Testicular development in male lambs prenatally exposed to a high-starch diet

Questa è la versione Post print del seguente articolo:

Original

Testicular development in male lambs prenatally exposed to a high-starch diet / Mossa, Francesca; Bebbere, Daniela; Ledda, Antonello; Burrai, Giovanni P; Chebli, Imane; Antuofermo, Elisabetta; Ledda, Sergio; Cannas, Antonello; Fancello, Francesco; Atzori, Alberto S. - In: MOLECULAR REPRODUCTION AND DEVELOPMENT. - ISSN 1040-452X. - 85:5(2018), pp. 406-416. [10.1002/mrd.22974]

Availability: This version is available at: 11388/203446 since: 2022-05-24T12:24:50Z

Publisher:

Published DOI:10.1002/mrd.22974

Terms of use:

Chiunque può accedere liberamente al full text dei lavori resi disponibili come "Open Access".

Publisher copyright

note finali coverpage

(Article begins on next page)

1	Testicular development in male lambs prenatally exposed to a high-starch diet
2	Francesca Mossa ^{1*} , Daniela Bebbere ¹ , Antonello Ledda ² , Giovanni P. Burrai ¹ , Imane Chebli ³ ,
3	Elisabetta Antuofermo ¹ , Sergio Ledda ¹ , Antonello Cannas ² , Francesco Fancello ² , Alberto S. Atzori ²
4	
5	¹ Department of Veterinary Medicine, University of Sassari, via Vienna 2, 07100, Sassari, Italy;
6	² Department of Agricultural Sciences, University of Sassari, viale Italia 39, 07100, Sassari, Italy; ³
7	Department of Biology, Faculty of Science, University of Djillali Liabes, 22000 Sidi Bel Abbes,
8	Algeria.
9	
10	
11	*Corresponding author: Francesca Mossa, Department of Veterinary Medicine, University of
12	Sassari, via Vienna 2, 07100, Sassari, Italy; phone: 0039079229413; email: fmossa@uniss.it
13	
14	Running title: dietary programming of testicular development
15	
16	Keywords: programming, DOHaD, gonad, sheep, gene expression.
17	
18	Abbreviations
19	<i>AMH</i> = Anti-Müllerian hormone
20	AR = androgen receptor
21	BCS = body condition score
22	DM = dry matter
23	DMI = dry matter intake
24	F = fiber diet
25	F147 = fiber diet for the entire gestation
26	F75 = fiber diet for the last 75 days of gestation
27	FSHR = follicle stimulating hormone receptor
28	HSD17B3 = hydroxysteroid (17-beta) dehydrogenase 3

- 29 *IGF1* = insulin-like growth factor 1
- 30 *IGF2* = insulin-like growth factor 2
- 31 *IGF2R* = insulin-like growth factor 2 receptor
- 32 *LHCGR* = luteinizing hormone/choriogonadotropin receptor
- 33 NDF = neutral detergent fiber
- 34 *RPL19* = ribosomal protein L19
- 35 S = starch diet
- 36 S147 = starch diet for the entire gestation
- 37 S75 = starch diet for the last 75 days of gestation
- 38 SDHA = succinate dehydrogenase complex flavoprotein subunit A
- 39 SEM = standard error of the mean
- 40 STAR = steroidogenic acute regulatory protein
- 41 *VEGFA* = vascular endothelial growth factor A
- 42 YWHAZ = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
- 43

44 **Funding:** This work was funded by the Italian Ministry of University and Research (MIUR), Grants:

45 FIR 2013 and Rita Levi Montalcini 2010.

46

47 SUMMARY

48 Maternal nutrition during critical gestation periods impacts on offspring in later life; effects of high-49 starch maternal diet on testicular development in lambs were addressed. Dairy ewes were fed 50 diets providing either 27% (Starch, S) or 11% (Fiber, F) of starch from mating to lambing (~147 51 days; S147, F147) or for the last 75 days of gestation (S75, F75). Testes of single male lambs 52 were measured and then sampled for histological and gene expression analyses at selected ages. 53 Testicular dimensions and weight were similar among groups, but the total area of seminiferous 54 tubules increased with age and tended to be higher (P = 0.057) in lambs from starch- than fiber-fed 55 ewes. Sertoli and germ cells number increased with age, but was not influenced by maternal diet. 56 Transcript abundances of and rogen receptor (AR), insulin-like growth factor 1 (IGF1) and

57 hydroxysteroid (17-beta) dehydrogenase 3 (HSD17B3) was similar between S147 and F147 lambs 58 (P > 0.1). Abundance of luteinizing hormone/choriogonadotropin receptor (LHCGR) and 59 steroidogenic acute regulatory protein (STAR) was higher in young vs older lambs, whereas 60 insulin-like growth factor 2 (IGF2) levels increased with age. The expression of vascular 61 endothelial growth factor A (VEGFA), Anti-Müllerian hormone (AMH), IGF1, follicle stimulating 62 hormone receptor (FSHR) and insulin-like growth factor 2 receptor (IGF2R) was not influenced by 63 maternal diet or lamb age (P > 0.1). In conclusion, a high-starch maternal diet did not influence 64 gene expression, but may have affected testicular structure in infant offspring, as seen by an 65 increase in the total area of seminiferous tubules.

66

67 **INTRODUCTION**

68 Cumulative evidence indicates that fetal life environment markedly influences development, 69 physiological function and risk of disease in adult mammals (Barker 2007; Langley-Evans and 70 McMullen 2010). Animal and human studies have shown that nutrient imbalance during fetal life is 71 positively associated with subsequent diseases, such as hypertension, diabetes and obesity 72 (Heindel et al. 2015; Langley-Evans 2006). Accumulating evidence suggests that maternal 73 nutritional status can also impact on the developmental programming of the reproductive system in 74 female (Bernal et al. 2010; Borwick et al. 1997; Mossa et al. 2013; Rae et al. 2001; Sloboda et al. 75 2009; Sullivan et al. 2009) and male offspring (Alejandro et al. 2002; Kotsampasi et al. 2009; Rae 76 et al. 2002b) and that the observed effects depend on the severity, duration and timing of 77 nutritional perturbation (reviewed in (Chadio and Kotsampasi 2014; Mossa et al. 2017; Zambrano 78 et al. 2014)).

79

The hypothesis of a negative impact of maternal undernutrition on female reproductive capacity is supported by several studies conducted in rodents (Bernal et al. 2010; Sloboda et al. 2009), sheep (Borwick et al. 1997; Rae et al. 2001) and cattle (Mossa et al. 2013). In rats, female offspring born to mothers undernourished during pregnancy or throughout pregnancy and lactation had low mRNA abundancy of genes critical for follicular maturation and ovulation (*FSHR*, *GDF9*, *ER* and

85 CPY17A1) (Bernal et al. 2010). In sheep, in utero undernutrition increased the expression of 86 apoptotic genes in fetal ovaries at day 110 of gestation (Lea et al. 2006) and the number of 87 oogonia in fetal ovaries at day 47 and 65 of gestation (Borwick et al. 1997; Rae et al. 2001). 88 Periconceptional undernutrition from estrus to day 7 of gestation resulted in a greater total 89 population of oocytes in 30-day-old-lambs (Abecia et al. 2014b) and similarly maternal 90 undernutrition from mating to day 15 of pregnancy increased the guantity of oocytes in 60-day-old 91 female lambs (Abecia et al. 2014a). Further, female progeny of ewes undernourished from mating 92 to day 95 of gestation had reduced ovulation rates at 20 months of age (Rae et al. 2002a). In 93 cattle, female calves born to nutritionally restricted mothers during the first trimester of pregnancy 94 had a reduced ovarian reserve as assessed by a reduced antral follicle count, lower peripheral 95 concentrations of anti-Müllerian hormone and increased follicle-stimulating hormone serum 96 concentrations, both before and after puberty (Mossa et al. 2013). Heifers exposed to a low-protein 97 and low-energy diet during early pregnancy followed by a high-protein diet during the second 98 trimester of gestation had a reduction in primordial and primary follicles and healthy antral follicles 99 as adults (Sullivan et al. 2009).

100

101 Few studies have examined the effects of maternal undernutrition on the development and function 102 of the reproductive system in male offspring. In sheep, maternal undernutrition from mating to day 103 110 of gestation had no effect on the number of Sertoli cells and on the expression of gene 104 products that regulate apoptosis in fetal testes (Andrade et al. 2013). Nonetheless, ewe 105 undernutrition from mating until day 50 of gestation increased the mRNA abundance of 106 steroidogenic acute regulatory protein (STAR), a protein involved in transport of cholesterol to 107 mitochondria for steroidogenesis, in fetal testes (Rae et al. 2002b). Furthermore, a reduction in the 108 number of Sertoli cells was observed at birth in lambs undernourished in utero from week 10 of 109 gestation until parturition (Alejandro et al. 2002) and at ten months of age in lambs undernourished 110 from day 31 to 100 of pregnancy (Kotsampasi et al. 2009), respectively.

111

On the other hand, the number of studies investigating the possible link between maternal overnutrition and fertility in female and male progeny is limited. In rats, maternal consumption of a high-fat diet during pregnancy and/or lactation advanced the age at puberty in female offspring (Sloboda et al. 2009). In sheep, a high nutrient intake during different windows of gestation (from mating to day 103 or 131 of gestation) impaired the number of follicles in female fetuses (Da Silva et al. 2002; Da Silva et al. 2003). In cattle, a high maternal dietary intake impaired the total number of follicles, upregulated the expression of genes involved in ovarian folliculogenesis,

steroidogenesis and pro-apoptosis (*P450* aromatase, *STAR*, *BMPR2*, *TGFBR1*, *GDF9*, *FSHR Bax*and *CASP3* genes) in the ovaries and increased the expression of *FSHB* in the pituitary gland of

121 female fetuses at day 139, 199 and 241 of gestation (Weller et al. 2016).

122 In rabbits, a dietary-induced maternal hyperlipidemia and hypercholesterolemia administered from 123 preconception to lactation led to male offspring with lighter testes and epididymis and decreased 124 testosterone concentrations as adults compared with offspring born to control dams (Dupont et al. 125 2014). Finally, in cattle maternal high intake reduced the diameter and length of the seminiferous 126 cords and decreased the expression of genes involved in steroidogenesis and in the development 127 and function of the gonad (STAR, HSD17B3, IGF1, IGF2 and IGF1R) in fetal testes (Weller et al. 128 2016). These studies, although limited in number, suggest that both female and male offspring of 129 overnourished mothers may have compromised reproductive potential.

130

131 Although a global maternal nutrient imbalance (restriction or excess) can program the offspring 132 phenotype, the diet composition during pregnancy may also have effects on the progeny (Indrio et 133 al. 2017). For example, evidence indicates that maternal isocaloric diets with different protein 134 composition may impact differently on offspring reproductive development (Sui et al. 2014a; Sui et 135 al. 2014b; Zambrano et al. 2005). Newborn female piglets born to mothers exposed to protein 136 restriction throughout gestation had lighter ovaries, higher circulating estradiol concentrations, 137 greater expression of genes involved in folliculogenesis (BAX/Bcl-2, BMP4, PCNA) and lower 138 mRNA abundancy of steroidogenic genes (FSHR and CYP19A1) in the ovaries, as compared to 139 offspring of sows fed an isocaloric diet with higher protein content (Sui et al. 2014b). In addition,

maternal low protein diet during gestation and lactation disrupted the ovarian follicular
development in prepubertal (6-month-old) gilts, as assessed by a decrease in the number of
primordial and Graafian follicles associated with an increased number of secondary follicles (Sui et
al. 2014a). In rats, maternal protein restriction during pregnancy and/or lactation caused a
reduction in LH and testosterone concentrations, as well as reduced fertility rates and sperm
counts in adult male offspring (Zambrano et al. 2005).

146 Energy intake is a primary limiting factor of milk yield in dairy ruminants and is determined by the 147 net energy content of the diet and dry matter intake (DMI). For this reason, starchy feeds are 148 commonly fed to dairy ruminants during lactation to increase energy intake. Forages are replaced 149 with grains rich in starch, so that the concentration of dietary neutral detergent fiber (NDF) 150 decreases and DMI increases (Allen 2000). At similar energy and nitrogen intakes, diets rich in 151 starch increased milk nitrogen efficiency (the proportion of feed nitrogen recovered in milk) and 152 improved mammary amino acid utilization compared with diets rich in fiber (Cantalapiedra-Hijar et 153 al. 2015; Huhtanen and Hristov 2009). Nevertheless, the potential long-term consequences of a 154 high-starch diet on the progeny are unknown. We hypothesize that a maternal diet rich in starch 155 influences gonadal development of the male offspring. To test this hypothesis, the ovine model 156 was used to determine whether a diet rich in starch fed during the entire gestation or during the 157 last 75 days of pregnancy: 1) alters the development of the seminiferous tubules and the number 158 of Sertoli and germ cells and 2) affects the expression of critical genes involved in testicular 159 development and function in infant lambs, as compared to a fiber-based diet.

160

161 **RESULTS**

162 Maternal Body Weight and BCS During Gestation

- 163 Thirteen days before mating, the ewes that would subsequently receive a Starch (S147, *n* = 8) and
- 164 a Fiber (F147, n = 10) diet for the entire pregnancy weighed 36.1 ± 1.1 and 36.8 ± 1.0 kg,
- 165 respectively (P = 1). Body weight increased during gestation in all these pregnant ewes (P <
- 166 0.001), but no difference was detected between S147 and F147 groups throughout pregnancy. At
- 167 lambing S147 and F147 ewes weighed 54.2 ± 1.9 and 52.8 ± 1.7 kg, respectively (P = 1). Body

168 condition score (BCS) was similar between S147 and F147 ewes before conception (S147 2.8 \pm 169 0.04; F147 2.9 \pm 0.1; *P* = 0.96) and at lambing (S147 3 \pm 0.1; F147 2.9 \pm 0.1; *P* = 1) and did not 170 vary during gestation (*P* = 0.90).

- 171 Pregnant ewes that were fed the experimental diets during the last 75 days of gestation (S75, *n* =
- 172 5; F75, n = 7) had a similar weight at the start of the experimental diet (S75 42.8 ± 1.4; F75 44.1 ±
- 173 1.1 kg; P = 1). All these ewes gained weight during pregnancy (P < 0.001), but no effect of diet
- 174 was detected. At lambing S75 and F75 weighed 51.1 ± 0.9 and 50.9 ± 1.3 kg, respectively (P = 1).
- 175 BCS was similar between groups at the start of the experimental diet (S75 2.7 ± 0.05; F75 2.7 ±
- 176 0.05; *P* = 1) and was not affected by diet or day of gestation. At lambing, the BCS of S75 and F75

177 was 2.7 ± 0.05 and 2.7 ± 0.1 , respectively (*P* = 1).

178

179 Phenotypic Measurements of the Lambs

180 Maternal nutritional regime did not affect body weight (S147 3900.6 ± 153.2; F147 4023.5 ±150.6; 181 S75 4214.8 \pm 306.1; F75 4346.4 \pm 71.3 g; P = 0.40) or height at withers (S147 36.9 \pm 0.3; F147 182 37.8 ± 0.3 ; S75 37.9 ± 0.9 ; F75 38.1 ± 0.3 cm; P = 0.40) of the male offspring at birth. At slaughter, 183 live weight, height at withers, girth circumference and scrotal circumference were similar among 184 lambs born to mothers fed the different diets (Table 1). Furthermore, length, width and weight of 185 each testis were not different among dietary groups. As expected, all phenotypic measurements 186 increased with age (P < 0.001), but no interaction was detected between maternal diet and age at 187 sampling (Table 1).

188

189 Testicular Development in Lambs Exposed to a High-Starch versus a Fiber-Based Diet

190 During the Entire Gestation or During the Last 75 Days of Pregnancy

- 191 The total area of the seminiferous tubules tended to be higher in offspring of ewes fed a high-
- starch diet (*P* = 0.057), increased with age (*P* = 0.026) and no interaction between maternal diet
- 193 and age at sampling was detected (Figure 2). In agreement with this findings, the percentage of
- 194 interstitial tissue tended to be higher in lambs born to mothers fed a fiber based diet (*P* = 0.057)
- and decreased with age (*P* = 0.026; Figure 2). The mean number of seminiferous tubules per field

decreased with age at sampling (P < 0.001), whereas the average area, and the maximum and minimum internal diameters of circular tubules increased with age (P < 0.0001). None of these parameters where affected by maternal diet (Table 2). The mean number of Sertoli cells did not vary with age, whereas the mean number of germ cells was lower in young vs older lambs (P =0.005), but no effect of maternal diet was detected (Table 2).

201

Variations in mRNA Abundance of Key Genes in Testes of Lambs Born to Mothers Fed a High-Starch versus a Fiber-Based Diet Throughout Gestation

204 Transcript abundance of all analysed genes was not influenced by maternal diet (P > 0.1; Figure 205 3). Transcript abundance of steroidogenic acute regulatory protein was higher (STAR; P < 0.05) in 206 testes of lambs sacrificed at 7-14 days of age compared to lambs aged 25 to 41 days, whereas 207 luteinizing hormone/choriogonadotropin receptor (LHCGR) and insulin-like growth factor 2 (IGF2) 208 levels were lower (P < 0.05) in younger than in older lambs. The expression of vascular endothelial 209 growth factor A (VEGFA), IGF1, Anti-Müllerian hormone (AMH), follicle stimulating hormone 210 receptor (FSHR) and insulin-like growth factor 2 receptor (IGF2R) did not vary with maternal diet or 211 age of the lamb.

212

213 **DISCUSSION**

214 To our knowledge, this is the first study to investigate the impact of isoenergetic maternal diets that 215 differ in starch concentration on the gonadal development in male offspring. Specifically, the high-216 starch diet consisted of 26.7% of starch and sugars on dry matter, whereas the fiber diet had 217 10.7% of starch and sugars. Results indicate that a high-starch maternal diet during the entire 218 gestation or during the last 75 days of pregnancy did not impair testicular development of infant 219 male offspring in sheep. Testicular physiology depends on the integrated function of the tubular 220 and interstitial compartment. Spermatozoa are produced in the seminiferous tubules, whereas the 221 interstitial tissue contains Leydig cells that secrete testosterone, blood and lymphatic vessels and 222 macrophages (Senger 2003). In our study, lambs born to ewes fed a high-starch diet tended to 223 have a greater proportion of seminiferous tubules as compared to the progeny of dams fed a fiber-

224 based diet. This may be interpreted as a positive effect of a high-starch diet on testicular 225 development of the offspring, because a larger seminiferous area may result in greater sperm 226 production during adulthood. In rats, maternal protein restriction during pregnancy and/or lactation 227 reduced seminiferous tubule diameter in prepubertal offspring (Rodríguez-González et al. 2012); 228 and this change, coupled with the impairment of total germ cell and Sertoli cell, may be responsible 229 for the lower fertility rate observed in adulthood (Zambrano et al. 2005). A smaller seminiferous 230 tubules diameter was also found in adult male sheep born to ewes nutritionally restricted in the 231 second part of gestation (Kotsampasi et al. 2009). In cattle, fetuses of overfed mothers had a 232 decrease in diameter, length and volumetric proportions of the seminiferous cords as compared to 233 fetuses of mothers fed a moderate intake of the same diet (Weller et al. 2016). Thus, the potential 234 positive effect of a maternal high-starch diet on testicular development merits further investigation, 235 possibly via a long-term study that monitors lamb growth until puberty. Furthermore, in the present 236 study the percentage of total seminiferous tubules was affected by maternal diet, but no interaction 237 between maternal diet and age at sampling was detected. This finding may indicate that maternal 238 diet did not alter the physiological growth of the seminiferous tubules and that the starch diet did 239 not delay testicular development during infancy.

240

241 Maternal diet did not significantly influence the number of Sertoli and germ cells as well as the 242 expression of the analyzed genes, probably because the two diets were designed to provide the 243 same level of energy intake and consequently dams in the two experimental groups were similar in 244 body weight and body condition score throughout gestation. Thus, the maternal endocrine and 245 metabolic environment may have been similar for fetuses in the two groups. An alternative 246 explanation is that the potential long-term effects of the maternal diet on testicular gene expression 247 may manifest in older offspring. For instance, the long-term effects of maternal diet on the 248 testicular expression of genes involved in steroidogenesis (AR, FSHR, LHCGR, STAR) may be 249 detected at puberty, when increasing androgen concentrations stimulate the maturation of the 250 reproductive system (Senger 2003; Yarney and Sanford 1989).

251 The transcript of IGF1 was similar in lambs born to mothers exposed to a high-starch and to a 252 fiber-based diet. IGF1 is synthesized by almost all tissues in the body; in the testis it is 253 predominantly expressed in Leydig cells and, to a lesser extent, in Sertoli and germ cells (Vannelli 254 et al. 1988) and it is pivotal for the development and function of the male gonad (Froment et al. 255 2004; Griffeth et al. 2014). For example, Igf1-null male mice are infertile dwarfs and exhibit a 256 reduction greater than 80% in both spermatogenesis and serum testosterone concentrations 257 (Baker et al. 1996). Also, insulin and *Iqf1* regulate Sertoli cell proliferation in mice (Pitetti et al. 258 2013), thus the lack of maternal diet effect on both IGF-1 expression and Sertoli cells reported in 259 the present work are in accordance. In cattle, IGF1, IGF2, and IGF2R had a lower expression in 260 fetal testis derived from cows fed a high compared with a moderate intake of the same diet 261 suggesting a detrimental effect of maternal overnutrition on the development of the male gonad in 262 the offspring (Weller et al. 2016). Nevertheless, in our study the abundance of IGF2 and IGF2R 263 was not influenced by maternal diet and IGF2 expression significantly increased with age. IGF2 is 264 considered essential for embryonic and fetal development, (Griffeth et al. 2014) and Igf2-deficient 265 mice show defects that are associated with intra-uterine growth restriction (Randhawa and Cohen 266 2005). Taken together, these findings indicate that maternal diet did not alter the expression of the 267 insulin family of growth factors which provide essential signals for the control and development of 268 the male gonad (Griffeth et al. 2014).

269

270 Maternal diet did not impact on the expression of AR; AR is found in Sertoli cells and is involved in 271 the normal development and function of postnatal testis by actions of androgens (Collins et al. 272 2003; Ruwanpura et al. 2010), which are also known to be essential for the completion of 273 spermatogenesis in mammals (Courot et al. 1979; Parvinen 1982). The expression of HSD17B3, a 274 gene that codes for an enzyme which catalyses the reduction of androstenedione to testosterone 275 (Ge and Hardy 1998), was also not influenced by maternal diet. Further, mRNA abundance of 276 STAR, LHCGR and FSHR, other genes essential for steroidogenesis, was not influenced by 277 maternal diet. Taken together these results indicate that maternal dietary composition did not alter 278 the pathways involved in the regulation of androgen synthesis and activity. An increase in the

expression of *STAR* was reported in 50-day-old male fetuses of undernourished ewes, suggesting that maternal undernutrition may upregulate steroidogenesis (Rae et al. 2002b). On the other hand, maternal overnutrition decreased the testicular expression of *STAR* and *HSD17B3* in 139 and 199-day-old bovine male foetuses (Weller et al. 2016). These two studies indicate that both maternal under and overnutrition may impact the expression of genes involved in steroidogenesis in male fetuses. Our maternal dietary treatment did not cause such variation in mRNA abundance, probably because the diets provided the same energy level.

286

287 The concept that the nutritional management of the pregnant dam may affect the development and 288 function of the male offspring is supported by growing evidence in rodents (Genovese et al. 2010), 289 sheep (Alejandro et al. 2002), horse (Robles et al. 2017) and cattle (Weller et al. 2016). However, 290 these studies investigated the impact of nutritional restriction or excess. Here, we compared two 291 diets that provided the same energy, but differed in their carbohydrate composition. It should be 292 noted that we only included singleton pregnancies to exclude the confounding effect of singleton vs 293 twin placental growth, which may result in different body composition of the offspring (Symonds et 294 al. 2016). Based on the observed lack of significant changes in testicular biometry (dimensions and 295 weight), and in the expression of several genes involved in gonadal development and function 296 (AMH, AR, FSHR, HSD17B3, IGF1, IGF2, IGF2R, LHCGR, STAR, VEGFA) in infant testes, we 297 conclude that a high-starch maternal diet did not impact on testicular development in prepubertal 298 offspring. Nevertheless, because lambs born to ewes fed a high-starch diet showed a strong 299 tendency to have a greater proportion of seminiferous tubules, a positive effect of a high-starch 300 diet on testicular structure (proportion of seminiferous tubules and interstitial tissue) may be 301 present and a longer study investigating the potential effects of a high-starch diet in sexually 302 mature offspring would be beneficial.

303

304 MATERIALS AND METHODS

Animals were located in a commercial farm located in the area of Porto Torres, north of Sardinia,
Italy (40°50′13″ N 8°24′05″ E). All animal experiments were performed in accordance with DPR

27/1/1992 (Animal Protection Regulations of Italy) in conformity with European Community
regulation 86/609. This research is a part of a larger project titled "Permanent effects of starch and
fiber supplied during uterine and postnatal life of dairy sheep on gastrointestinal microbiota and
energy partitioning between milk production and fat deposition". All chemicals were purchased
from Sigma Chemical CO. (St. Louis, MO, USA) unless otherwise stated.

312

313 Maternal Diet

314 Sarda dairy ewes (n = 30; age = 2.6 ± 0.4 years; parity = 1.6 ± 0.4) were randomly allocated to one 315 of two experimental diets: Starch (S; n = 13) and Fiber (F; n = 17) consisting of 26.7% and 10.7% 316 of starch and sugars on total dry matter (DM), respectively and crude protein content of 14% on 317 DM (Table 3). The diets were fed as a conventional diet based on ryegrass hay (55%) and a 318 commercial concentrate mix (45%). The same hay was used for all dietary groups. For the S diet 319 the concentrate was mainly based on corn grain and barley, whereas in the F diet part of starch 320 was composed of high digestible fiber from soyhulls to achieve the desired level of dietary starch. 321 Dietary intake of the ewes was adjusted to cover animal requirements during different stages of 322 gestation, whereas the proportion of forages and concentrate was maintained to keep constant the 323 percentage of starch in each diet. Ewes were fed the assigned diet three times a day (9:30, 15:30 324 and 19:30 h) from mating to lambing (approximately 147 days; S147, n = 8; F147, n = 10) or from 325 day 90 of gestation to lambing (approximately 75 days; S75, n = 5; F75, n = 7). Water was offered 326 ad libitum. Ewes were naturally mated and pregnancy was diagnosed via transabdominal 327 ultrasonography (MyLabOneVet, Esaote, Genoa, Italy) approximately 25 days after the 328 introduction of rams in the flock. Live weight and body condition score (BCS) were assessed every 329 fortnight.

330

331 Phenotypic Measures of the Male Offspring and Tissue Collection

All ewes lambed a healthy single male lamb (n = 30). To investigate whether maternal diet
influenced body growth in neonatal offspring, live weight and height at withers were recorded at
birth. Lambs were fed with milk replacers until slaughter. To evaluate the effects of maternal diet

335 on the progeny growth, lambs were slaughtered in a commercial abattoir at selected ages: Day 1 336 (n = 3), Days 7-14 (n = 11), Days 21-25 (n = 9) and Days 25-41 (n = 7). At slaughter live weight, 337 height at withers, thoracic and scrotal circumference at the largest circumference of the scrotum 338 were measured with a measuring tape. Testes were removed, cleaned of the surrounding tissues 339 and then circumference, length, height and weight were recorded for each testis. Testes were cut 340 along the longest axis with a sterile surgical blade. The right testis of the lambs of the four groups 341 (S147, F147, S75, F75) was 10% formalin fixed and stored at room temperature for histological 342 analysis, whereas the left testis of the lambs of the S147 and F147 groups was immersed in RNA 343 later, snap frozen in liquid nitrogen and stored at -80°C for RNA isolation.

344

345 **Testicular Histology**

346 To determine whether maternal diet impacted on testicular morphology, testicular samples (n = 30)347 were fixed in 10% neutral-buffered formalin for at least 24 hours at room temperature, then 348 dehydrated in a graded ethanol series and embedded in paraffin for stereological analysis. Two 349 serial sections (3 µm-thick) per sample were cut at 50 µm intervals and stained with hematoxylin 350 and eosin. Sections were analyzed under a light microscope and high power field 351 photomicrographs from 5 randomly selected microscopic fields for each section (resulting in a total 352 of 10 fields per testis) were acquired with a Nikon Digital Sight DS-U1 camera mounted on a Nikon 353 80-i microscope (Nikon Instruments Spa, Florence, Italy). Images were processed with Fiji ImageJ 354 software (Schneider et al. 2012). Histological analysis focused on previously described parameters 355 (Montoto et al. 2012; Rojas-García et al. 2013). In each field, all the cross-sections of the tubules 356 were counted (number of tubules per field) and their circumference was traced (Figure 1). Cross 357 sections where perpendicular diameters of seminiferous tubules did not differ by more than 20% 358 were defined as circular tubules and their individual area (area of individual tubule), maximum 359 (maximum internal diameter) and minimum (minimum internal diameter) internal diameter were 360 measured and then averaged (average internal diameter). In each field, the total area occupied by 361 the seminiferous tubules was calculated by tracing the circumference of all the cross-sections of

the tubules (both circular and asymmetrical) in the image. The total interstitial area was calculated
 as the difference between the field area and the area of all seminiferous tubules.

To estimate the number of Sertoli and germ cells, 74 circular seminiferous tubules were analyzed
 on average per lamb. Cells with basal, leptochromatic (lax chromatin), ovoid or pyriform nuclei
 were considered Sertoli cells, whereas non-Sertoli cells were considered germ cells (Hoffman et al.
 2018; Wrobel et al. 1995).

368

369 Gene Expression Analysis

370 The expression pattern of a panel of genes involved in testicular development and function was

analyzed in the testes of lambs born to mothers fed the experimental diets: Starch (S147, n = 8) or

Fiber (F147; *n* = 10) for the entire gestation (Table 4). The genes analyzed were: Anti-Müllerian

373 hormone (AMH), androgen receptor (AR), follicle stimulating hormone receptor (FSHR),

374 hydroxysteroid (17-beta) dehydrogenase 3 (HSD17B3), insulin-like growth factor 1 (IGF1), insulin-

375 like growth factor 2 (*IGF2*), insulin-like growth factor 2 receptor (*IGF2R*), luteinizing hormone

376 /choriogonadotropin receptor (LHCGR), steroidogenic acute regulatory protein (STAR) and

377 vascular endothelial growth factor A (*VEGFA*). Details on gene expression analysis by real-time

378 PCR are described according to the MIQE guidelines (Bustin et al. 2009).

379

380 Total RNA Isolation and Reverse Transcription

381 All tissue samples were immediately plunged into RNALater (Qiagen, Hilden, Germany) after 382 collection and stored at -80°C until RNA isolation. Total RNA was isolated using TRIzol reagent 383 (Invitrogen Corporation, Carlsbad, CA) at 1 ml per 50 mg tissue and treated with DNase I 384 (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's protocols. Resulting RNA 385 quantity and purity was spectroscopically checked with NanoDropLite (Fisher Scientific S.A.S., 386 France). One µg total RNA from each sample was reverse transcribed in a 20 µL reaction with 50 387 mM Tris HCI (pH 8.3), 75 mM KCI, 3 mM MgCl2, 5 mM DTT, 1 mM dNTPs, 2.5 µM Random 388 Hexamer primers, 20 U of RNase OUT[™] and 100 U of SuperScript[™] III RT (all provided by 389 Invitrogen Corporation, Carlsbad, CA). Negative control reactions (without the enzyme) were

390 carried out to confirm the absence of genomic DNA contamination. The reaction tubes were 391 incubated at 25°C for 10 min, at 42°C for 1 h and finally at 70°C for 15 min to inactivate the 392 reaction.

393

(SDHA).

394 **Real Time-Polymerase Chain Reaction**

395 Primers for all genes studied are listed in Table 1. Relative guantification of transcripts was 396 performed by real-time polymerase chain reaction (RT-PCR) in a 7900HT Fast Real-Time PCR 397 System (Applied Biosystems, Foster City, CA), as previously described (Bebbere et al. 2014). The 398 PCR was performed in a 15 µL reaction volume containing 7.5 µL 2× SYBR Green PCR Master 399 Mix (Applied Biosystems, Foster City, CA), 200 nM of each primer and cDNA equivalent to ~50 ng 400 RNA. The PCR protocol consisted of two incubation steps (50°C for 5 min and 95°C for 2 min), 401 followed by 40 cycles of amplification program [95°C for 15 s, gene specific annealing temperature 402 (see Table 1) for 30 s], a melting curve programme (65–95°C, starting fluorescence acquisition at 403 65°C and taking measurements at 10-s intervals until the temperature reached 95°C) and finally a 404 cooling step to 4°C. Fluorescence data were acquired during the elongation step.

405 To minimise handling variation, all samples to be compared were run on the same plate using a 406 PCR master mix containing all reaction components apart from the sample. The PCR products 407 were analysed by generating a melting curve to check the specificity and identity of the 408 amplification product. For each primer pair, the efficiency of the PCR reaction was determined by 409 building a standard curve with serial dilutions of a known amount of template, covering at least 3 410 orders of magnitude, so that the calibration curve's linear interval included the interval above and 411 below the abundance of the targets. Only primers achieving an efficiency of reaction between 90 and 110% (3.6 > slope > 3.1) and a coefficient of determination $r^2 > 0.99$ were used for the 412 413 analysis. Messenger RNAs of all evaluated genes were detected in all samples. Target gene 414 expression was normalized against the geometrical mean of three housekeeping gene expression 415 ribosomal protein L19 (RPL19), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase 416 activation protein zeta (YWHAZ) and succinate dehydrogenase complex flavoprotein, subunit A 417

419 Statistical Analysis

420 Statistical analysis was performed using SAS University Edition version 3.6 (SAS Institute Inc., 421 Cary, NC, USA; 2012-2016). Maternal body weight and BCS during gestation were analyzed with a 422 linear model (Proc MIXED of SAS with repeated measures) considering the main effects of diet 423 (Starch or Fiber; 2 levels) within the duration of dietary treatment (75 and 147 days of gestation; 2 424 levels), days of gestation and their interaction; the effect of ewe was considered as random. All 425 phenotypic measurements of the lambs (live weight, height at withers, girth circumference, scrotal 426 circumference and testicular length, width and weight) were analyzed with a general linear model 427 (Proc GLM of SAS) with the terms of diet (Starch or Fiber; 2 levels) within the duration of dietary 428 treatment (75 and 147 days of gestation; 2 levels), age classes (birth, 7-14 days, 21-25 days, 26-429 41 days; 4 levels) and their interaction. Testicular histology measurements and the number of 430 Sertoli and germ cells were analyzed with a linear model (Proc MIXED of SAS with repeated 431 measurements) considering the main effects of diet (Starch or Fiber; 2 levels) within the duration of 432 dietary treatment (75 and 147 days of gestation; 2 levels), age classes (birth, 7-14 days, 21-25 433 days, 26-41 days; 4 levels) and their interaction. The effects of lamb and visual field nested within 434 testicular section were tested as random effects. Variation in mRNA abundance of key genes was 435 analyzed with a general lineal model (Proc GLM of SAS) considering the main effect of diet (Starch 436 or Fiber; 2 levels), class of age at slaughtering (7-14 days, 25-41 days; 2 levels) and their 437 interaction. Tuckey test was used for comparisons in all the models. All results are expressed as 438 mean ± standard error of the mean (SEM). A value of $P \le 0.05$ was considered significant.

439

440 ACKNOWLEDGEMENTS

This work was funded by the Italian Ministry of University and Research (MIUR), Grants: FIR 2013 and Rita Levi Montalcini 2010. We thank Ledda Farm family members for their assistance with sample collection, M. Sanna for processing of histological samples and Dr. A. Dias Francesconi for editing the language and style of our manuscript. Authors declare no conflict of interest.

446 **REFERENCES**

- Abecia JA, Casao A, Pascual-Alonso M, Lobón S, Aguayo-Ulloa LA, Forcada F,
 Meikle A, Sosa C, Marín RH, Silva MA, Maria GA. 2014a. Periconceptional
 undernutrition increases quantity and quality of oocyte population, but not
 cognitive or emotional response of 60-day-old lambs. J Anim Physiol Anim
 Nutr (Berl).
- Abecia JA, Casao A, Pascual-Alonso M, Lobón S, Aguayo-Ulloa LA, Meikle A,
 Forcada F, Sosa C, Marín RH, Silva MA, Maria GA. 2014b. The effect of
 periconceptional undernutrition of sheep on the cognitive/emotional response
 and oocyte quality of offspring at 30 days of age. J Dev Orig Health Dis
 5(2):79-87.
- Alejandro B, Pérez R, Pedrana G, Milton JT, Lopez A, Blackberry MA, Duncombe
 G, Rodriguez-Martinez H, Martin GB. 2002. Low maternal nutrition during
 pregnancy reduces the number of Sertoli cells in the newborn lamb. Reprod
 Fertil Dev 14(5-6):333-337.
- Allen MS. 2000. Effects of diet on short-term regulation of feed intake by lactating
 dairy cattle. J Dairy Sci 83(7):1598-1624.
- Andrade LP, Rhind SM, Rae MT, Kyle CE, Jowett J, Lea RG. 2013. Maternal
 undernutrition does not alter Sertoli cell numbers or the expression of key
 developmental markers in the mid-gestation ovine fetal testis. J Negat
 Results Biomed 12:2.
- Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellvé AR, Efstratiadis A. 1996.
 Effects of an lgf1 gene null mutation on mouse reproduction. Mol Endocrinol 10(7):903-918.
- Barker DJ. 2007. The origins of the developmental origins theory. J Intern Med
 261(5):412-417.
- Bebbere D, Ariu F, Bogliolo L, Masala L, Murrone O, Fattorini M, Falchi L, Ledda S.
 2014. Expression of maternally derived KHDC3, NLRP5, OOEP and TLE6 is
 associated with oocyte developmental competence in the ovine species.
 BMC Dev Biol 14:40.
- Bernal AB, Vickers MH, Hampton MB, Poynton RA, Sloboda DM. 2010. Maternal
 undernutrition significantly impacts ovarian follicle number and increases
 ovarian oxidative stress in adult rat offspring. PLoS One 5(12):e15558.
- Borwick SC, Rhind SM, McMillen SR, Racey PA. 1997. Effect of undernutrition of
 ewes from the time of mating on fetal ovarian development in mid gestation.
 Reprod Fertil Dev 9(7):711-715.
- Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R,
 Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The
 MIQE guidelines: minimum information for publication of quantitative real time PCR experiments. Clin Chem 55(4):611-622.
- 486 Cantalapiedra-Hijar G, Ortigues-Marty I, Lemosquet S. 2015. Diets rich in starch
 487 improve the efficiency of amino acids use by the mammary gland in lactating
 488 Jersey cows. J Dairy Sci 98(10):6939-6953.

- Chadio S, Kotsampasi B. 2014. The role of early life nutrition in programming of
 reproductive function. J Dev Orig Health Dis 5(1):2-15.
- 491 Collins L, Lee H, Chen Y, Chang M, Hsu H, Yeh S, Chang C. 2003. The androgen
 492 receptor in spermatogenesis. Cytogenetic and Genome Research 103(3493 4):299-301.
- 494 Courot M, Hochereau-de-Reviers MT, Monet-Kuntz C, Locatelli A, Pisselet C, Blanc
 495 MR, Dacheux JL. 1979. Endocrinology of spermatogenesis in the
 496 hypophysectomized ram. J Reprod Fertil Suppl(26):165-173.
- Da Silva P, Aitken RP, Rhind SM, Racey PA, Wallace JM. 2002. Impact of maternal
 nutrition during pregnancy on pituitary gonadotrophin gene expression and
 ovarian development in growth-restricted and normally grown late gestation
 sheep fetuses. Reproduction 123(6):769-777.
- Da Silva P, Aitken RP, Rhind SM, Racey PA, Wallace JM. 2003. Effect of maternal
 overnutrition during pregnancy on pituitary gonadotrophin gene expression
 and gonadal morphology in female and male foetal sheep at day 103 of
 gestation. Placenta 24(2-3):248-257.
- 505 Dupont C, Ralliard-Rousseau D, Tarrade A, Faure C, Dahirel M, Sion B, Brugnon F,
 506 Levy R, Chavatte-Palmer P. 2014. Impact of maternal hyperlipidic
 507 hypercholesterolaemic diet on male reproductive organs and testosterone
 508 concentration in rabbits. J Dev Orig Health Dis 5(3):183-188.
- Froment P, Staub C, Hembert S, Pisselet C, Magistrini M, Delaleu B, Seurin D,
 Levine JE, Johnson L, Binoux M, Monget P. 2004. Reproductive
 abnormalities in human insulin-like growth factor-binding protein-1 transgenic
 male mice. Endocrinology 145(4):2080-2091.
- Ge RS, Hardy MP. 1998. Variation in the end products of androgen biosynthesis
 and metabolism during postnatal differentiation of rat Leydig cells.
 Endocrinology 139(9):3787-3795.
- 516 Genovese P, Núñez ME, Pombo C, Bielli A. 2010. Undernutrition during foetal and 517 post-natal life affects testicular structure and reduces the number of Sertoli 518 cells in the adult rat. Reprod Domest Anim 45(2):233-236.
- 519 Griffeth RJ, Bianda V, Nef S. 2014. The emerging role of insulin-like growth factors 520 in testis development and function. Basic Clin Androl 24:12.
- 521 Heindel JJ, Balbus J, Birnbaum L, Brune-Drisse MN, Grandjean P, Gray K,
- 522 Landrigan PJ, Sly PD, Suk W, Cory Slechta D, Thompson C, Hanson M.
- 523 2015. Developmental Origins of Health and Disease: Integrating
- 524 Environmental Influences. Endocrinology 156(10):3416-3421.
- Hoffman F, Boretto E, Vitale S, Gonzalez V, Vidal G, Pardo MF, Flores MF, Garcia
 F, Bagnis G, Queiroz OCM, Rabaglino MB. 2018. Maternal nutritional
 restriction during late gestation impairs development of the reproductive
 organs in both male and female lambs. Theriogenology 108:331-338.
- Huhtanen P, Hristov AN. 2009. A meta-analysis of the effects of dietary protein
 concentration and degradability on milk protein yield and milk N efficiency in
 dairy cows. J Dairy Sci 92(7):3222-3232.
- Indrio F, Martini S, Francavilla R, Corvaglia L, Cristofori F, Mastrolia SA, Neu J,
 Rautava S, Russo Spena G, Raimondi F, Loverro G. 2017. Epigenetic

534 Matters: The Link between Early Nutrition, Microbiome, and Long-term 535 Health Development. Front Pediatr 5:178. Kotsampasi B, Balaskas C, Papadomichelakis G, Chadio SE. 2009. Reduced 536 537 Sertoli cell number and altered pituitary responsiveness in male lambs undernourished in utero. Anim Reprod Sci 114(1-3):135-147. 538 Langley-Evans SC. 2006. Developmental programming of health and disease. Proc 539 Nutr Soc 65(1):97-105. 540 Langley-Evans SC, McMullen S. 2010. Developmental origins of adult disease. 541 Med Princ Pract 19(2):87-98. 542 Lea RG, Andrade LP, Rae MT, Hannah LT, Kyle CE, Murray JF, Rhind SM, Miller 543 544 DW. 2006. Effects of maternal undernutrition during early pregnancy on apoptosis regulators in the ovine fetal ovary. Reproduction 131(1):113-124. 545 Montoto LG, Arregui L, Sánchez NM, Gomendio M, Roldan ER. 2012. Postnatal 546 547 testicular development in mouse species with different levels of sperm competition. Reproduction 143(3):333-346. 548 Mossa F, Carter F, Walsh SW, Kenny DA, Smith GW, Ireland JL, Hildebrandt TB, 549 Lonergan P, Ireland JJ, Evans AC. 2013. Maternal undernutrition in cows 550 551 impairs ovarian and cardiovascular systems in their offspring. Biol Reprod 88(4):1-9. 552 553 Mossa F, Walsh S, Evans A, Jimenez-Krassel F, Ireland J. 2017. Early 554 Developmental Programming of the Ovarian Reserve, Ovarian Function. Animal Models and Human Reproduction: Wiley. p 91-108. 555 Parvinen M. 1982. Regulation of the seminiferous epithelium. Endocr Rev 3(4):404-556 417. 557 558 Pitetti JL, Calvel P, Zimmermann C, Conne B, Papaioannou MD, Aubry F, 559 Cederroth CR, Urner F, Fumel B, Crausaz M, Docquier M, Herrera PL, Pralong F, Germond M, Guillou F, Jégou B, Nef S. 2013. An essential role for 560 insulin and IGF1 receptors in regulating sertoli cell proliferation, testis size, 561 and FSH action in mice. Mol Endocrinol 27(5):814-827. 562 Rae MT, Kyle CE, Miller DW, Hammond AJ, Brooks AN, Rhind SM. 2002a. The 563 effects of undernutrition, in utero, on reproductive function in adult male and 564 565 female sheep. Anim Reprod Sci 72(1-2):63-71. Rae MT, Palassio S, Kyle CE, Brooks AN, Lea RG, Miller DW, Rhind SM. 2001. 566 Effect of maternal undernutrition during pregnancy on early ovarian 567 development and subsequent follicular development in sheep fetuses. 568 Reproduction 122(6):915-922. 569 Rae MT, Rhind SM, Fowler PA, Miller DW, Kyle CE, Brooks AN. 2002b. Effect of 570 maternal undernutrition on fetal testicular steroidogenesis during the CNS 571 572 androgen-responsive period in male sheep fetuses. Reproduction 124(1):33-39. 573 574 Randhawa R, Cohen P. 2005. The role of the insulin-like growth factor system in prenatal growth. Mol Genet Metab 86(1-2):84-90. 575 576 Robles M, Gautier C, Mendoza L, Peugnet P, Dubois C, Dahirel M, Lejeune JP, Caudron I, Guenon I, Camous S, Tarrade A, Wimel L, Serteyn D, Bouraima-577 578 Lelong H, Chavatte-Palmer P. 2017. Maternal Nutrition during Pregnancy

- Affects Testicular and Bone Development, Glucose Metabolism and
 Response to Overnutrition in Weaned Horses Up to Two Years. PLoS One
 12(1):e0169295.
- Rodríguez-González GL, Vigueras-Villaseñor RM, Millán S, Moran N, Trejo R,
 Nathanielsz PW, Larrea F, Zambrano E. 2012. Maternal protein restriction in
 pregnancy and/or lactation affects seminiferous tubule organization in male
 rat offspring. J Dev Orig Health Dis 3(5):321-326.
- Rojas-García PP, Recabarren MP, Sir-Petermann T, Rey R, Palma S, Carrasco A,
 Perez-Marin CC, Padmanabhan V, Recabarren SE. 2013. Altered testicular
 development as a consequence of increase number of sertoli cell in male
 lambs exposed prenatally to excess testosterone. Endocrine 43(3):705-713.
- 590Ruwanpura SM, McLachlan RI, Meachem SJ. 2010. Hormonal regulation of male591germ cell development. J Endocrinol 205(2):117-131.
- 592 Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of 593 image analysis. Nat Methods 9(7):671-675.
- Senger P. 2003. Pathways to pregnancy and parturition. Pullma, WA, USA: Current
 Conceptions Inc.
- Sloboda DM, Howie GJ, Pleasants A, Gluckman PD, Vickers MH. 2009. Pre- and
 postnatal nutritional histories influence reproductive maturation and ovarian
 function in the rat. PLoS One 4(8):e6744.
- Sui S, He B, Jia Y, Li R, Cai D, Li X, Song H, Jia L, Zhao R. 2014a. Maternal
 protein restriction during gestation and lactation programs offspring ovarian
 steroidogenesis and folliculogenesis in the prepubertal gilts. J Steroid
 Biochem Mol Biol 143C:267-276.
- Sui S, Jia Y, He B, Li R, Li X, Cai D, Song H, Zhang R, Zhao R. 2014b. Maternal
 Low-protein Diet Alters Ovarian Expression of Folliculogenic and
 Steroidogenic Genes and Their Regulatory MicroRNAs in Neonatal Piglets.
 Asian-Australas J Anim Sci 27(12):1695-1704.
- Sullivan TM, Micke GC, Greer RM, Irving-Rodgers HF, Rodgers RJ, Perry VE.
 2009. Dietary manipulation of Bos indicus x heifers during gestation affects
 the reproductive development of their heifer calves. Reprod Fertil Dev
 21(6):773-784.
- Symonds ME, Dellschaft N, Pope M, Birtwistle M, Alagal R, Keisler D, Budge H.
- 612 2016. Developmental programming, adiposity, and reproduction in ruminants.
 613 Theriogenology 86(1):120-129.
- Vannelli BG, Barni T, Orlando C, Natali A, Serio M, Balboni GC. 1988. Insulin-like
 growth factor-I (IGF-I) and IGF-I receptor in human testis: an
 immunohistochemical study. Fertil Steril 49(4):666-669.
- Weller MM, Fortes MR, Marcondes MI, Rotta PP, Gionbeli TR, Valadares Filho SC,
 Campos MM, Silva FF, Silva W, Moore S, Guimarães SE. 2016. Effect of
 maternal nutrition and days of gestation on pituitary gland and gonadal gene
 expression in cattle. J Dairy Sci 99(4):3056-3071.
- 621 Wrobel KH, Bickel D, Kujat R, Schimmel M. 1995. Evolution and ultrastructure of
- the bovine spermatogonia precursor cell line. Cell Tissue Res 281(2):249-259.

- Yarney TA, Sanford LM. 1989. Pubertal changes in the secretion of gonadotropic
 hormones, testicular gonadotropic receptors and testicular function in the
 ram. Domest Anim Endocrinol 6(3):219-229.
- Zambrano E, Guzmán C, Rodríguez-González GL, Durand-Carbajal M, Nathanielsz
 PW. 2014. Fetal programming of sexual development and reproductive
 function. Mol Cell Endocrinol 382(1):538-549.
- 630 Zambrano E, Rodríguez-González GL, Guzmán C, García-Becerra R, Boeck L,
- 631 Díaz L, Menjivar M, Larrea F, Nathanielsz PW. 2005. A maternal low protein
- 632 diet during pregnancy and lactation in the rat impairs male reproductive 633 development. J Physiol 563(Pt 1):275-284.
- 634

636 Figure Legends



- 637
- 638 Figure 1. Representative photographs of hematoxilin and eosin-stained testicular section (left
- 639 image) and measurements of the total area of the seminiferous tubules (ST), area of the interstitial
- 640 tissue (IA), major (D) and minor (d) internal diameter of a seminiferous tubule (right image). The
- total area of the seminiferous tubules was obtained by summing the area of the individual tubules,
- 642 whereas the area of the interstitial tissue was calculated by subtracting the area of the
- 643 seminiferous tubules from the total area of the image. Scale bar = 10 μ m.
- 644



Figure 2. Percentage of total seminiferous tubules (top panel) and percentage of interstitial tissue
(bottom panel) in the testes of lambs born to mothers fed a diet with high starch vs a fiber diet for
the entire gestation (S147, closed circles; F147, open circles) or for the last 75 days of pregnancy
(S75, closed triangles; F75, open triangles), respectively.



- Figure 3. Testicular expression of selected genes in male lambs (aged 7 to 14 days and 25 to 41
- days) born to mothers fed high-starch (S147, n = 8) versus fiber-based (F147, n = 10) diet for the
- 654 entire gestation. Genes analyzed were: Anti-Müllerian hormone (AMH), androgen receptor (AR),
- 655 follicle stimulating hormone receptor (*FSHR*), hydroxysteroid (17-beta) dehydrogenase 3
- 656 (HSD17B3), insulin-like growth factor 1 (IGF1), insulin-like growth factor 2 (IGF2), insulin-like
- 657 growth factor 2 receptor (*IGF2R*), luteinizing hormone/choriogonadotropin receptor (*LHCGR*),
- 658 steroidogenic acute regulatory protein (STAR) and vascular endothelial growth factor A (VEGFA).
- 659
- 660

Table 1. Effect of maternal diet on postnatal growth of their male offspring.

662

Maternal diet	S75		F75		S147		F147		P			
											value	
Age at sampling (d)	1	21-25	1	21-25	7-14	26-41	7-14	26-41	SEM	Diet	Age	Diet x Age
Live weight (g)	4100	9682.50	4159.5	10266	5666.20	9978.33	5719.92	10107	484.48	0.79	< 0.001	0.95
Height at withers (cm)	37.5	42.25	37.5	-	40.4	47.7	41.0	46.6	0.96	0.98	< 0.001	0.47
circumference (cm)	38	50.0	37.50	-	42.5	50.8	42.9	51.5	0.91	0.70	< 0.001	0.93
Scrotal cirumference (cm)	9	11.38	7.70	11.40	7.64	11.0	7.95	9.75	0.364	0.75	< 0.001	0.22
Left testis												
Circumference (cm)	3.0	3.1	3.75	3.86	2.98	3.67	29.8	3.8	0.09	0.78	< 0.001	0.90
Lenght (cm)	1.3	1.9	1.3	2.0	1.58	2.1	1.63	2.12	0.06	0.91	< 0.001	0.96
Width (cm)	1.0	1.18	0.85	1.28	0.94	1.3	1.03	1.40	0.04	0.42	< 0.001	0.48
Weight (g)	0.58	1.67	-	1.72	0.96	2.1	1.1	2.22	0.19	0.83	< 0.001	0.93
Right testis												
Circumference (cm)	2.9	3.90	3.35	3.78	3.07	3.70	3.05	3.92	0.09	0.58	< 0.001	0.29
Lenght (cm)	1.3	1.83	1.3	1.98	1.65	2.1	1.68	2.08	0.06	0.91	< 0.001	0.89
Width (cm)	1.0	0.85	1.28	1.22	1.0	1.3	1.05	1.47	0.44	0.45	< 0.001	0.45
Weight (g)	0.58	1.62	-	1.69	0.92	2.03	1.0	2.40	0.13	0.59	< 0.001	0.52

664 Table 2 Effect of maternal diet on the testicular development of the	heir male offspring.
--	----------------------

Maternal diet	S75		F75		S147		F147		P value			
Age at sampling (d)	1	21-25	1	21-25	7-14	26-41	7-14	26-41	SEM	Diet	Age	Diet x Age
Number of tubules per field	15.7	10.33	12.8	9.60	11.62	10.03	11.53	11.15	0.141	0.178	<0.001	0.422
Area of individual tubule* (µm ²)	1108.19	1562.92	1062.32	1621.33	1313.02	1797.6	1378.75	1505.58	0.944	0.578	< 0.001	0.242
Maximum internal diameter*(µm)	35.09	44.54	34.81	44.78	39.81	50.01	39.77	45.43	0.183	0.406	<0.0001	0.417
Minimum internal diameter*(µm)	31.78	40.74	31.01	41.39	36.52	45.06	36.38	40.19	0.883	0.353	<0.0001	0.378
Average internal diameter*(µm)	33.44	42.64	32.91	43.07	38.17	47.54	38.08	42.81	0.908	0.370	<0.0001	0.389
Number of Sertoli cells*	9.64	9.87	8.32	9.40	9.04	9.77	8.36	9.26	0.16	0.136	0.088	0.782
Number of germ cells*	3.19	3.97	3.36	4.46	3.36	5.18	3.57	4.61	0.18	0.806	0.005	0.630
Sertoli/germ cells ratio*	3.02	2.55	2.48	2.21	2.75	1.95	2.40	2.24	0.10	0.571	0.145	0.467

665 *Data referred to circular tubules with < 20% difference between maximum and minimum internal
 666 diameter