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# Effects of selection for litter size variability on ovarian folliculogenesis, ovarian cell proliferation, apoptosis, and production of regulatory peptides in rabbits

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## ABSTRACT

The present study aimed to identify the novel mechanisms regulating rabbit fecundity. For this purpose, the association between litter size variability, fecundity, ovarian morphology, and markers of proliferation, apoptosis, steroidogenesis, and the presence of regulatory proteins were examined in ovarian cells from two rabbit lines divergently selected for low (LL) and high (HL) variability in litter size throughout sixteen generations. Ovaries and uteri were isolated from multiparous non-lactating female rabbits from each line at sixteen generations of selection. One part of the ovary was subjected to histomorphometric analysis of folliculogenesis. From the rest of ovary, ovarian granulosa cells (OGCs) were isolated, cultured, and cell viability, proliferation (accumulation of PCNA and of cyclin B1, and BrdU-positive cells), and apoptosis (accumulation of caspase 3, bax and DNA fragmentation) were evaluated by the Trypan blue exclusion test and BrdU, quantitative immunocytochemistry, and cell death detection assays. Furthermore, OGCs were subjected to proteomic analysis by using the nano HPLC-Chip-MS/MS method. The release of progesterone and oestradiol was measured by ELISA. The LL had more than one kit per litter than the HL (7.6 kits vs. 6.5 kits, $p \leq 0.05$). No differences were found in the diameter of primordial and primary ovarian follicles, theca, and granulosa thickness, but the diameter of oocytes in the primary and secondary follicles was higher in the LL than in the HL (88.49 μm in the LL vs. 77.86 μm in the HL for oocytes of primary follicles, $p \leq 0.05$; 122.10 μm in the LL vs. 109.87 μm in the HL for oocytes of secondary follicles, $p \leq 0.05$). Preovulatory follicles were presented only in the ovaries of the LL. The LL had higher incorporation of BrdU and reduced accumulation of bax within OGCs (0.92% in the LL vs. 0.44% in the HL for incorporation of BrdU, $p \leq 0.05$; 41% in the LL vs. 48% in the HL for accumulation of bax, $p \leq 0.05$). Ovarian fragments from the LL produced less progesterone and oestradiol than those of the HL (12 ng/mg tissue/day in the LL vs, 45 ng/mg tissue/day in the HL for progesterone, $p \leq 0.05$; 11 ng/mg tissue/day in the LL vs. 30 ng/mg tissue/day in the HL for oestradiol, $p \leq 0.05$). Besides, the OGCs from the LL produced a higher number of specific regulatory proteins involved in cell differentiation, proliferation, and adhesion than the HL (50 vs 38, $p \leq 0.05$). In conclusion, higher prolificacy in the LL line would be caused by: (1) the selection of growing primordial ovarian follicles; (2) better transformation to preovulatory follicles; (3) increased cytoplasmic maturation of oocytes; (4) increased DNA synthesis and decreased cytoplasmic apoptosis in OGCs; (5) changes in ovarian steroidogenesis; and (6) changes in the number of peptides involved in cell differentiation, proliferation, and adhesion.

## HIGHLIGHTS

- Assessing fecundity in two divergently rabbit lines for variability in litter size.
- Fecundity and its variability are related to the diameter and quality of oocytes.
- Selection and better growth of preovulatory follicles have been related to fecundity.

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## Introduction

Increasing productivity has been the main objective of animal breeding programs in recent decades (see review by Simianer et al. 2021), and selection programs have successfully achieved increased milk production in dairy cattle (Miglior et al. 2017), improved feed conversion ratio and meat yield in pigs (Hermesch et al. 2015), in broilers (Hartcher and Lum 2020), and in rabbits (Blasco et al. 2018), as well as egg production in laying hens (Poggenpoel et al. 1996) and litter size in rabbits (Garcia and Argente 2021). However, this genetic progress has been accompanied by a decrease in the robustness and resilience of animals (Knap 2009). This could be a problem in intensive systems with highly productive animals, which may be impaired in their ability to regulate their homeostatic balance, leading to pathologies and reduced animal welfare (Rauw 2009; Rauw et al. 1998). In this scenario, the livestock industry is increasingly interested in having animals that are not only highly productive but also more resilient to environmental challenges (Brito et al. 2020; Hermesch et al. 2015). Selection for environmental variability in physiological traits has been proposed as an indirect selection methodology for improving resilience and welfare (see review by Lung et al. 2020). Several divergent selection studies have been successfully conducted for variability in weight (Bolet et al. 2007 in rabbits; Formoso-Rafferty et al. 2022 in mice) and in litter size (Blasco et al. 2017 in rabbits).

In the divergent selection for litter size variability, Blasco et al. (2017) reported after ten generations of selection, that the line selected for low variability in litter size (LL) showed 1.67 kits$^2$ less in selection criteria than the line selected for high variability in litter size (HL), which is 45% of the original mean. Both lines differed not only by variability but also by size of litters: 8.5 kits at birth in the LL vs. 7.8 kits at birth in the HL. The difference in fecundity between the lines was associated with a difference in embryo implantation rate (11.7 embryos in the LL vs. 10.4 embryos in the HL) (Argente et al. 2017). In turn, a lower number of embryo losses at implantation in the LL was related to an embryo development more advanced in the first hours of gestation in this line (Calle et al. 2017; García et al. 2016). Embryo viability has been directly related to oocyte quality (Lasienë et al. 2009; Wang and Sun 2007). Quality of oocytes is directly related to oocyte growth, nuclear maturation, and cytoplasmic maturation -accumulation of nutrients and regulatory molecules (Bartkova et al. 2020; Sirotkin 2014; Soler et al. 2020). These processes are regulated by the state of ovarian follicular cumulus and granulosa cells surrounding oocyte. The proportion of proliferating and apoptotic cells defines viability of follicular cell population, growth, development, ovulation, and atresia of ovarian follicles, and finally the fecundity (Sirotkin 2014; Webb and Campbell 2007). Our working hypothesis is that the greater embryo development and survival in the LL might be explained by higher quality of their oocytes. Nevertheless, it remains unclear, whether differences in variability and fecundity rate between the LL and HL rabbits are defined by a different growth pattern in follicles and oocytes or by a different ovarian cell activity (proliferation, apoptosis, viability, production of hormones, and other regulatory molecules). Understanding these differences could be helpful for understanding similar differences in fecundity in other farm animal species, and humans.

The available model enables us to understand the general extra- and intracellular mechanisms defining female fecundity in various species. The present study was to identify factors defining differences in rabbit fecundity. The concrete aim was to compare the state and functions of ovarian cells and ovarian follicles in rabbit does of LL and HL groups. The following parameters have been analysed: weight and lengths of ovaries and uterus, histomorphological evaluation of ovarian follicles and their compartments, viability, proliferation, apoptosis, production of steroid hormones, and regulatory peptides responsible for the control of the main functions of ovarian cells.

## Material and methods

### *Animal material*

The rabbits belonged to a divergent selection experiment for litter size variability. Litter size variance was calculated within females with all their litters, and low (LL) and high (HL) lines were generated by selecting females with lower and higher litter size variance, respectively. Litter variability was estimated with litter size pre-correlated by season-year and lactation status effects (see more details of the experiment in Blasco et al. 2017). Each divergent line had approximately 120-130 females and twenty to twenty-five males per generation. Selection pressure on females was approximately thirty percent in each line. Females were first mated at eighteen weeks of age and at twelve days postpartum. Females that were not receptive were mated again the following week. The kits were weaned at thirty days of age. The mean number of litters per female was 4.5. This study used a subset of 16 non-lactating multiparous rabbit females (8 per

line) of the sixteen generations of the experiment described above. Females were bred and kept in individual cages (37.5 cm × 33 cm × 90 cm) under standard conditions (photoperiod 16 h:8 h, temperature 25.2 °C, air humidity 53%) at the Universidad Miguel Hernández de Elche. Spain. Feeding was *ad libitum* with a commercial feed (16.3% crude protein, 15% crude fibre, 3.2% ether extract, 8.9% ash, 0.56% phosphor, and 0.23% sodium; Cunilactal, NANTA S.A., Las Palas, Murcia, Spain). Means and standard deviation of weight were and 3517 g and 562 g for LL and 3387 g and 235 g for HL.

Fecundity was estimated in the subset of sixteen multiparous rabbit females as the average number of offspring produced in the first five deliveries. Females that had finished the fifth lactation were euthanised one month later by intravenous administration of sodium thiopental in a dose of 50 mg/kg of body weight (see the procedure in García et al. 2016). The entire reproductive tract was immediately removed. The ovaries were weighed, and the uterine horns were weighed and their length measured. Then, ¾ part of the ovaries were submerged in physiological saline solution, and ¼ part of the ovaries were submerged in ten percent formalin solution (Sigma-Aldrich Corp., St. Louis, MO, USA) for twenty-four hours until subsequent analysis. Body weight was 3,309 g in the LL females and 3,293 g in the HL females at the end of the fifth lactation, and 3,542 g in the LL females and 3,505 g in the HL females on the day of the euthanasia.

### Ovarian histological analysis

Following the procedure of Šedová et al. (2021), the fixed ovarian samples in ten percent formalin solution were processed by standard histological methods using an automated tissue processor (Leica ASP6025, Leica Microsystems, Wetzlar, Germany) and then embedded in paraffin blocks using a Leica EG 1150H paraffin embedding station (Leica Microsystems, Wetzlar, Germany). Slices from three to five μm-thick were cut from each sample using a microtome (Leica RM2255, Leica Microsystems, Wetzlar, Germany) and mounted on standard glass slides. The slices were stained with haematoxylin-eosin (Bamed S.R.O., Czech Republic). The primary and secondary ovarian follicles, along with large non-ovulated haemorrhagic follicles, were identified, classified, and counted in the manner outlined by Sirotkin et al. (2013). The quantity of follicles observed in a single section ranged from one to fifty per section. Primary ovarian follicles were differentiated from secondary follicles based on the quantity of granulosa cell layers. The structure of the ovaries was examined, and the thickness of primary, secondary, and preovulatory follicles was determined per Baláži et al. (2019). Haemorrhagic follicles were excluded from the analysis. Follicles with morphological signs of degeneration (deformation of follicle, follicular cells and their nuclei, blebbing, pyknosis and vacuolisation of cytoplasm, and appearance of apoptotic bodies) were considered as degenerated/atretic. The measurements were conducted with an Axio Scan.Z1 slide scanner (Zeiss, Oberkochen, Germany), and histological evaluation was carried out with a Carl Zeiss Axio Scope A1 microscope (Zeiss). Each follicle was measured twice, and only follicles containing clearly visible oocytes were measured, as described by Loncová et al. (2023).

### Ovarian cell viability, proliferation, apoptosis, and hormone released analysis

Ovarian granulosa cells were carefully removed from the inner surface of cleaned ovarian follicles using a lancet and then separated through centrifugation (ten minutes at 1,500 xg) (Sirotkin et al. 2024). Subsequently, the cells were suspended again and cultured in aseptic DMEM/F12 1:1 medium (BioWhittaker; Lonza, Verviers, Belgium), with ten percent foetal calf serum (Bio-West Inc., Logan, UT, USA) and one percent antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA) (Loncová et al. 2023). An automated cell counter from Thermo Fisher Scientific Inc. in Waltham, MA, USA was used to count cells and to adjust their concentration to $10^6$ cells/mL of medium. The cell suspension was distributed into twenty-four-well culture plates (NuncTM, Roskilde, Denmark, 1 mL per well) for Trypan blue staining and ELISA procedures, ninety-six-well culture plates (Brand®, Wertheim, Germany; 100 μL per well) for BrdU and cell death detection assays, or sixteen-well chamber slides (Nunc, Inc., International, Naperville, IL, USA; 100 μL per well) for immunocytochemistry. The cells were grown in a medium at 37.5 °C with five percent CO2 until a seventy-five percent confluent monolayer was achieved (2-3 days). The identification of cell viability (Trypan blue exclusion test; Sirotkin et al. 2024), proliferation markers (PCNA, marker of S-phase of cell cycle; Moldovan et al. 2007 and cyclin B1, marker of- M and G-phase of mitosis; Dai et al. 2019; Ligasová et al. 2023) and apoptosis (bax and caspase 3, markers of cytoplasmic/mitochondrial apoptosis; Spitz and Gavathiotis 2022) were detected *via*

immunocytochemistry as previously described by Fabová et al. (2022), Fabová et al. (2023) and Sirotkin et al. (2024).

To study the hormones released by ovarian follicular fragments, the tissue surrounding the ovary was removed, and the ovaries were cut open with scissors at the location where blood vessels enter. The lancet was used to gently break the connective tissue in the ovary to reach the follicles. Follicles were pushed down and isolated from the nearby connective tissues. After that, the follicular wall was sliced into slender lengthwise pieces measuring between 2-4 mm in diameter. Afterward, the fragments were rinsed three times in sterile DMEM/F12 1:1 medium (BioWhittaker), then placed in the same medium with ten percent foetal bovine serum (Bio-West) and one percent antibiotic-antimycotic solution (Sigma-Aldrich) in twenty-four-well culture plates (Nunc, 1 ml/fragment/well). Follicular pieces were incubated in a solution at a temperature of 37.5 °C with five percent CO2 for a duration of two days (more details of the procedure in Sirotkin et al. 2024). The concentrations of progesterone and *17β*-oestradiol in ovarian granulosa cells and follicular fragments were determined by using an enzyme-linked immunosorbent assay following the procedure of Fabová et al. (2023). The characteristics of these assays are presented in Table 1.

## Proteomic analysis

For nano HPLC-Chip-MS/MS analysis, cells from each experimental group were harvested using Accutase (Sigma-Aldrich) and washed twice in PBS. The cell suspension with a concentration of $10^6$ cells/mL was lysed in 200 μL of urea lysis buffer containing 8 M urea, 0.1 M DTT, and protease inhibitors (1x Roche complete) by shaking for one hour at 37 °C. Immediately after lysis, samples were cooled down to 4 °C and centrifuged at 16,000 x g for five minutes. The supernatant was transferred to 3 kDa molecular weight cut-off filter plates (Amicon Ultra-0.5 ml 3K, Millipore) and centrifuged at 14,000 x g for forty minutes. A modified filter-assisted sample preparation protocol (FASP), according to Wiśniewski et al. (2009), was used for the extraction of tryptic peptides from complex protein mixtures. Detergent removal by buffer exchange was performed in two sequential washes of the filter plates with 200 μL of 8 M urea in 0.1 M Tris/HCl pH 8.5 (UA), each followed by centrifugation at 14,000 x g for forty minutes. The flow-through of the collection tubes was removed. Proteins were then alkylated in 100 μL of 0.05 M iodoacetamide in UA by mixing at 600 xg in a thermomixer for one minute and incubating without mixing for twenty minutes. After centrifugation at 14,000 x g for thirty minutes, two additional washes using 100 μL of 0.05 M NH4HCO3, with ten minutes of centrifugation at 14,000 x g after each wash, were included to remove excess urea and prepare the proteins for digestion. Protein digestion was performed by adding 2 μg of trypsin (Trypsin Gold, Promega) in 0.05 M NH4HCO3 and incubating at 37 °C overnight. Peptides were recovered by spinning the filter plates upside down at 14,000 x g for forty minutes. The mixture was dried under vacuum, and the precipitate was resuspended in 50 μL mobile phase (ninety-seven percent water, three percent acetonitrile). The procedure is described in Bartkova et al. (2020) and Bartkova et al. (2024). The tryptic peptides were loaded into the 40 nL enrichment column (filled with Zorbax SB C18, 5 μm) of Agilent 1260 ChipCube MS Interface by Agilent 1260 Capilary Pump (Agilent Technologies, Palo Alto, USA). Subsequent to the loading and desalting of the sample on the enrichment column of the chip, the peptides were extracted and transferred to the analytical column at a flow rate of 600 nanolitres per minute using an Agilent 1260 Nano pump. This procedure was carried out in accordance with an increasing percentage of the organic phase. The mobile phase comprised an aqueous solution (A) or acetonitrile solution (B)

**Table 1.** Characteristics of the immunoassays used in experiments.

| Substance assayed | Specificity of assay (cross-reactivity of antiserum) | Sensitivity of assay (ng/mL) | Coefficient of variation (%) | |
|---|---|---|---|---|
| | | | Intra-assay | Inter-assay |
| **Progesterone** | ≤1.1 % with 11-desoxycorticosterone, ≤0.35 % with pregnenolone, ≤0.30 % 17α-OH with progesterone, ≤0.20 % with corticosterone, <0.10 % with oestriol, 17β-oestradiol, testosterone, cortisone and 11-desoxycortisol, <0.02 % with DHEA-S and cortisol | 0.045 | 5.4 | 5.59 |
| ***17β*-oestradiol** | ≤9.5 % with fulvestrant, ≤4.2 % with oestrone, ≤3.8 % with E2-3-glucuronide, ≤3.6 % with E2-3-sulphate, ≤0.4 % with oestriol, <0.1 % with androstenedione, 17-hydroxyprogesterone, corticosterone, pregnenolone, E2-17-glucuronide, progesterone, and testosterone | 0.0062 | 6.4 | 4.5 |

containing 0.1% formic acid (v/v). Chromatographic separation was achieved with a gradient elution using the following schedule: 0 min, three percent B; two minutes, three percent B; twenty-five minutes, fifty percent B; thirty minutes, fifty percent B; thirty-five minutes, ninety-five percent B; 40 min, ninety-five percent B; forty-five minutes, three percent B, followed by ten minutes for column re-equilibration. The analytical column was linked in accordance with the instructions for to the Agilent 6500 series Q-TOF mass spectrometer. A voltage of 1850 V was applied to the electrodes of the nanospray ionisation chamber. The use of high-purity nitrogen (99.99999%) as the collision gas was accompanied by a variable collision energy, which was dependent on the precursor ion's mass and charge. MS/MS spectra were acquired by automatically switching between MS and MS/MS modes (auto MS/MS mode). The acquired MS/MS data were analysed using the SpectrumMill search engine (Agilent Technologies, Palo Alto, USA). Database searches were performed against the self-built database from the UniProt rabbit (Oryctolagus cuniculus, Thorbecke inbred) proteome (UP000001811). The following autovalidation criteria of SpectrumMill software were used to validate the identified proteins and peptides: the minimum score for proteins was ten, and the minimum scores for spectra resulting from fragmentation of 2+, 3+, and 4+ precursor ions were 8, 7 and 9 respectively, with a scored peak intensity value of at least sixty percent. The procedure is described in Bartkova et al. (2020) and Bartkova et al. (2024).

### Statistical analysis

The experiment was performed on eight animals per group. Fecundity was estimated as the average number of kits per litter in the first four deliveries. Each experimental group was represented by eight culture wells containing ovarian granulosa cells. For the Trypan blue exclusion test, viability rates were estimated from at least one hundred cells per well. For the immunocytochemical analysis, the proportion of cells containing antigens was estimated to be at least 1,000 cells per well. The ELISA was performed by subtracting the blank control readings from the corresponding readings obtained on cells in the media to eliminate non-specific background (less than ten percent of the total readings). Substance secretion rates were calculated per $10^6$ viable cells/day. Three replicates in each proteomic analysis were performed. The protein was considered as specific for a particular line if proteomic analysis detected it in one group but not in other group of samples during three consecutive cycles. Fecundity, weight of the ovary and uterine horn, as well as the length of the uterine horn, diameter of follicle and oocyte, thickness of theca and granulosa, percentage of degenerated follicles, percentage of viable cell, accumulation of PCNA, cyclin B1, BrdU, bax, caspase 3, DNA fragmented cells, release of progesterone and oestradiol, and proteomic profile are presented as the mean ± SEM. The variables were tested for their normality (Shapiro-Wilk test) before being analysed by one-way analysis of variance (ANOVA) following by Tukey's test; $p \leq 0.05$ was consider significant. Statistical analysis was performed using the SigmaPlot 11.0 program (Systat Software, GmbH, Erkrath, Germany).

## Results

### Reproductive traits

Figure 1 shows the differences between the LL and HL lines in reproductive traits such as ovarian weight, uterine horn weight and length, and average number of kits per litter up the first four deliveries. We found that there were no differences between the lines in the weight of the ovary (0.38 g in the LL vs. 0.34 g in the HL) and uterine horn (4.35 g in the LL vs. 3.65 g in the HL), as well as in the length of the uterine horn (7.47 cm in the LL vs. 7.41 cm in the HL). However, female rabbits of the LL had on average more than one kit per litter compared to those in the HL (7.6 kits vs. 6.5 kits, $p \leq 0.05$).

### Histomorphometric traits of the ovaries

Table 2 shows the proportion of dominant and growing primordial follicles and primary (preantral), secondary (antral), and preovulatory follicles, as well as the diameter of follicles and oocytes and the thickness of theca and granulosa. The ovary of the LL had a similar percentage of dormant and growing primordial follicles and primary follicles as the HL. However, the proportion of the secondary follicles in the ovaries of does from the LL line was significantly lower, and the proportion of preovulatory follicles was higher than in the ovaries isolated from animals of HL (6.2% in the LL line vs. 18.7% in the HL line for secondary follicles, $p \leq 0.05$; 1.50% in the LL line vs. 0% in the HL line for preantral follicles, $p \leq 0.05$). No significant differences between the lines in the diameters of dormant and growing primordial follicles or primary (preantral) follicles were found, but ovaries isolated from LL animals had a lower diameter of secondary (antral) follicles

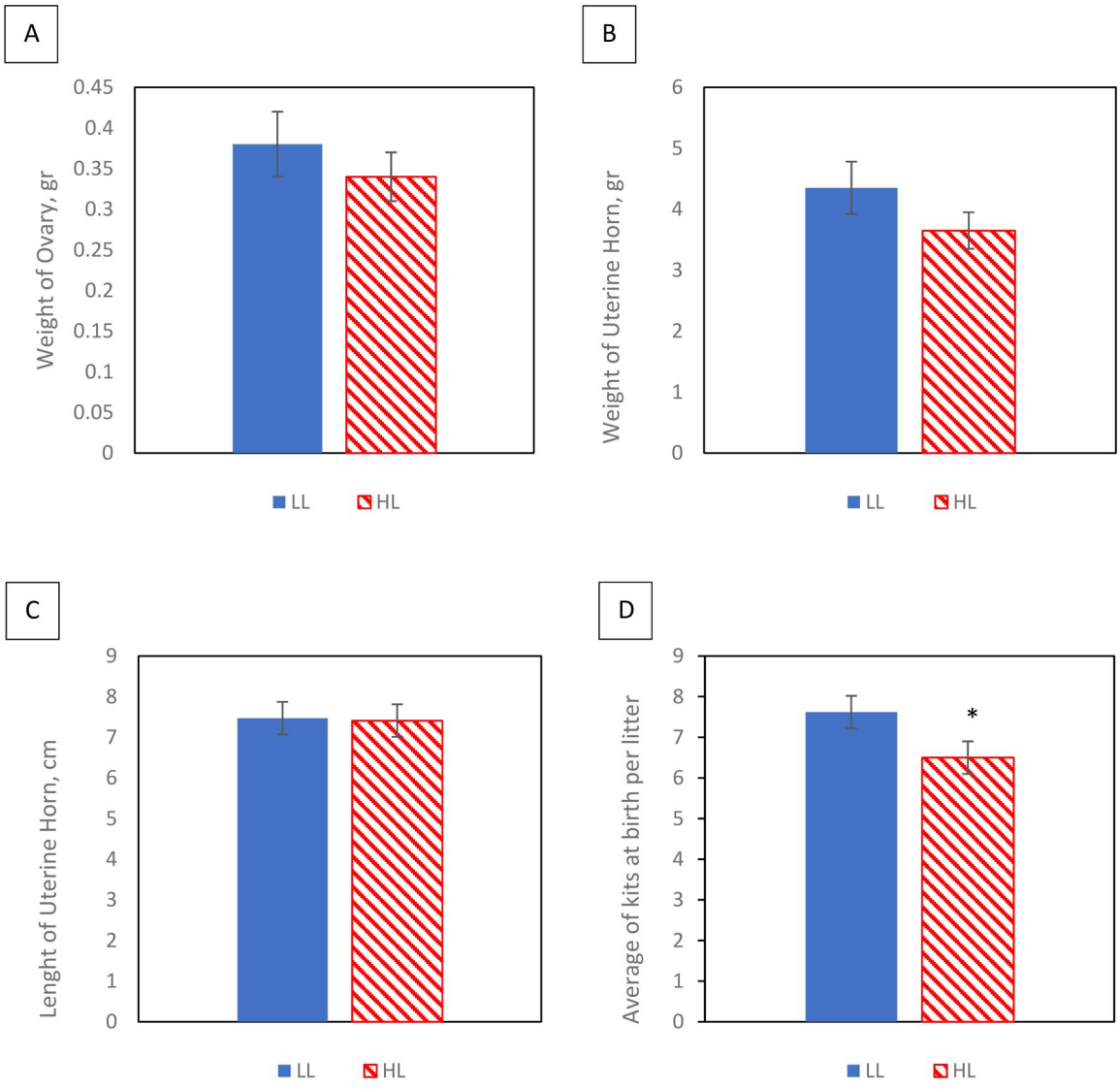


**Figure 1.** The effect of selection for litter size variability on ovarian weight (A), uterine horn weight (B), uterine horn length (C), and average number of kits per litter up the first four deliveries (D) in the low (LL) and in the high (HL) lines. Values represent means ± SEM. * indicates statistically significant differences ($p \leq 0.05$).

than ovaries of the HL group (358.96 μm in the LL line vs. 416.80 μm in the HL line, $p \leq 0.05$). Furthermore, the ovaries of animals from the LL contained preovulatory follicles, which were absent in HL.

In the preantral, secondary, and preovulatory follicles granulosa, and theca cells were distinguishable. The thickness of both theca and granulosa of primary (preantral) follicles of the LL and HL animals was similar, but the thickness of both theca and granulosa of secondary (antral) follicles in LL animals was significantly lower than in HL animals (38.49 μm in the LL line vs. 42.69 μm in the HL for thickness of theca, $p \leq 0.05$; 79.94 μm in the LL line vs. 110.8 μm in the HL for thickness of granulosa, $p \leq 0.05$).

No differences between the lines in the diameter of oocytes in primordial follicles occurred, but primary (preantral) and secondary (antral) follicles of LL animals had significantly larger oocytes than those of HL animals (88.49 μm in the LL line vs. 77.86 μm in the HL line for oocytes of primary follicles, $p \leq 0.05$; 122.10 μm in the LL line vs. 109.87 μm in the HL line for oocytes of secondary follicles, $p \leq 0.05$).

No significant differences in the proportion of follicles with morphological signs of degeneration (atresia) between the main groups were observed. On the other hand, a substantial proportion of primordial follicles of LL ovaries expressed signs of degeneration, but these follicles in the HL group did not show such

**Table 2.** The results of morphometric analysis of folliculogenesis and oogenesis in ovaries from females rabbit of the low (LL) and the high (HL) line selected for litter size variability.

| Ovarian structures and their parameters | LL | HL |
|---|---|---|
| **Dormant primordial follicles** | | |
| Number | 52 | 74 |
| Proportion in relation to all detected follicles (%) | 40.0 | 49.3 |
| Diameter of follicle (μm) | 30.17 ± 0.75 | 30.89 ± 0.56 |
| Diameter of oocyte (μm) | 23.45 ± 0.60 | 23.04 ± 0.46 |
| % of degenerated follicles | 0 | 0 |
| **Growing primordial follicles** | | |
| Number | 38 | 26 |
| Proportion in relation to all detected follicles (%) | 29.2 | 17.3 |
| Diameter of follicle (μm) | 57.39 ± 2.22 | 53.03 ± 2.69 |
| Diameter of oocyte (μm) | 36.43 ± 1.45 | 33.98 ± 2.35 |
| % of degenerated follicles | 26.3 | 0* |
| **Primary (preantral) follicles** | | |
| Number | 30 | 22 |
| Proportion in relation to all detected follicles (%) | 23.1 | 14.7 |
| Diameter of follicle (μm) | 185.74 ± 10.76 | 177.52 ± 10.35 |
| Thickness of theca (μm) | 17.79 ± 0.54 | 17.35 ± 1.25 |
| Thickness of granulosa (μm) | 30.84 ± 1.50 | 32.46 ± 2.01 |
| Diameter of oocyte (μm) | 88.49 ± 3.41 | 77.86 ± 3.87* |
| % of degenerated follicles | 6.7 | 7.6 |
| **Secondary (antral) follicles** | | |
| Number | 8 | 28* |
| Proportion in relation to all detected follicles (%) | 6.2 | 18.7* |
| Diameter of follicle (μm) | 358.96 ± 40.07 | 416.80 ± 15.71* |
| Thickness of theca (μm) | 38.49 ± 1.30 | 42.69 ± 1.97* |
| Thickness of granulosa (μm) | 79.94 ± 9.74 | 110.8 ± 1.71* |
| Diameter of oocyte (μm) | 122.10 ± 5.66 | 109.87 ± 2.86* |
| % of degenerated follicles | 37.5 | 31.8 |
| **Preovulatory follicles** | | |
| Number | 2 | 0* |
| Proportion in relation to all detected follicles (%) | 1.5 | 0* |
| Diameter of follicle (μm) | 429.53 + 63.47 | 0* |
| Thickness of theca (μm) | 33.27 ± 1.60 | 0* |
| Thickness of granulosa (μm) | 124.52 ± 6.12 | 0* |
| Diameter of oocyte (μm) | 113.93 ± 11.12 | 0* |
| % of degenerated follicles | 0 | 0 |

Values represent means ± SEM. * indicate statistically significant ($p \leq 0.05$) differences between the ovaries isolated from low (LL) and high (HL) line animals.

signs (26.3% in the LL line vs. 0% in the HL line, $p \leq 0.05$).

## *Viability, proliferation, apoptosis, and release of hormones by ovarian cells in rabbit does from the low line (LL) and the high line (HL)*

The Figures 2, 3 and 4 show several markers of viability, proliferation, apoptosis, and steroidogenesis in isolated and cultured ovarian cells. The percentage of viable cells was similar between the LL and the HL (Figure 2A). Regarding cell proliferation, no differences were found in PCNA and cyclin B1 accumulation in both lines (Figures 2B and 2C), but incorporation of BrdU was markedly higher in the LL than in the HL (0.92% in the LL line vs. 0.44% in the HL line, $p \leq 0.05$; Figure 2D).

In connection with apoptosis, no difference was found between the lines in accumulation of caspase 3 (Figure 3A). However, the LL showed a lower accumulation of bax (forty-one percent in the LL line vs. forty-eight percent in the HL line, $p \leq 0.05$; Figure 3B), and the index of apoptotic cell death also showed a tendency to be lower in the LL compared to the HL (0.82 OD in the LL line vs 1.33 OD in the HL line; Figure 3C), but the P value was less than or equal to 0.10. There was no difference between lines in the release of progesterone and oestradiol by granulosa cells (Figure 4A and 4B), but the release of progesterone and oestradiol by ovarian fragments was less in the LL than in the HL (12 ng/mg tissue/day in the LL line and 45 ng/mg tissue/day in the HL line for progesterone, $p \leq 0.05$; 11 ng/mg tissue/day in the LL line and 30 ng/mg tissue/day in the HL line for oestradiol, $p \leq 0.05$; Figure 4C and 4D).

## *Proteomic evaluation of the low and the high lines*

The proteomic profile of rabbit ovarian granulosa cells was different between the LL and the HL. The total number of identified proteins was 471, while we have

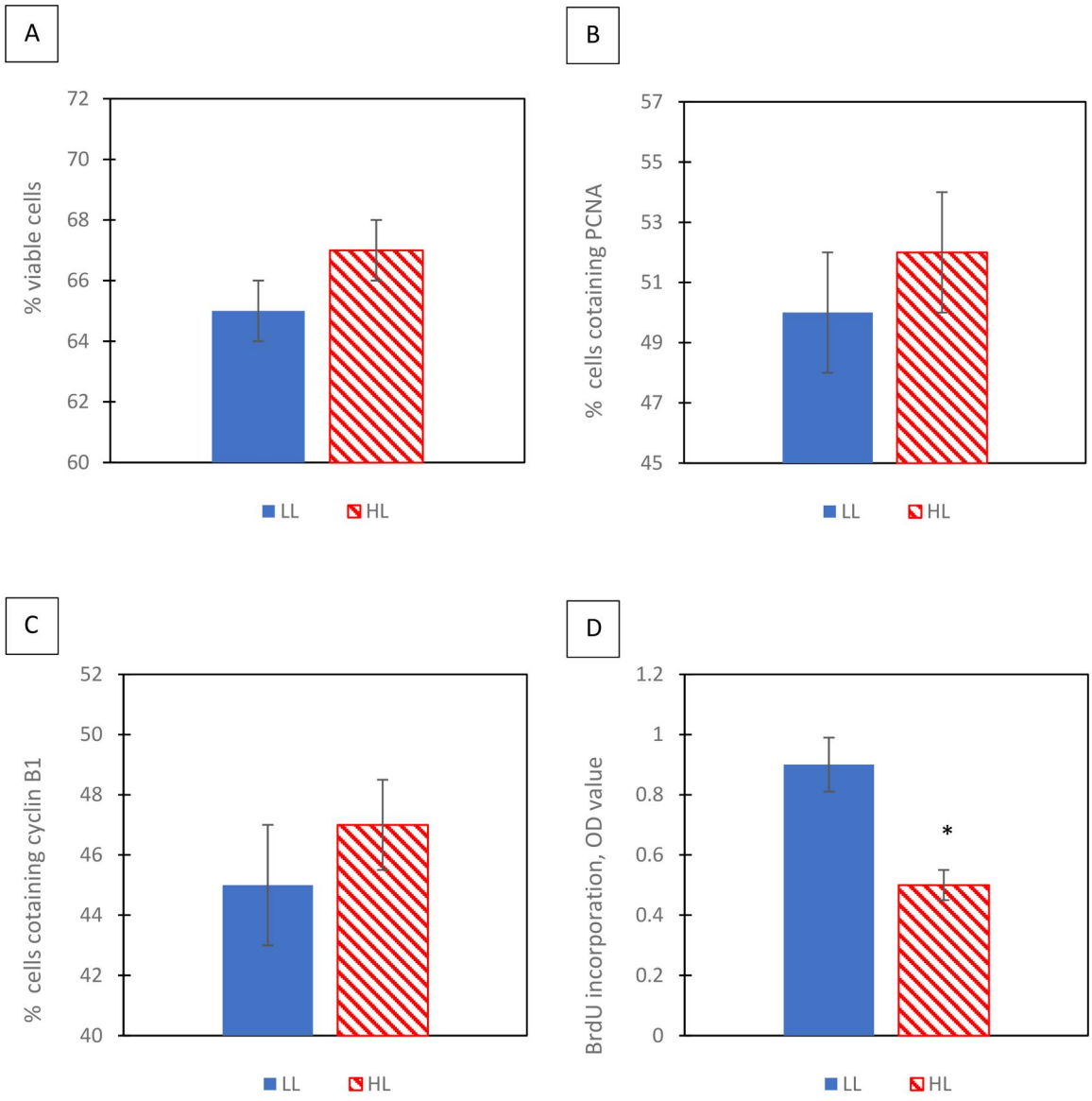


**Figure 2.** The percentage of viable cells (A), accumulation of PCNA (B), cyclin B1 (C), and BrdU (D) in rabbit ovarian granulosa cells isolated from ovaries of the low (LL) and the high (HL) lines. Values represent means ± SEM. * indicates statistically significant differences ($p \leq 0.05$).

identified a higher number of specific proteins in the granulosa cells of animals from the LL line than those of the HL line (50 vs. 38, respectively, $p < 0.05$; Figure 5). Cells isolated from the LL animals expressed more specific peptides involved in cell differentiation, adhesion, cell proliferation/division (cytoskeletal proteins), and fewer (below the detection level) proteins involved in control of cell cycle, chromatin organisation and hormone reception (Table 3).

## Discussion

We have successfully conducted a unique divergent selection experiment for environmental variability in litter size over sixteen generations using the rabbit as the experimental species. Divergent lines provide exceptional biological material for genetic studies, as by sharing the same environment, differences in the selected trait and correlated traits can be attributed to the fact that the selected trait is heritable and genetically correlated with the other traits (Falconer and Mackay 1996). In agreement with a previous study (Argente et al. 2017), we consistently found a higher number of kits at birth in the line selected to decrease the variability in litter size (LL). A higher prolificity in the LL line has been related to a larger number of implanted embryos (Argente et al. 2017), as a consequence of a more advanced embryonic development in early gestation (García et al. 2016; Calle et al. 2017). According to this finding, selection for uniformity in

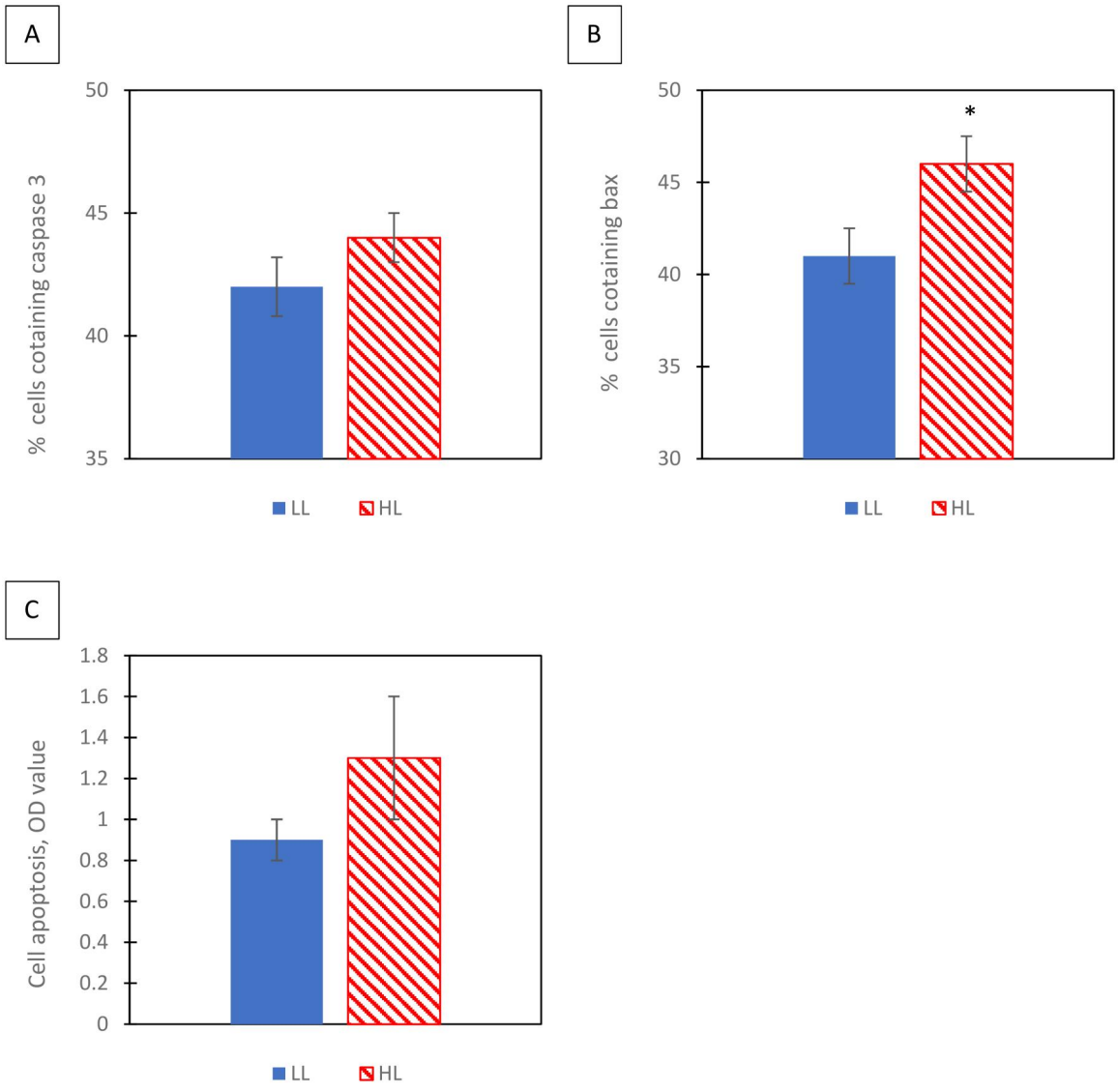


**Figure 3.** Accumulation of caspase 3 (A), bax (B) and index of apoptotic cell death in rabbit ovarian granulosa cells (C) from the low (LL) and the high (HL) line. Values represent means ± SEM. * indicates statistically significant differences ($p \leq 0.05$).

litter size improves female reproductive performance and reduces embryo losses, which results in larger litter sizes at birth -a finding that has also been reported in relation to the selection for uniformity weight in mice (Formoso-Rafferty et al. 2023)-. These authors reported that uniformity in birth weight is directly related to the robustness of females (Formoso-Rafferty et al. 2019). On the other hand, embryo losses are directly related to oocyte quality (Wang and Sun 2007; Lasienë et al. 2009). Oocyte growth, nuclear maturation, and cytoplasmic maturation -the build-up of nutrients and regulatory molecules- all have a direct impact on the quality of oocytes (Sirotkin 2014; Bartkova et al. 2020; Soler et al. 2020). In the present study, we have approached the study of the growth of follicles and oocytes and ovarian cell status (proliferation, apoptosis, viability, hormone production, and other regulatory molecules) in both LL and HL lines as adequate tools to assess the viability and quality of oocytes.

## *Growth pattern in follicles and oocytes in the LL and HL lines*

To understand the underlying biological mechanisms that control the difference between the lines in early embryonic development, we performed an analysis of histomorphometry in the ovaries from both lines.

To maintain the length of female reproductive life, the majority of primordial follicles must be preserved in a quiescent state (McGee and Hsueh 2000). Our study was carried out in multiparous rabbits after the

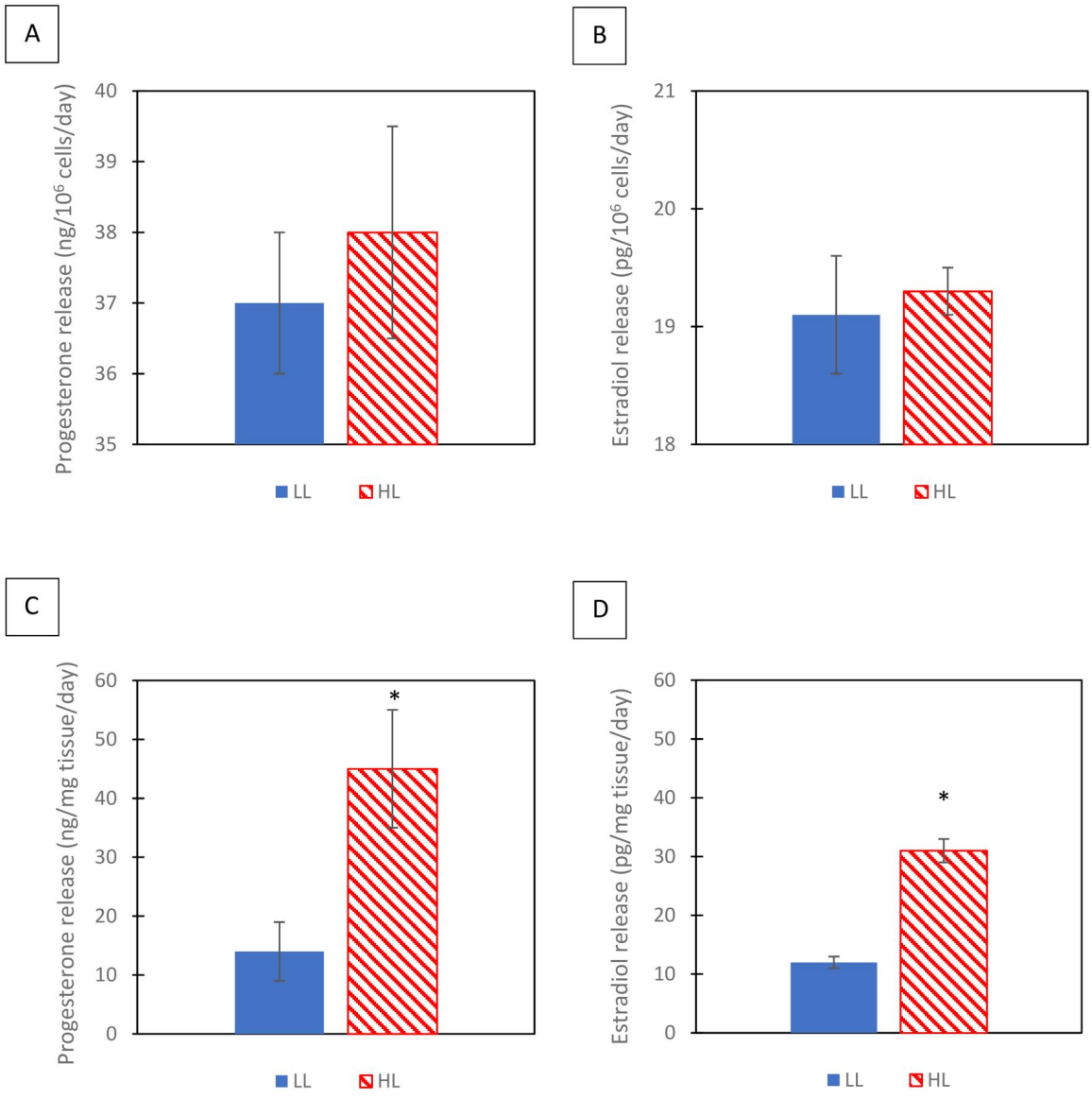


**Figure 4.** Release of progesterone (A) and oestradiol (B) in rabbit ovarian granulosa cells, and release of progesterone (C) and oestradiol (D) in ovarian fragments from the low (LL) and the high (HL) line. Values represent means ± SEM. * indicates statistically significant differences ($p \leq 0.05$).

fifth delivery. At this stage of reproductive life, we found a high and similar percentage of dormant and growing primordial follicles in both lines. Therefore, the fifth delivery in rabbits was far from being the end point of a female's reproductive lifespan. No differences between the lines in the diameter of primordial follicles and oocytes suggest, that at this stage no differentiation in their growth occurs. On the other hand, the occurrence of degenerated growing primordial follicles in the LL, but not in the HL indicates the start of a process of follicular atresia and selection in the LL rabbits.

Lack of differences between the line in the proportion, diameter of primary (preantral) follicles, and thickness of their compartments indicates a lack of divergence between the lines in primary follicle growth. On the other hand, the significantly larger diameter of oocytes in primary follicles in the LL suggests the more intensive growth of oocytes in these animals.

The trend to higher growth of oocytes in the LL continues in secondary (antral) follicles. Higher growth of oocytes in the LL animals could indicate more intensive cytoplasmic maturation (accumulation of nutrients and regulatory molecules, Sirotkin 2014) in oocytes in the LL in comparison with the HL. Better oocyte growth/cytoplasmic maturation in these animals could explain their higher and less variable fertility in the LL does. On the other hand, in the LL does the percentage of secondary follicles and the thickness

of their compartments were lower than in the HL animals. These differences might be explained by a more intensive transformation of secondary to preovulatory follicles. This hypothesis was confirmed by the presence of preovulatory follicles in the ovaries of the LL but not of the HL animals.

Fecundity and its variability are the defined by diameter and quality of oocytes (Lasienė et al. 2009), as well as growth, differentiation, and selection of ovarian follicles (Webb and Campbell 2007; Sirotkin 2014). The present observations explain the differences in rate and variability in fecundity of rabbits of the two lines by morphological development of ovarian follicles and their oocytes. Higher fecundity of LL versus HL could be caused by (1) selection of primordial follicles at the stage of their growth, (2) better transformation of secondary to preovulatory follicles, and (3) more intensive growth (cytoplasmic maturation) of oocytes in ovaries of low line does.

These differences could be due to differences in proliferation, apoptosis, and production of steroid hormones and regulatory proteins by ovarian cells (see below).

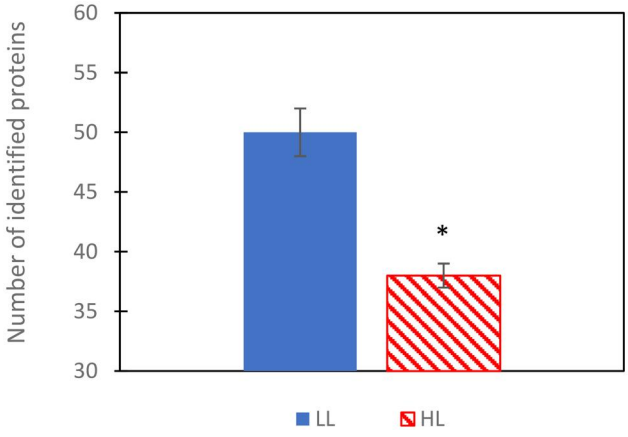


**Figure 5.** Number of identified proteins from ovarian granulosa cells from the low (LL) and the high (HL) lines. Values represent means ± SEM. * indicates statistically significant differences ($p \leq 0.05$) between the ovaries isolated from the LL and the HL lines for three independent proteomic analyses (sample preparation and protein identification by tandem mass spectrometry).

## *Ovarian cell activity in the LL and HL lines*

As it was mentioned in Materials and Methods, in the present study, the following markers and regulators of the cell functions were analysed: BrdU incorporation (cell proliferation; Ligasová et al. 2023), PCNA (S-phase of mitosis; Moldovan et al. 2007), cyclin B1 (M and G-phase of mitosis; Dai et al. 2019; Ligasová et al. 2023), DNA fragmentation (extrinsic apoptosis, nuclear DNA fragmentation; Mirzayans et al. 2016), bax (cytoplasmic/mitochondrial apoptosis; Spitz and Gavathiotis 2022), and progesterone and oestradiol (ovarian cell proliferation, apoptosis, ovarian follicullo-, oo- and luteogenesis; Sirotkin et al. 2017; Chou and Chen 2018; Chen et al. 2022).

The observed differences between isolated and cultured ovarian cells isolated from the ovaries of does from the two lines suggest that the causes of

**Table 3.** List of 25 selected proteins from spectrum mill – peptide/summary involved in different cell functions/activities.

| Protein function | Accession number* | Protein name | High | Low |
|---|---|---|---|---|
| Cell differentiation | G1SD59 | ADAM metallopeptidase with thrombospondin type 1 motif 15 | – | + |
| | G1SMB4 | Teneurin transmembrane protein 4 | – | + |
| Cell adhesion | A0A5F9DGZ2 | Laminin subunit alpha 3 | – | + |
| | G1U334 | von Willebrand factor | – | + |
| | G1U985 | Stabilin 1 | – | + |
| | A0A5F9C0S1 | ABI family member 3 binding protein | + | – |
| | A0A5F9C343 | Periostin | + | – |
| | A0A5F9C3C4 | Integrin beta | + | – |
| Cytoskeletal activity | A0A5F9CD17 | Myosin heavy chain 7B | – | + |
| | A0A5F9CEM2 | Myosin IIIA | – | + |
| | A0A5F9CNK3 | Dystonin | – | + |
| | A0A5F9CT68 | Cytoskeleton associated protein 5 | – | + |
| | A0A5F9D1T9 | phosphatidylinositol-4,5-bisphosphate 3-kinase | – | + |
| | A0A5F9CYW9 | Spectrin beta, non-erythrocytic 1 | – | + |
| | A0A5F9DLP9 | Dynein cytoplasmic 2 heavy chain 1 | – | + |
| | G1SU35 | Dynein axonemal heavy chain 1 | + | – |
| | G1T5K0 | Hemicentin 1 | + | – |
| | A0A5F9C6V3 | protein-tyrosine-phosphatase | – | + |
| | A0A5F9CVG4 | Lysyl oxidase homolog | – | + |
| | A0A5F9C3K1 | ADAM metallopeptidase with thrombospondin type 1 motif 20 | + | – |
| Molecular transducer activity | P49260 | Secretory phospholipase A2 receptor | + | – |
| | A0A5F9C0Z2 | Insulin like growth factor 2 receptor | + | – |
| GTP-ase activity | A0A5F9C3J4 | DENN domain containing 4 C | – | + |
| Cell cycle regulation | A0A5F9C604 | Zinc finger FYVE domain-containing protein 26 | + | – |
| Chromatin organisation | A0A5F9DIU5 | Jumonji domain containing 1 C | + | – |

*Protein accession number referring to UniProt database. + detected, – undetected.

differences in number and variability in litter size could be caused by differences in some processes related to proliferation, apoptosis, and steroidogenesis. Granulosa cells of the LL animals expressed a higher incorporation of BrdU (marker of DNA synthesis, Ligasová et al. 2023), but not the accumulation of PCNA (marker of S-phase of mitosis; Moldovan et al. 2007) or cyclin B1 (marker of M- and G-phase of mitosis; Dai et al. 2019; Ligasová et al. 2023), than the ovarian cells of the HL animals. These data suggest that higher fecundity and promoted folliculogenesis of the LL animals could be due to increased synthesis of DNA, but not to the transition between the particular phases of mitosis.

Furthermore, granulosa cells of does from the LL contained less bax (marker of cytoplasmic/mitochondrial apoptosis; Spitz and Gavathiotis 2022), but not of caspase 3 or marker of DNA fragmentation (Mirzayans et al. 2016). Therefore, the promoted fecundity and folliculogenesis in the LL animals could be explained not only by increased DNA synthesis but also by reduced DNA fragmentation.

A comparison of the ability of granulosa cells to secrete steroid hormones did not show significant differences between the lines. On the contrary, comparison of the secretory activity of cultured ovarian fragments demonstrated that ovaries of the LL animals released less progesterone and oestradiol than ovaries of the HL animals. The differences between steroid release by granulosa and ovarian fragments confirm the "two cells, two gonadotropins" theory. According to this theory, physiological regulation of steroid synthesis by ovarian follicles requires cooperation between granulosa and theca cells (Sirotkin 2014; Li et al. 2021). The impaired release of steroid hormones by ovarian fragments of the LL does might be explained by the reduced number of secondary follicles in their ovaries - the main source of ovarian steroid hormones during the follicular phase of the ovarian cycle (Sirotkin 2014). The functional interrelationships between different ovarian events in the studied animals require further elucidation. Nevertheless, the present observations indicate that differences in ovarian folliculogenesis, steroidogenesis and ovarian cell proliferation and apoptosis can be a cause of differences between the studied lines in litter size and variability.

Production of biologically active proteins (mainly enzymes) regulating cellular growth, survival, proliferation, differentiation and migration, gametogenesis, and response to hormones can affect embryogenesis and fecundity. For example, the absence of Cytoskeleton-associated protein 5, Phosphatidylinositol 3-kinase and Protein-tyrosine-phosphatase in knockout mice has been related to impaired early embryonic development (O'Neill 2008; Lu et al. 2017; Idrees et al. 2020), while the absence of ADMATS15 protease, Teneurin transmembrane protein 4, Laminin alpha3 submit, VWF, Stabilin-1 and Lysyl oxidase homolog in knockout mice prevent implantation due to poor placental development (Tucker and Chiquet-Ehrismann 2006; Hamill et al. 2010; Segond et al. 2013; Nandadasa et al. 2014; Randi and Laffan 2017; Kim et al. 2020).

The present proteomic analysis demonstrated that ovarian granulosa cells of the LL animals produced more specific proteins than the HL, including two peptides involved in cell differentiation, five for adhesion, nine for cell proliferation/division (cytoskeletal proteins), and one for GTP-ase activity. On the other hand, the cells of these animals did not produce a detectable amount of proteins involved in chromatin organisation and hormone reception. These observations indicate that the litter size and its variability in rabbits could be regulated by a number of peptides regulating different biological events. Moreover, the absence of some specific regulatory proteins either in the LL or the HL animals indicates that fecundity can be regulated not only by the presence but also by the absence or underproduction of some proteins stimulating or inhibiting various cellular functions. The present results suggest the multiple regulation of fecundity in rabbits. On the other hand, association between fecundity and production of particular proteins provides only indirect evidence for involvement of these proteins in ovarian functions and fecundity. Further studies are required to understand the role and functional interrelationships between the detected proteins and their targets.

## Conclusion

Selection of rabbits to reduce the variability in litter size resulted in increased fecundity (litter size). A higher prolificacy in the LL animals would be caused by: (1) selection of the growing primordial ovarian follicles; (2) better transformation of secondary to preovulatory follicles; (3) increased growth (cytoplasmic maturation) of oocytes; (4) increased DNA synthesis and decreased cytoplasmic apoptosis in ovarian granulosa cells; (5) changes in ovarian steroidogenesis; and (6) up- and down-regulation of a number of peptides involved in cell differentiation, proliferation/division (cytoskeletal proteins) and adhesion. Understanding

the hierarchical functional interrelationships between these regulators and their target processes requires further studies, although the available evidence enables us to hypothesise some regulatory axis. For example, steroid hormones can control ovarian cell proliferation, apoptosis, growth, differentiation, and selection of ovarian follicles, oocyte maturation, and maintenance of gravidity - processed defining fecundity and its variability. Cell viability and growth and development of ovarian follicles are defined by ovarian cell proliferation and apoptosis rate. Peptides involved in ovarian remodelling can affect ovarian folliculogenesis, ovulation, the quality of oocytes, and the success of fertilisation. GTP-ase can affect the reception of hormones and the subsequent post-receptory events. The functional interrelationships between numerous regulators of female reproduction require further elucidation. Nevertheless, the model of two lines of rabbits with different variability and sizes of litter developed previously and studied here enabled us to outline several extra - and intracellular mechanisms regulating fecundity in rabbits and other mammals including humans.

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## Disclosure statement

No potential conflict of interest was reported by authors.

## Ethics approval

All experimental procedures were approved by the Miguel Hernández University of Elche Research Ethics Committee, according to Council Directives 98/58/EC and 2010/63/EU (reference number 2023/VSC/PEA-0079).

## Author contributions

Conceptualisation, A.V.S and M.J.A.; Methodology, A.V.S., M.M. and pm; Laboratory analysis, I.H., Z.F., B.L., M.M., and pm; Statistical analysis, I.H., Z.F., M.L.G., B.L., M.M., pm, and I.A.; Data curation I.H., Z.F., and B.L.; Writing – original draft, **I.H**.; Writing-review & editing, I.H., Z.F., M.L.G., B.L., M.M., pm, I.A., A.V.S., and M.J.A. All authors have read and agreed to the published version of manuscript.

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## Data availability statement

None of the data were deposited in an official repository. All data are available upon request.

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