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ORIGINAL ARTICLE

Periconceptional undernutrition increases quantity and quality of oocyte population, but not cognitive or emotional response of 60-day-old lambs

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Summary

Maternal periconceptional undernutrition is associated with altered development and increased risks of adverse outcomes in the offspring. The aim of this work was to determine the effect of periconceptional undernutrition on behavioural and reproductive aspects of the offspring. Fifty ewes were synchronized in oestrus (day 0) and allocated to two groups ($n = 25$) to be fed diets that provided 1.5 (C) or 0.5 (L) times the requirements for maintenance until day 15. Ewes were mated and fed the control diet from day 16 until lambing. Two months after lambing, 26 lambs were exposed to tests to determine their cognitive/emotional responses. Six ewe lambs were euthanized and *in vitro* oocyte maturation and fertilization procedures performed. The experimental diets produced no changes of mean live weight (LW) of C ewes, L ewes presenting a reduction in their initial LW with significant differences at day 15, in comparison with C ewes ($p < 0.05$). L ewes experienced a significant reduction in their body condition (BC) in comparison with C ewes ($p < 0.05$). Fourteen days after the onset of the experimental diets, mean LW and BC of L ewes was significantly lower than those of C ewes ($p < 0.05$). Undernourished ewes presented a trend to a reduction of prolificacy and fecundity ($p < 0.10$) in comparison with C ewes. Emotional and cognitive test revealed a similar response between groups. Ewe lambs from the undernourished ewes presented a population of oocytes 1.7 times higher than ovaries from control ewe lambs (66.0 ± 0.73 vs. 113.7 ± 15.6 oocytes; $p < 0.05$) and had more oocytes in the 'good' ($p < 0.05$) and 'healthy' ($p < 0.05$) categories. In conclusion, a low plane of nutrition around conception significantly increases quantity and quality of the oocyte population of 60-day-old female descendants. Modifications of the cognitive and emotional responses of the progeny have not been evidenced.

Keywords sheep, periconceptional, undernutrition, offspring, oocyte

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Introduction

Terms as 'Foetal Programming', 'The Barker Hypothesis' or 'developmental origins of health and disease' (Caton and Hess, 2010) have been described as the programming of various bodily systems and processes by a stressor of the maternal system during pregnancy or during the neonatal period. It is also known as 'developmental programming' (Reynolds et al., 2010).

In some animal species, the effects of this process are evident in the adult life of the offspring, through changes in litter size, sex ratios, foetal or neonatal development and in key organ systems and functions, especially reproductive capacity, health and behaviour (Ashworth et al., 2009). One of these stressors is maternal nutrition, especially around conception, which is known as the 'periconceptional period'. It has been reviewed (Bloomfield, 2011) that maternal periconceptional undernutrition is related with

altered development and enlarged danger for adverse neurodevelopmental and metabolic consequences in childhood and later life, suggesting that environmental signals acting during early development may result in epigenetic changes which may play role acting between early life vulnerability and adult phenotype.

The effects of a maternal undernutrition around conception on several aspects of the offspring physiology are abundant. Thus, a low level of nutrition from −45 days to 6 days after conception is sufficient to change the amount of key factors regulating cardiac growth and metabolism, and this may increase the capacity to develop cardiovascular diseases in later life (Lie et al., 2013) or cause a suppression of the pituitary glucocorticoid receptor expression at the end of pregnancy (Zhang et al., 2013). At the reproductive level, maternal feed restriction has originated a delay in foetal ovarian development in sheep (Rae et al., 2001).

Nutritional conditions experienced during early development have consequences for the reproductive performance of humans. Thus, reproductive success of men and women born around the time of the Dutch famine of 1944–1945 was studied by Painter et al. (2008), concluding that poor nutrition during foetal development followed by improved nutrition after birth can give rise to a female phenotype characterized by greater reproductive success. Undernutrition before conception and during the periconceptional period can also alter the behaviour of the resultant offspring. In animal models, a significant decrease in voluntary physical activity in adult offspring after a periconceptional undernutrition of sheep has been observed (Donovan et al., 2013). Moreover, offspring from undernourished ewes suppress behavioural reactions and cortisol secretion in response to isolation stress in the offspring into adulthood and that these effects differ between males and females (Hernandez et al., 2010). When ewes were undernourished from 61 days before until 30 days after conception, offspring plasma cortisol was suppressed, producing a prolonged, sex-dependent effect on adrenal function in the offspring (Oliver et al., 2012). Such observations confirm the importance of the nutritional status of the oocyte and/or early cleavage-stage embryo on post-natal behaviour. It has been suggested that prenatal undernutrition can have long-term adverse effects on the animals' responses to normal husbandry procedures, in which pregnant ewes are often subject to periods of low food supply and have the potential to produce offspring with higher levels of emotional reactivity (Erhard et al., 2004).

The aim of this work was to determine the effect of periconceptional undernutrition on behavioural characteristics of the offspring and on their reproductive traits in sheep. Thus, some cognitive and emotional responses of the offspring and the number and quality of the oocytes recovered from female offspring born as a result of the window under study have been examined. Moreover, the capacity of these oocytes to become embryos after *in vitro* maturation (IVM) and fertilization (IVF) techniques was also investigated. Thus, it is hypothesized that undernutrition at the very early stages of development produces long-term negative effects on the adult animal life, having the potential to produce offspring with lower reproductive performances and higher levels of emotional reactivity.

Material and methods

The study was conducted at the experimental farm of the University of Zaragoza, Spain (latitude 41°N). All procedures were carried out under Project License PI05/10 approved by the in-house Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

Animals and experimental procedures

In mid-October, 50 mature Rasa Aragonesa ewes were synchronized in oestrus (day 0) using intravaginal sponges that contained 30 mg fluorogestone acetate (Chronogest; MSD Salud Animal, Madrid, Spain), which were inserted for 14 days. Upon sponge withdrawal, 480 IU eCG (Folligon; MSD Salud Animal) were administered. Ten fertile rams were introduced into the flock 24 h after sponge removal and kept for 72 h. At the time of sponge insertion, ewes were allocated to one of two groups to be fed diets that provided either 1.5 [control group (C); $n = 25$] or 0.5 [low group (L); $n = 25$] times the daily requirements for maintenance. Diets consisted of 0.80 or 0.50 kg of barley straw and 0.55 or 0.10 kg of pellets, for C and L groups respectively. The pelleted diet consisted of barley (85%) and soy bean (15%). The animals had unrestricted access to water and mineral supplement. At day 15, all ewes were regrouped and fed the control diet until lambing.

Live weight (LW) and body condition (BCS) (on a scale of 0–5, where 0 = emaciated and 5 = obese

(Russel et al., 1969)) were determined at time of sponge insertion (day −14), at withdrawal (day −1) and at day 15, the end of the differential diet period. The reproductive parameters assessed at lambing were fertility rate (number of ewes lambing per 100 ewes presented to rams), prolificacy (mean number of live and dead lambs born per ewe lambing) and fecundity (number of live and dead lambs born per ewe presented to the ram). Ratio male/female born and mean pregnancy lengths were also calculated. Lambs were weighed at lambing and weaning (45 days of age).

Approximately 2 months after lambing, 26 lambs (C: five males, eight females; L: five males, eight females) with a mean (±SEM) LW of 17.4 ± 0.5 kg (C: 18.8 ± 0.2; L: 18.2 ± 1.0 kg) and a mean age (±SEM) of 51.6 ± 0.1 days (C: 52.1 ± 0.1; L: 51.1 ± 0.1 days) were selected to be exposed to T-maze, isolation and novel object tests. Lambs were selected from those born in a 7-day interval. A blood sample was collected 48 h before the tests to determine plasma concentrations of some metabolic indicators of stress and energetic activity (cortisol, glucose, creatine kinase (CK) and lactate).

Seven days after the behavioural and cognitive tests, six ewe lambs (3 C, 3 L) born the same day, with a mean LW (±SEM) of 19.0 ± 0.6 kg (C: 19.2 ± 0.6; L: 18.9 ± 0.5 kg) and a mean age (±SEM) of 60.0 ± 0.0 days, were euthanized using an i.v. injection of a commercial euthanizer (T-61; MSD, Salamanca, Spain). Ewe lambs were selected on the basis of a similar LW, being born the same day and being twins. None of them participated in the behavioural tests. Their ovaries were recovered and stored at 39°C until they were processed, not later than half an hour after ovariectomy.

T-maze test

Lambs were subjected to a cognitive test in two consecutive rounds. A T-maze adapted for lambs (Marín et al., 1997) built with plastic panels 1.40 m in height (Fig. 1) was used. The T-maze test was developed as a learning paradigm for young chicks (Gilbert, 1989) and to assess emotionality on the basis of escape behaviour (Marin and Arce, 1996). It consisted of a start box and an isolation chamber (2 × 2 m) joined

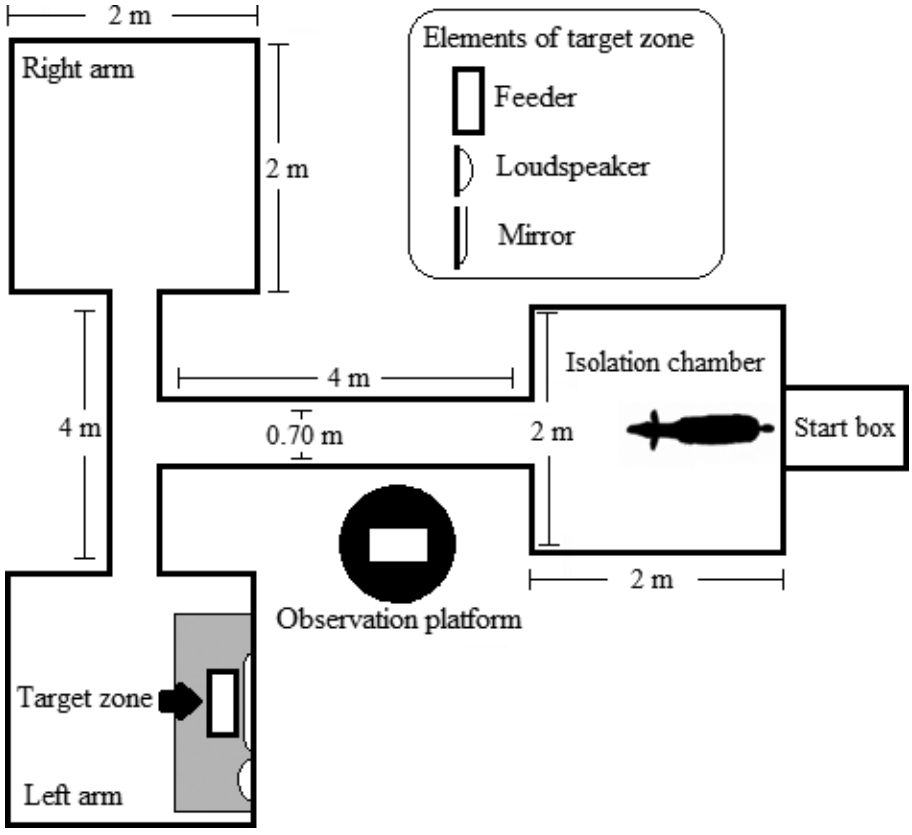

Fig. 1 T-maze apparatus used in the experiment. Dimensions are expressed in metres. The discontinuous lines create the imaginary divisions used to count traversed areas.

on one of its sides to a T-corridor. The start box was fully closed but large enough to enable an individual to move around. The T-corridor consisted of a 2 × 0.80 m path linked to two perpendicular arms (1.65 × 1.65 m each). A mirror (70 × 30 cm) and a loudspeaker were located in the target zone on the left arm. An observation platform was placed 3 m above the ground adjacent to the T-maze apparatus, so as not to influence animal movement (Ortiz-Plata et al., 2012). The apparatus was kept in a soundproof room (9 × 6 m) maintained at constant temperature and humidity during the test.

The sounds used in the experiment were a playback of ewe and lamb vocalizations during a short-time separation. They were recorded at a distance of 50 cm from the sound source using a Handy Recorder H1 (Zoom Corporation, Tokyo, Japan) numeric recorder (sampling rate: 44.1 kHz). Sounds were then imported into a computer at a sampling rate of 44.1 kHz and saved in WAV format at 16-bit amplitude resolution (Briefer and McElligott, 2012). The Audacity® audio software (Audacity, Pittsburgh, PA, USA) was used to prepare the sound sequences that were played back. A sample of these sounds was combined to produce a 5 min segment, and a random portion of this segment was played at each trial. The noise was played and measured using a Bioblock Scientific Sound Level Meter, type 50517, at a set volume that ensured lambs were exposed to 81 dB of intensity through the majority of the T-maze. For each trial, the sounds were played back using a Handy Recorder H1 connected to a loudspeaker located at floor level on the left arm in the target zone. Before entering the T-maze, the lambs were kept in a holding pen for 60 min. After this adaptation period, each individual was separated from the group and moved to the entrance of the test pen. The procedure was designed to ensure that the handling protocol applied to the animals between the holding and test pen was as standardized as possible and was performed quietly by the same person each time to avoid arousal before the test. Lambs from different treatment groups were tested alternately, so that the treatment factor was not confounded with the order of testing. All of them stayed in the start box for 20 s before a guillotine door was lifted to allow entrance into the arena. After the lamb left the start box, the guillotine door was quietly closed (Langbein, 2012) and recorded sounds were played back. Each individual was filmed, and the time taken in reaching the target zone (always on the left side), with a mirror (social clue) and the sound source (sound clue), was recorded. The latency to leave the start box and the number of areas traversed (Fig. 1) were calculated. Each animal was given a maximum of 5 min to solve the maze. No animal exceeded that time limit.

Isolation test

Lambs underwent an isolation test to measure fear in novel environments and response to separation. The test pen represented a novel environment in which the tested animal was visually isolated from other members of the group. The lambs were tested in an arena measuring 4 × 4 m, marked out in a grid of 0.50 × 0.50 m. Water and familiar food were placed in the arena in a familiar bucket against the wall facing the entrance door. Before testing, groups of lambs were moved to a holding pen, where they stayed for 30 min. After this adaptation period, each individual was separated from the group and moved to the entrance of the test pens. The procedure was designed to ensure that the handling protocol applied to the animals between the holding and test pen was standardized as far as possible and was performed quietly by the same person each time to avoid arousal before the test. Lambs from different treatment groups were tested alternately, so that the treatment factor was not confounded with the order of testing. All of them stayed in the start box for 20 s before a guillotine door was lifted to allow entrance into the arena. After the lamb left the start box, the guillotine door was quietly closed (Langbein, 2012). The lamb to be tested entered the arena, remaining there for a 5 min period. All behavioural responses were recorded on videotape using an overhead colour camera, and a microphone was used to record vocalizations. The time each animal spent walking, exploring, standing, attempting to escape (i.e. jumps: four legs leaving the ground), the latency to leave the start box, the latency to leave the test once the test was over and the number of bleats were recorded.

Novel object test

This test was designed to examine the initial reaction of a lamb when exposed to a novel object (1 min) and when exposed to the same object for a second time (1 min), one minute after the first exposure. Five minutes after opening the swing doors, right after the end of the isolation test, a novel object consisting of a blue plastic ball connected to a rope was lowered from the ceiling to the floor at the centre of the arena. When it hit the floor, it was left in that position for the first 1 min novel object test; then it was lifted up, and after 1 min, the same procedure was repeated. The distance to the novel object in the 30th second of the test (two

measurements) and the time taken to approach the novel object in each exposure were measured. The total number of times an animal touched the object during the whole test was also recorded.

In vitro procedures

A combination of puncture and slicing techniques was used to collect oocytes, which were classified based on their cumulus cells and cytoplasm morphology as follows: good (all oocytes with a lot of complete layers of granulose cells and homogeneous cytoplasm), fair (all oocytes with few or incomplete layers of granulose cells and homogeneous cytoplasm) or poor (oocytes with few or absence of granulose cells and non-homogeneous cytoplasm). Only good and fair oocytes (healthy oocytes) were selected for IVM. At the end of IVM, the oocytes were denuded from the cumulus cells and transferred to the fertilization medium. On the same day of fertilization, the semen collected from two rams was pooled, diluted 1:10 in a saline medium and kept at 15 °C until IVF. Highly motile spermatozoa were selected by swim-up technique and added to the fertilization medium that contained the oocytes at a final concentration of 1×10^6 spermatozoa/ml, covered with mineral oil and incubated for 24 h at 39 °C in an atmosphere of 5% CO_2 and saturated humidity. After 24 and 36 h, presumptive zygotes were assessed for cleavage. Non-cleaved oocytes were observed to assess their maturation stage. Oocytes showing the first polar body were considered matured, and oocytes with two polar bodies were considered fertilized but not cleaved. After fertilization, cleaved embryos were placed in a culture medium for 8 days. Media used for oocyte collection and IVM, IVF and embryo culture have been previously described (Forcada et al., 2011).

Plasma assays

Plasma glucose (g/l) and creatine kinase (CK) (UI/l) concentrations were determined with a Multichannel Technicon Analyser (RA-500), using reagents for RA Technicon systems (Bayer Diagnostics, Barcelona, Spain) (glucose, Ref. T01-1492-56; CK Ref. T01-1885-01). Plasma cortisol levels (ng/ml) were determined in duplicate by enzyme immunoassay (EIA; Chacon et al., 2004). The concentration of lactate (g/l) was determined in fluoride oxalate plasma using a Sigma Diagnostic Kit (Lactate no 735-10) and a spectrophotometer (Lamda 5; Perkin Elmer, Waltham, MA, USA). The intra-assay coefficients of variation were 10, 8, 14 and 5% for glucose, CK, cortisol and lactate respectively.

Statistical analysis

The rate of matured and fertilized oocytes, cleaved embryos and blastocysts was expressed as a percentage for each group. Maturation and cleavage rates were calculated over the number of healthy oocytes, fertilization rate was based on the number of matured oocytes, and blastocyst rates were based on the number of cleaved embryos or oocytes. They were evaluated statistically using chi-square or Fisher Exact tests, as appropriate. To integrate the percentages in the model, to determine whether they were influenced by the effects considered, individual proportions were arcsine-transformed before being subjected to statistical analysis. Total number and classification of oocytes recovered and number of healthy oocytes selected for maturation and the rest of the reproductive and growth parameters were also subjected to ANOVA. T-maze variables were subjected to repeated-measures ANOVA that examined the main effects of treatment (control and undernourished), T-maze trial (1st and 2nd T-maze escape; the repeated measure) and their interaction. To better fit the assumptions of the ANOVA, latency to leave the start box data was transformed to ranks. A one-way ANOVA was used to determine differences between control and undernourished lambs in open-field variables. Within novel object test, the distance to the novel object and the time to approximate the object were subjected to a repeated-measures ANOVAS that examined the main effects of treatment (control and low), novel object trial (1st and 2nd; the repeated measure) and their interaction. Because the number of times the object was touched was evaluated only once, differences between control and undernourished lambs were evaluated using a one-way ANOVA. Where appropriate, Fisher LSD tests were used for post hoc comparisons of means. Results were expressed as mean ± standard error of the mean (SEM). The probability level for statistical significance was set to $p < 0.05$ and trend to significance to $p < 0.10$.

Results

Live weight and body condition

During the experimental period, the mean LW of C ewes did not change; L ewes presented a reduction in their initial LW (Fig. 2), with significant differences at day 15, in comparison with C ewes ($p < 0.05$). Accordingly, L ewes experienced a significant reduction in their BC in comparison with C ewes ($p < 0.05$). After pessary withdrawal (14 days after

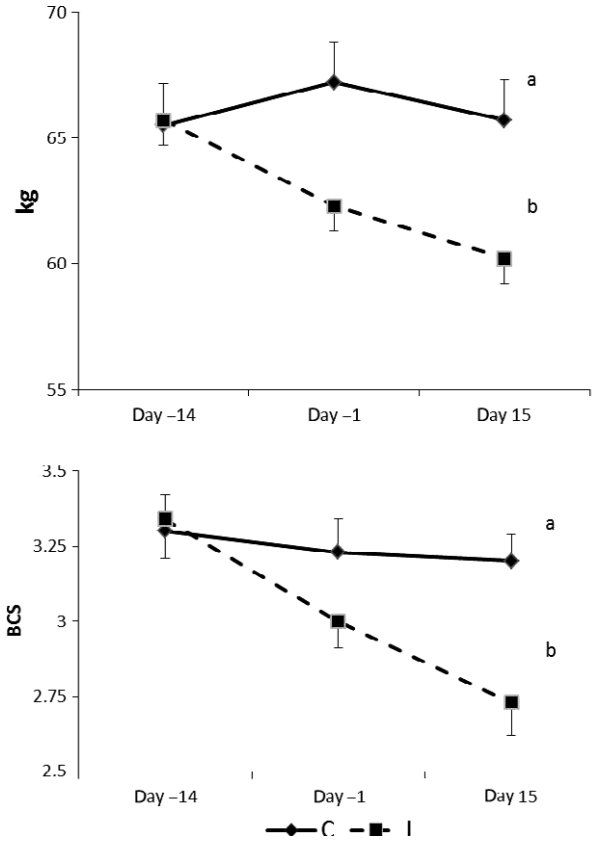

Fig. 2 Mean liveweight (LW) (upper panel) and body condition score (BCS) (lower panel) (±SEM) at the onset (day −14) and end of oestrous synchronization treatment (day −1) and at the end of the experimental diet period (day 15) of ewes, fed to provide 1.5 (C) or 0.5 (L) times the daily requirements for maintenance from sponge insertion to day 15 (oestrus = day 0). a, b: $p < 0.05$

the onset of the experimental diets), mean LW and BC of L ewes was significantly lower than those of C ewes ($p < 0.05$).

Reproductive parameters

A trend to reduce fertility ($p < 0.10$) and fecundity ($p < 0.10$) by periconceptional undernutrition up to the 15th day of pregnancy was observed, in comparison with the control group (Table 1). Fertility rate of the L group was 20% lower than the C group. No significant differences between groups were observed for mean pregnancy length and the ratio male/female born (Table 1).

Lamb growth

Mean LW at lambing of lambs born from L mothers presented a trend to be higher ($p = 0.10$) when compared to C lambs (Table 1). When male and females were compared separately, the differences between groups presented a statistically significant trend for females lambs ($p = 0.06$). Mean live weight at weaning and mean growth rate was not different between groups.

Plasma concentrations

No significant differences between groups for plasma glucose, CK, cortisol and lactate concentrations were observed (glucose: 0.96 ± 0.01 vs. 0.93 ± 0.01 g/l; CK: 189.4 ± 46.0 vs. 270.5 ± 50.3 UI/l; lactate: 0.18 ± 0.01 vs. 0.18.3 ± 0.02 g/l; cortisol: 86.2 ± 5.8 vs. 66.9 ± 4.2 ng/ml, for C and L lambs respectively).

T-maze, isolation and novel object tests

No significant differences between groups were observed in the T-maze test performed by the lambs (Table 2). Both the latency to leave the start

	C	L	Sig.
Fertility (% ewes lambing)	84.0% (25)	64.0% (25)	p = 0.10
Prolificacy (lambs/lambing)	1.84 ± 0.12	1.79 ± 0.15	NS
Fecundity (lambs/ewe)	1.52 ± 0.18	1.09 ± 0.21	p = 0.10
Pregnancy length (days)	150.1 ± 0.2	149.4 ± 0.4	NS
Ratio male/female	41/59	36/64	NS
Live weight at lambing (kg)	4.10 ± 0.13 (32)	4.40 ± 0.14 (25)	p = 0.10
Male lambs (kg)	4.32 ± 0.19 (13)	4.40 ± 0.19 (9)	NS
Female lambs (kg)	3.95 ± 0.17 (19)	4.41 ± 0.15 (16)	p = 0.06
Live weight at weaning (kg)	14.06 ± 0.28	14.50 ± 0.55	NS
Male lambs (kg)	15.59 ± 0.42	15.78 ± 0.38	NS
Female lambs (kg)	12.78 ± 0.31	13.87 ± 0.38	NS
Daily growth (g/day)	0.24 ± 0.03	0.24 ± 0.02	NS
Male lambs (g/day)	0.28 ± 0.01	0.26 ± 0.03	NS
Female lambs (g/day)	0.22 ± 0.05	0.24 ± 0.10	NS

Table 1 Reproductive performances of ewes synchronized in oestrus and fed to provide 1.5 (C) or 0.5 (L) times the daily requirements for maintenance from the onset of oestrous synchronization treatment to day 15 (oestrus = day 0) and live weights and growth rate of the offspring (mean ± SEM) (number of animals)

Table 2 Results of the T-maze and novel object tests performed by lambs born from ewes synchronized in oestrus and fed to provide 1.5 (C) or 0.5 (L) times the daily requirements for maintenance from the onset of oestrous synchronization treatment to day 15 (oestrus = day 0) (mean ± SEM)

	C (n = 13)		L (n = 13)		Sig.		
	1st trial	2nd trial	1st trial	2nd trial	Treatment	Trial	Treat*Trial
			T-maze test				
Latency to leave the start box (s)	5.8 ± 0.9	6.83 ± 5.03	8.0 ± 4.0	2.4 ± 0.7	NS	NS	NS
Total time (s)	141.5 ± 40.1	63.8 ± 32.7	172.4 ± 33.5	98.4 ± 37.7	NS	$p < 0.01$	NS
Number of areas traversed	13.25 ± 3.85	5.25 ± 0.41	17.4 ± 4.3	10.2 ± 2.02	NS	$p < 0.05$	NS
			Novel object test				
Distance to the novel object (cm)	74.0 ± 22.3	85 ± 16.5	73.0 ± 16.9	116.2 ± 18.4	NS	NS	NS
Time to approximate the object (s)	5.4 ± 1.7	4. 3 ± 1.2	5.6 ± 1.5	16.0 ± 6.4	NS	NS	NS
Number of times the object is touched	6.6 ± 0.7		5.1 ± 0.4		$p = 0.10$	–	–

box and the total time spent in the maze were significantly lower in the second trial and similar in both groups. Results of the isolation test reveal no differences between groups for the parameters under study (Table 3). Regarding the novel object tests, no differences between groups were detected (Table 2).

Oocyte quality

Ovaries from ewe lambs born from undernourished ewes had a total population of oocytes that was 1.7 times higher than that of the ovaries from control ewe lambs (Table 4; $p < 0.05$). Furthermore, they had more oocytes in the 'good' ($p < 0.05$) and 'healthy' ($p < 0.05$) categories.

Table 3 Results of the isolation test performed by 2-month-old lambs born from ewes synchronized in oestrus and fed to provide 1.5 (C) or 0.5 (L) times the daily requirements for maintenance from the onset of oestrous synchronization treatment to day 15 (oestrus = day 0) (mean ± SEM)

	C (n = 13)	L (n = 13)	Sig.
Latency to leave the start box (s)	4.7 ± 0.9	6.4 ± 1.4	NS
Walk (s)	112.5 ± 13.9	116.4 ± 11.2	NS
Explore (s)	23.8 ± 5.6	20.4 ± 5.5	NS
Scape attempts	16.7 ± 5.6	28.3 ± 8.0	NS
Stand (s)	142.8 ± 17.7	129.1 ± 16.5	NS
Latency to leave the pen at the end of the test (s)	29.3 ± 7.15	29.5 ± 8.3	NS
Number of bleats	83.4 ± 8.2	82.1 ± 10.3	NS

IVM and IVF

Results of the IVF procedures revealed a lower percentage of maturation ($p < 0.05$) and cleavage rates ($p < 0.05$) of oocytes from L ewe lambs (Table 4). In spite of these differences, blastocyst rate were similar between groups.

Discussion

The most interesting result of the present experiment is the significantly higher amount of oocytes and better quality showed by ewe lambs from undernourished dams in comparison with control animals. There are few comparable studies that have examined the effect of maternal nutrition on the quality of the offspring's oocyte. It is possible that maternal undernutrition has a direct effect on the ovarian development of the offspring. Supporting this hypothesis, Rae et al. (2001) observed a delay in foetal ovarian development after maternal feed restriction in sheep. As this effect was not limited to cells and tissues present only during the period of underfeeding, it was concluded that a nutritional restriction imposed at an early stage of foetal development can have effects at later stages. Moreover, ovaries of foetuses of undernourished dams at 47 days of pregnancy contained significantly more oocytes than those of control foetuses (Borwick et al., 1997), suggesting that the process of oogonial degradation, and the associated reduction in germ cell concentration, may have been reduced or delayed in the ovaries of this foetus. The degree of staining of the tis-

	C (n = 3)	L (n = 3)	Sig.
Good (%)	17.0 ± 6.03 (25.7%)	53.0 ± 8.4 (46.4%)	$p < 0.05$
Fair (%)	4.3 ± 2.0 (6.1%)	15.0 ± 3.8 (13.2%)	$p < 0.10$
Poor (%)	44.7 ± 6.9 (68.2%)	45.7 ± 4.3 (40.4%)	NS
Total	66.0 ± 0.73	113.7 ± 15.6	$p < 0.05$
Healthy (good + fair) (%)	21.3 ± 7.9 (31.8%)	68.0 ± 12.1 (59.6%)	$p < 0.05$
IVM	57/63 (90.5%)	154/203 (75.9%)	$p < 0.05$
IVF	56/57 (98.2%)	148/154 (96.1%)	NS
Cleavage rate 48 h	55/63 (87.3%)	136/203 (67.0%)	$p < 0.05$
Blastocysts rate (blastocyst/embryos)	3/55 (5.5%)	18/136 (13.2%)	NS
Blastocysts rate (blastocyst/oocyte)	3/63 (4.8%)	18/203 (8.8%)	NS

Table 4 Number (mean ± SEM) of good, fair or poor oocytes and results of the *in vitro* maturation (IVM) and fertilization (IVF) procedures per ewe lamb, recovered from ovaries of 2-month-old ewe lambs born from ewes synchronized in oestrus and fed to provide 1.5 (C) or 0.5 (L) times the daily requirements for maintenance from the onset of oestrous synchronization treatment to day 15 (oestrus = day 0)

sues at day 62 of gestation observed by these authors indicated that there was greater nuclear DNA activity in the undernourished ovaries than in the control ones, which in turn suggests that the arrest of meiotic activity had been delayed in the ovaries. It has also been concluded (Kelly et al., 2005) that maternal nutrition during pregnancy significantly influences the number of oocytes harvested from resulting lambs as well as the *in vitro* developmental capacity of these oocytes. As they compared several diets through the whole pregnancy, they concluded that these effects were a consequence of significant interactions that occur between diet and stage of pregnancy indicating that nutrition plays a complex role in the regulation of foetal oogenesis and foliculogenesis.

Undernourished ewes suffered a reduction of their reproductive parameters, which was accompanied with a significant reduction of both LW and BC throughout the experimental period. A similar decrease has been previously observed by our group, applying exactly the same experimental diets with the same breed (Sosa et al., 2006, 2009; Abecia et al., 2013). We have also reported that this level of undernutrition increases NEFA and decreases leptin concentrations in the undernourished ewes, indicating an increase in the lipolytic activity (Abecia et al., 2013).

Lambs born from undernourished ewes were slightly heavier at lambing than C lambs. In sheep, the effects of maternal undernutrition on birthweight and foetal adipose tissue mass have been inconsistent and depend on the timing, level and length of dietary restriction (Bispham et al., 2003). Other studies have documented that total visceral (Gardner et al., 2005) and perirenal adiposity (Ford et al., 2007) of the offspring of undernourished mothers are increased, which is accompanied by insulin resistance compared with progeny from well-nourished ewes (Gardner et al., 2005; Ford et al., 2007; Caton and Hess, 2010). Supporting these observations, Jaquiery et al. (2012) observed that a brief undernutrition around the time of conception in sheep increases the relative amount of body fat of the offspring. No differences in the ratio male/female born have been detected in the present experiment. An influence of maternal nutrition on the sex ratio of the offspring has been described (Rosenfeld and Roberts, 2004), so that dams in poor body condition give birth to proportionately more females. It seems that sons require higher nourishment from mothers compared with daughters starting from earliest stages of gestation, and factors that adversely affect maternal condition could cause excess of male foetal mortalities (Cagnacci et al., 2003). However, evidence for influence of parental condition on offspring sex has been controversial for humans (Cameron, 2004).

The cognitive and emotional tests have not shown differences between groups. Donovan et al. (2013) demonstrated that periconceptional undernutrition in sheep from 60 days before conception to 30 days of pregnancy leads to a significant decrease in voluntary locomotor activity of 18 months of age lambs in a natural environment. The absence of differences in the present experiment is in agreement with previous works (Simitzis et al., 2009), where it was concluded that prenatal undernutrition during different periods of pregnancy has no effect on fear-related behaviour. This is also supported by the absence of differences in the plasmatic hormonal and metabolic indicators measured in this experiment, mean age and LW being also similar between groups, indicating that lambs from both groups reached the behavioural tests in similar physiological conditions.

In conclusion, a low plane of nutrition around conception significantly increases quantity and quality of the oocyte population of 60-day-old female offspring

in the sheep model. Modifications of the cognitive and emotional responses of the offspring have not been evidenced.

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