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Abbreviations

AMH = Anti-Müllerian hormone

AR = androgen receptor

BCS = body condition score

DM = dry matter

DMI = dry matter intake

F = fiber diet

F147 = fiber diet for the entire gestation

F75 = fiber diet for the last 75 days of gestation

FSHR = follicle stimulating hormone receptor

HSD17B3 = hydroxysteroid (17-beta) dehydrogenase 3

IGF1 = insulin-like growth factor 1

IGF2 = insulin-like growth factor 2

IGF2R = insulin-like growth factor 2 receptor

LHCGR = luteinizing hormone/choriogonadotropin receptor

NDF = neutral detergent fiber

RPL19 = ribosomal protein L19

S = starch diet

S147 = starch diet for the entire gestation

S75 = starch diet for the last 75 days of gestation

SDHA = succinate dehydrogenase complex flavoprotein subunit A

SEM = standard error of the mean

STAR = steroidogenic acute regulatory protein

VEGFA = vascular endothelial growth factor A

YWHAZ = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

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SUMMARY

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

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

INTRODUCTION

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

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

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

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

112 On the other hand, the number of studies investigating the possible link between maternal
113 overnutrition and fertility in female and male progeny is limited. In rats, maternal consumption of a
114 high-fat diet during pregnancy and/or lactation advanced the age at puberty in female offspring
115 (Sloboda et al. 2009). In sheep, a high nutrient intake during different windows of gestation (from
116 mating to day 103 or 131 of gestation) impaired the number of follicles in female fetuses (Da Silva
117 et al. 2002; Da Silva et al. 2003). In cattle, a high maternal dietary intake impaired the total number
118 of follicles, upregulated the expression of genes involved in ovarian folliculogenesis,
119 steroidogenesis and pro-apoptosis (*P450* aromatase, *STAR, BMPR2, TGFBR1, GDF9, FSHR Bax*
120 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).

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

RESULTS

Maternal Body Weight and BCS During Gestation

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

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

Pregnant ewes that were fed the experimental diets during the last 75 days of gestation (S75, $n = 5$; F75, $n = 7$) had a similar weight at the start of the experimental diet (S75 42.8 ± 1.4; F75 44.1 ± 1.1 kg; $P = 1$). All these ewes gained weight during pregnancy ($P < 0.001$), but no effect of diet was detected. At lambing S75 and F75 weighed 51.1 ± 0.9 and 50.9 ± 1.3 kg, respectively ($P = 1$). BCS was similar between groups at the start of the experimental diet (S75 2.7 ± 0.05; F75 2.7 ± 0.05; $P = 1$) and was not affected by diet or day of gestation. At lambing, the BCS of S75 and F75 was 2.7 ± 0.05 and 2.7 ± 0.1, respectively ($P = 1$).

Phenotypic Measurements of the Lambs

Maternal nutritional regime did not affect body weight (S147 3900.6 ± 153.2; F147 4023.5 ±150.6; S75 4214.8 ± 306.1; F75 4346.4 ± 71.3 g; $P = 0.40$) or height at withers (S147 36.9 ± 0.3; F147 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, live weight, height at withers, girth circumference and scrotal circumference were similar among lambs born to mothers fed the different diets (Table 1). Furthermore, length, width and weight of each testis were not different among dietary groups. As expected, all phenotypic measurements increased with age ($P < 0.001$), but no interaction was detected between maternal diet and age at sampling (Table 1).

Testicular Development in Lambs Exposed to a High-Starch versus a Fiber-Based Diet During the Entire Gestation or During the Last 75 Days of Pregnancy

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 and age at sampling was detected (Figure 2). In agreement with this findings, the percentage of 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

196 decreased with age at sampling ($P < 0.001$), whereas the average area, and the maximum and
197 minimum internal diameters of circular tubules increased with age ($P < 0.0001$). None of these
198 parameters where affected by maternal diet (Table 2). The mean number of Sertoli cells did not
199 vary with age, whereas the mean number of germ cells was lower in young vs older lambs ($P =$
200 0.005), but no effect of maternal diet was detected (Table 2).
201
202 **Variations in mRNA Abundance of Key Genes in Testes of Lambs Born to Mothers Fed a**
203 **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-

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

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

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

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

279 expression of *STAR* was reported in 50-day-old male fetuses of undernourished ewes, suggesting
280 that maternal undernutrition may upregulate steroidogenesis (Rae et al. 2002b). On the other
281 hand, maternal overnutrition decreased the testicular expression of *STAR* and *HSD17B3* in 139
282 and 199-day-old bovine male foetuses (Weller et al. 2016). These two studies indicate that both
283 maternal under and overnutrition may impact the expression of genes involved in steroidogenesis
284 in male fetuses. Our maternal dietary treatment did not cause such variation in mRNA abundance,
285 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**
305 Animals were located in a commercial farm located in the area of Porto Torres, north of Sardinia,
306 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.

Maternal Diet

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

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

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

Testicular Histology

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

362 the tubules (both circular and asymmetrical) in the image. The total interstitial area was calculated
363 as the difference between the field area and the area of all seminiferous tubules.
364 To estimate the number of Sertoli and germ cells, 74 circular seminiferous tubules were analyzed
365 on average per lamb. Cells with basal, leptochromatic (lax chromatin), ovoid or pyriform nuclei
366 were considered Sertoli cells, whereas non-Sertoli cells were considerd germ cells (Hoffman et al.
367 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
371 analyzed in the testes of lambs born to mothers fed the experimental diets: Starch (S147, *n* = 8) or
372 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 HCl (pH 8.3), 75 mM KCl, 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

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

Real Time-Polymerase Chain Reaction

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

To minimise handling variation, all samples to be compared were run on the same plate using a PCR master mix containing all reaction components apart from the sample. The PCR products were analysed by generating a melting curve to check the specificity and identity of the amplification product. For each primer pair, the efficiency of the PCR reaction was determined by building a standard curve with serial dilutions of a known amount of template, covering at least 3 orders of magnitude, so that the calibration curve's linear interval included the interval above and 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 analysis. Messenger RNAs of all evaluated genes were detected in all samples. Target gene expression was normalized against the geometrical mean of three housekeeping gene expression ribosomal protein L19 (*RPL19*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and succinate dehydrogenase complex flavoprotein, subunit A (*SDHA*).

418

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 $P \leq 0.05$ was considered significant.

439

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Figure Legends

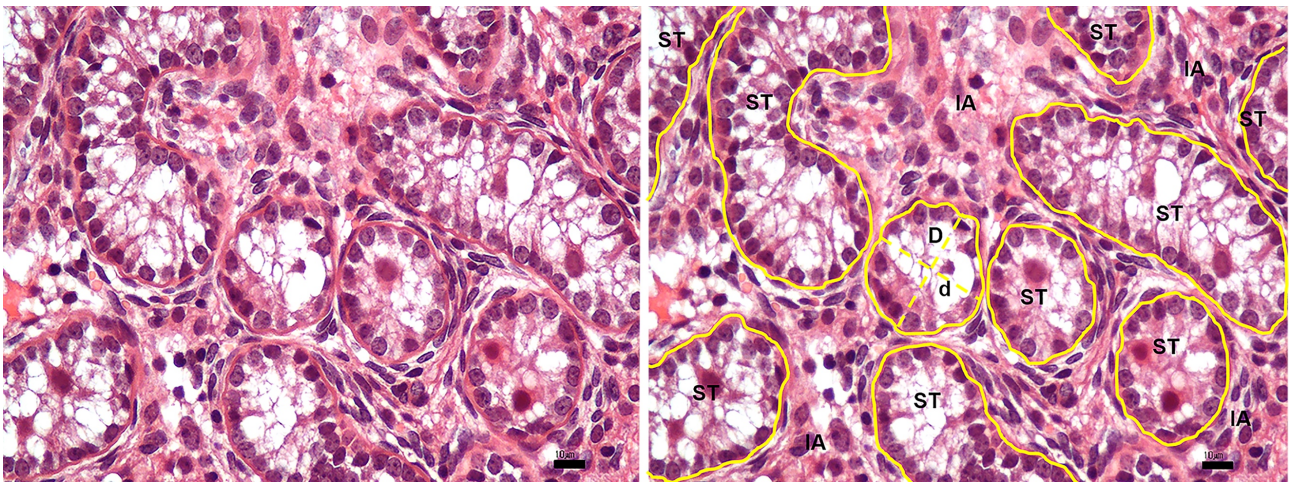
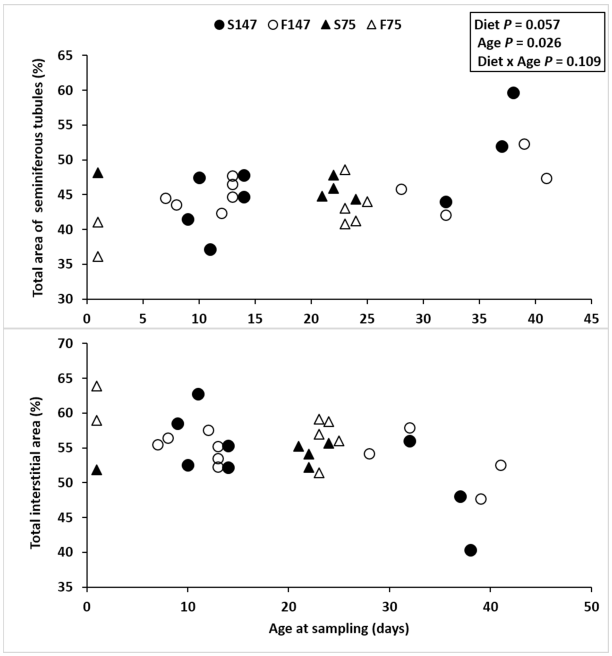


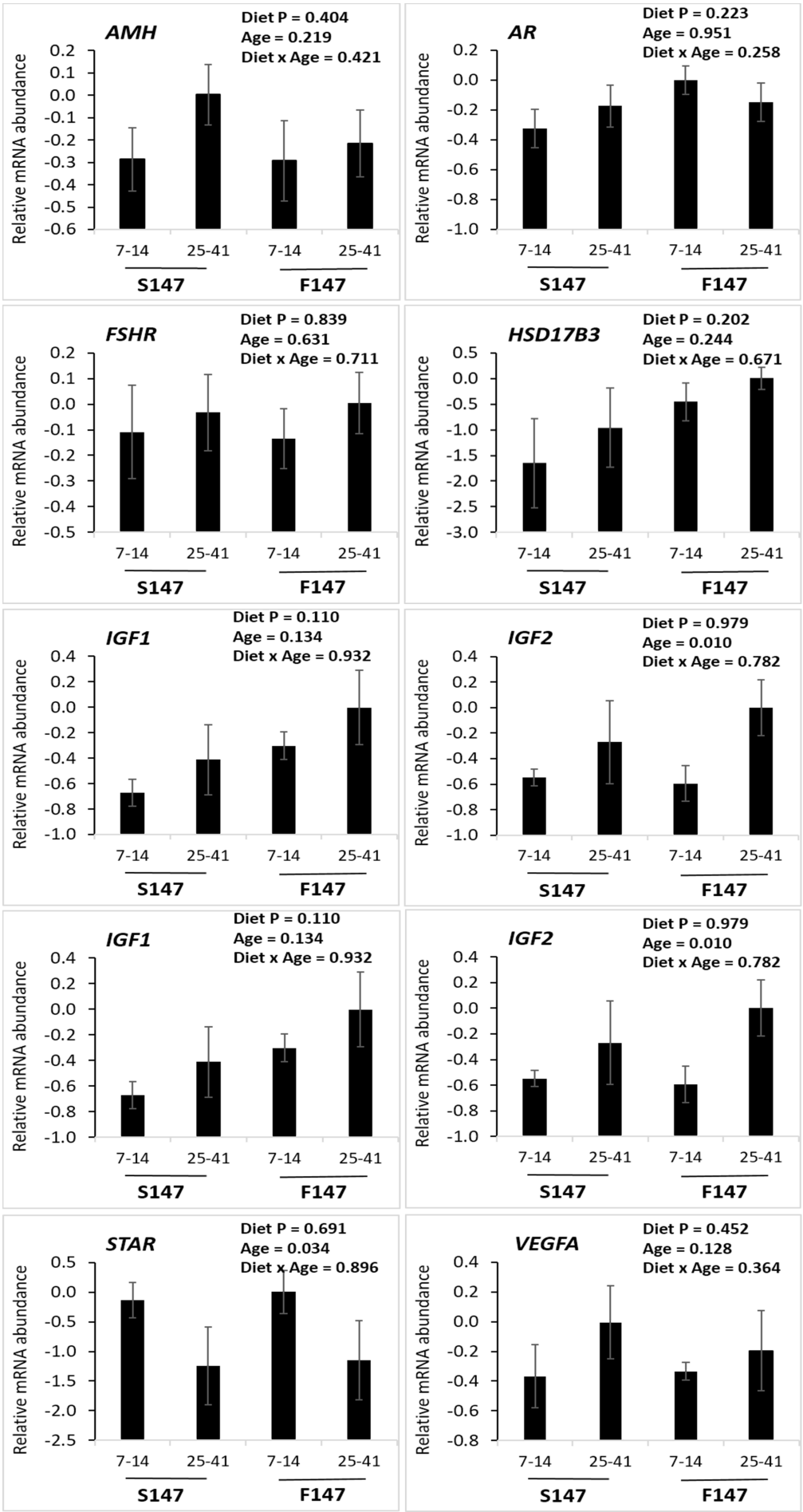
Figure 1. Representative photographs of hematoxilin and eosin-stained testicular section (left image) and measurements of the total area of the seminiferous tubules (ST), area of the interstitial tissue (IA), major (D) and minor (d) internal diameter of a seminiferous tubule (right image). The total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



645

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

650


AMH
Diet P = 0.404
Age = 0.219
Diet x Age = 0.421
AR
Diet P = 0.223
Age = 0.951
Diet x Age = 0.258
FSHR
Diet P = 0.839
Age = 0.631
Diet x Age = 0.711
HSD17B3
Diet P = 0.202
Age = 0.244
Diet x Age = 0.671
IGF1
Diet P = 0.110
Age = 0.134
Diet x Age = 0.932
IGF2
Diet P = 0.979
Age = 0.010
Diet x Age = 0.782
IGF1
Diet P = 0.110
Age = 0.134
Diet x Age = 0.932
IGF2
Diet P = 0.979
Age = 0.010
Diet x Age = 0.782
STAR
Diet P = 0.691
Age = 0.034
Diet x Age = 0.896
VEGFA
Diet P = 0.452
Age = 0.128
Diet x Age = 0.364
Relative mRNA abundance
7-14
25-41
S147
F147

651

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 entire gestation. Genes analyzed were: Anti-Müllerian hormone (*AMH*), androgen receptor (*AR*), follicle stimulating hormone receptor (*FSHR*), hydroxysteroid (17-beta) dehydrogenase 3 (*HSD17B3*), insulin-like growth factor 1 (*IGF1*), insulin-like growth factor 2 (*IGF2*), insulin-like growth factor 2 receptor (*IGF2R*), luteinizing hormone/choriogonadotropin receptor (*LHCGR*), steroidogenic acute regulatory protein (*STAR*) and vascular endothelial growth factor A (*VEGFA*).

Table 1. Effect of maternal diet on postnatal growth of their 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
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
Girth 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 their 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
667