parameters assessed and blastocyst rates. These findings clearly demonstrated that both seasonal and in vitro extreme temperatures significantly impaired oocyte quality and embryo production, emphasizing the need to develop strategies to reduce the impacts of heat stress and enhance reproductive outcomes in sheep.

Data availability

All data generated or analyzed during this study are included in this article.

Received: 6 December 2024; Accepted: 5 May 2025
Published online: 17 May 2025

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Author contributions

Conceptualization: AJS; methodology: AM-M, IS-A, MI-C, D-AM-C, CM and RF-S; formal analysis: AM-M and IS-A; resources: AJS and JJG; writing: AM-M, IS-A and AJS; original draft preparation: AM-M; writing—review and editing: AJS, JJG and MI-C; funding acquisition: AJS and JJG. All the authors have read and agreed to the published version of the manuscript.

Funding

This study was funded by the Spanish Ministry of Economy and Competitiveness (AGL2017-89017-R and PID2021-126988OB-I00). AM-M was supported by a Ministry of Economy and Competitiveness scholarship (PRE2018-084837). D-AM-C received a contract from UCLM cofinanced by the European Social Fund.

Declarations

Ethics approval and consent to participate

The adult sheep ovaries were collected from an authorized slaughterhouse, and sperm samples were obtained from the Germplasm Bank of the "Reproduction Biology Group", which is officially authorized for collecting and storing semen from sheep (ES07RS02OC).

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-01173-1.

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