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1 **Testicular Parameters and Spermatogenesis in Different Birth Weight Boars**

2

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24 **ABSTRACT**

25 The present study investigated the impact of birth weight on testicular development and
26 spermatogenesis in boars. Twenty four pairs of littermate boars were selected: one piglet
27 with the highest (HW) and the other with the lowest birth weight (LW) within the litter.
28 Two sub-sets of 12 pairs of male littermates from each experimental group were obtained
29 after selection: one sub-set was orchiectomized at eight days and the other at eight months
30 of age. HW boars had higher body and testicular weights at both ages ($P < 0.05$).
31 Testosterone concentrations and the relative expression of 17-alpha hydroxylase in testis
32 were similar between experimental groups. Birth weight affected somatic and germ cells
33 numbers in the neonatal testis, which were higher in HW boars ($P < 0.05$). Moreover, a
34 significant reduction in the number of pachytene spermatocytes and round spermatids was
35 observed in LW boars ($P < 0.05$) at 8 months of age, which caused a decrease in the total
36 number of elongated spermatids and daily sperm production ($P < 0.05$). Hence, HW boars
37 have the potential to produce more sperm and consequently more semen doses per
38 ejaculate, and would be very valuable to an industry that relies on artificial insemination.

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43 **KEYWORDS:** testis, birth weight, boar, spermatogenesis

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47 **INTRODUCTION**

48 In the swine industry, both the number of offspring born and their developmental
49 competence are critical. In this sense, breeding programs have focused on the increase of
50 ovulation rate, however there was evidence that maternal limitations (uterine capacity)
51 could affect both litter size and the average birth weight of the litter due to impaired
52 placental growth and efficiency (Town *et al.* 2004; Wu *et al.* 2006).

53 Placental insufficiency affects nutrient and oxygen supply, impairing fetal
54 development and growth (Pére and Etienne 2000; Wu *et al*. 2004; Town *et al*. 2004),
55 which is severely aggravated in contemporary highly prolific commercial sows (Town *et*
56 *al*. 2004). In fact, increased fetal number (uterine crowding) is not followed by an
57 increase in uterine blood flow (Pére and Etienne 2000), which will lead to slowing of fetal
58 growth and the birth of an individual with lower birth weight, which did not reach its full
59 growth potential (Martin-Gronert and Ozanne 2006).

60 Low birth weight piglets are a reality in commercial farms and have been
61 associated with functional disorders of several organs systems, resulting in deleterious
62 consequences during postnatal life. There is strong evidence that low birth weight pigs
63 present compromised postnatal growth and performance and poor meat quality (Gondret
64 *et al*. 2006; Beaulieu *et al*. 2010; Alvarenga *et al*. 2013). However, reports of birth weight
65 effects on the reproductive system are scarce, especially in boars (Almeida *et al*. 2009;
66 Lin *et al*. 2015).

67 The use of artificial insemination (AI) for breeding pigs has been instrumental for
68 facilitating global improvements in fertility, genetics, allocation of labour, and herd
69 health. The establishment of AI centers for management of boars and production of

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70 semen has allowed for selection of boars for fertility and sperm production using *in vitro*
71 and *in vivo* measures (Knox 2016). With respect to the boar, increased genetic indices,
72 fertility and high efficiency in the production of AI doses are main factors contributing to
73 the high performance of pig production (Knox 2014). Given the importance of boars as
74 semen donors in AI centers, it is essential to monitor fertility in these animals as they may
75 represent a limiting factor for the improvement of reproductive efficiency of the breeding
76 stock through the quality of the ejaculate (Waberski *et al*. 2008).
77 In this context, the effects of birth weight on testicular development and its
78 implications on sperm production in boars deserves further investigation. If such effects
79 exist, it would be essential to know if they could be identified at birth or would be
80 apparent only later during their reproductive life. Therefore, the aim of the present study
81 was to evaluate testicular parameters associated with spermatogenesis efficiency in
82 different birth weight boars.
83

84 **MATERIAL AND METHODS**

85 ***Animals and Experimental Design***

86 Forty-eight newborn male pigs Agroceres-PIC genotype (crossbred Landrace,
87 Large White and Duroc) from 24 litters, born to 4th- 6th parity sows, in litters of 10 to 15
88 total born, and mean litter birth weight from 1.25 to 1.65 kg, were selected immediately
89 after birth, before they had suckled colostrum, and identified as falling into two birth
90 weight categories: high (HW: birth weight range from 1.85 to 2.15 kg; n=24) and low
91 (LW: birth weight range from 0.85 to 1.15 kg; n=24) littermates. The pair selected
92 represented the highest and lowest birth weight boars from each litter. The criteria used at
93 selection were based on the concept of intra-uterine crowding as performed in the study

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94 of Alvarenga *et al.* (2013). Birth weight ranges for each experimental group were
95 determined as mean +1SD to mean + 2SD for the HW and mean – 2SD to mean – 1SD
96 for the LW groups, based on the average (mean) and standard deviation (SD) of birth
97 weights previously obtained from 1,000 newborn piglets of the same genetic line. Litters
98 containing runts, defined as piglets weighing less than 700 g, were avoided. Furthermore,
99 in order to overcome possible litter birth weight effects on fetal development (Foxcroft *et*
100 *al.* 2006), the piglets selected belonged to median birth weight litters, defined as the
101 average litter birth weight registered at the farm in the previous year. Hence, the average
102 litter birth weight range was 1.25 kg to 1.65 kg. At the end of selection, four experimental
103 groups were obtained: two sub-sets of 12 pairs of male littermates from each
104 experimental group which were bilaterally orchiectomized at eight days post-partum and
105 two sub-sets of 12 pairs of male littermates from each experimental group,
106 orchiectomized at eight months of age.

107 The surgical procedure used at both ages was the method of opened orchiectomy
108 described by Turner and McIlwaith (2002). To perform orchiectomy in the 8 day-old
109 boars, a local anesthetic (0.3 mL 2% lidocaine hydrochloride, Cristalia, Itapira, Brazil)
110 was applied in the incision line. In the post-pubertal boars, surgical procedure was
111 preceded by general anaesthesia using an intravenous injection of 2% xylazine
112 hydrochloride (1.0 mg/kg, Bayer, Sao Paulo, Brazil) and10% ketamine (5.0 mg/kg,
113 Agener União, Sao Paulo, Brazil). A local anesthetic (20 mL 2% lidocaine hydrochloride,
114 Cristalia, Itapira, Brazil) was also applied in the incision line. The experiment was
115 approved by the Ethical Committee in Animal Experimentation of the Federal University
116 of Minas Gerais (protocol # 65/2011).

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117 ***Biometrical data***

118 All pigs studied were weighed at birth and at orchiectomy. Immediately after
119 orchiectomy, testes were weighed without the epididymis and biometrical measurements,
120 including width, height, and length, were made to calculate testicular volume (cm^3),
121 assuming the shape of a prolate spheroid.

122

123 ***Tissue preparation***

124 Testicular samples were collected from the same area of the parenchyma (close to
125 the mediastinum) in the right and left testis in all animals with a razor blade and subjected
126 to different preparations according to the histomorphometrical, immunohistochemical,
127 gene expression, and sperm head count analysis. Samples of 1.0-2.0 mm thickness were
128 fixed through immersion in 5% glutaraldehyde in 0.05M phosphate buffer pH 7.3 for 24
129 hours, dehydrated in increasing concentrations of ethanol, embedded in glycol
130 methacrylate plastic resin (Historesin, Leica, Heidelberg, Germany), sectioned at 3 and 5
131 µm thicknesses and stained with toluidine blue sodium borate (Chiarini-Garcia *et al.*
132 2011), for all histomorphometrical evaluations. To perform immunohistochemistry,
133 samples were fixed in 4% paraformaldehyde in 0.05M phosphate buffer pH 7.3 for 24
134 hours and embedded in paraffin (Histosec, Merck, Darmstadt, Germany). Sections of 4
135 µm thickness were placed in silicanized slides.

136 For gene expression studies, fresh testes samples were preserved in RNA holder
137 (BioAgency, Sao Paulo, Brazil) for 24 hours overnight at 4°C and stored at -20°C.
138 Finally, for further sperm head count, other fresh testes samples were frozen at -20°C.

139

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140 ***Morphometric Methods***

141 For all the histomorphometrical evaluations, seven males were randomly selected within
142 the subsets of each experimental group.
143
144 *Seminiferous cord/tubule diameter and seminiferous epithelium height*
145 Seminiferous cord (8 days) and tubule (8 months) diameter and seminiferous
146 epithelium height (8 months) were measured using a graduated ruler fitted to an eyepiece
147 of an Olympus BX 41 light microscope (Olympus, Tokyo, Japan) calibrated with a Leitz
148 micrometer ruler. Ten round or nearly round seminiferous cords/tubules and their
149 epithelium heights were measured in the 5 μm tissue sections from the right and left
150 testes at a final magnification of 400X in 8-day old boars and 200X in the 8-month old
151 ones.
152
153 *Volume density of the testicular components*
154 The volume densities (Vv%) of the testicular components (seminiferous
155 cords/tubule and interstitium), tubular parameters (seminiferous epithelium, tunica
156 propria and lumen) and germ (gonocytes at 8 days and type A spermatogonia at 8
157 months) and somatic (Sertoli and Leydig) cells were obtained using a 441-point grid
158 placed in an eyepiece of the light microscope as described by Drumond *et al.* (2011a).
159 Ten fields (total of 4410 points) were randomly selected per animal in the 5 μm tissue
160 sections at 400X magnification.
161
162 *Cell counts per testis*

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163 The absolute number of Sertoli, Leydig and germ cells (gonocytes/type A
164 spermatogonia) per testis and per gram of testis were estimated based on their respective
165 volume density obtained previously, according to the method described by Drumond *et*
166 *al.* (2011a). The results are reported as total number of each cell type per testis and per
167 gram of testis (ratio between the absolute number per testis and the testicular weight).
168
169 *Cell number and spermatogenesis efficiency*
170 All germ cell nuclei and Sertoli cell nucleoli present at stage I of the seminiferous
171 epithelium cycle, according to the tubular morphology system (França and Cardoso
172 1998), were counted to evaluate spermatogenesis efficiency, as previously described by
173 Melo *et al.* (2014).Ten round or nearly round cross-sections of seminiferous tubules were
174 randomly selected per each animal at 1000X magnification. Cellular number per cross
175 section was corrected for section thickness (5 μm) and nucleus diameter according to
176 Abercrombie (1946) and modified by Amann and Almquist (1962). Nuclei diameter for
177 each cellular type was obtained by the average of 10 nuclei per animal at 1000X
178 magnification, using a graduated ruler fitted to an eyepiece and calibrated with a Leitz
179 micrometer ruler. Due to their ovoid and non-round shape, the sizes of Sertoli cells nuclei
180 at 8 days and type A spermatogonia nuclei at 8 months were obtained as the mean of their
181 larger and smaller diameters. Using the correct cell counts present at stage I (type A
182 spermatogonia, preleptotene primary spermatocytes, pachytene primary spermatocytes
183 and round spermatids), the following ratios were obtained: (1) *mitotic index:* number of
184 spermatocytes at preleptotene divided by the number of type A spermatogonia, to
185 determine the coefficient of efficiency of spermatogonial mitosis; (2) *meiotic index*:

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186 number of round spermatids divided by the number of pachytene spermatocytes, to obtain
187 the rate of germ cell loss during meiosis; (3) *Sertoli cell efficiency:* number of round
188 spermatids divided by the number of Sertoli cell nucleoli, to estimate the number of
189 sperm supported by each Sertoli cell and (4) *spermatogenesis efficiency*: number of round
190 spermatids divided by the number of type A spermatogonia at stage I, to determine the
191 number of spermatids after mitotic and meiotic processes, and estimate the overall rate of
192 spermatogenesis.
193
194 *Sperm counts and daily sperm production*
195 Homogenization-resistant testicular spermatids were counted as previously
196 described by Drumond *et al.* (2011b). Approximately 0.1 – 0.2 grams of testis, without
197 the albuginea tunica, was immersed in 1.0 mL of distillated water followed by sonication
198 for 2 minutes (Cole Parmer Ultrasonic Processor, Illinois, USA), keeping the samples on
199 ice. Sperm heads were counted in a Neubauer chamber (two fields per animal) with a
200 40X objective at phase contrast microscopy. Daily sperm production was estimated, as
201 described by Okwun *et al*. (1996), dividing the number of elongated spermatids
202 enumerated in the homogenate by 5.86, which is the number of days of the seminiferous
203 epithelium cycle in which these spermatids are present in the seminiferous epithelium.
204
205 *Immunohistochemistry*
206 The spermatogonial proliferation activity was evaluated by the
207 immunohistochemical detection of MCM7 (Minichromosome Maintenance Complex), a
208 nuclear protein that is part of a complex essential for chromosomal DNA replication

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209 (Pacek and Walter 2004). Slides were deparaffinized, rehydrated in decreasing ethanol
210 concentrations and subjected to heat-induced antigen retrieval (water bath at 98°C, 30
211 min) with citrate buffer solution at pH 6.0. The slides were blocked with 30% of BSA
212 (bovine serum albumin) at 0.3% in PBS for 60 min at 37ºC. All samples, except the
213 negative controls, were subjected to overnight incubation (16 to 18h at 4ºC) with primary
214 biotinylated antibody (mouse monoclonal anti-IgG anti-MCM7, clone 47DC141, 1:400
215 dilution, Abcam, Cambridge, United Kingdom). Negative control was maintained in PBS
216 at 4ºC. In order to block the endogenous peroxidase activity, the slides were incubated
217 with a solution of H_2O_2 (3%) in PBS for 30 minutes. All sections were incubated (30 min
218 at 37ºC) with the secondary anti-mouse biotinylated antibody (1:500) (Vector
219 Laboratories, Burlingames, California, USA), followed by incubation with avidin-
220 streptavidin-peroxidase complex (Vector Laboratories, Burlingames, California, USA),
221 both procedures for 30 minutes at 37ºC, and 3,30-diaminobenzidine tetrahydrochloride
222 was used as a chromogen (DAB substrate system, Dakocytomation). Slides were
223 counterstained with Mayer's hematoxylin and dehydrated in increasing ethanol
224 concentrations. As positive control, samples of adult rat testis previously tested were
225 used.

226 The proliferation activity index was calculated by the ratio between the number of
227 positive cells and the total number of cells (positive + negative), in 30 cross sections of
228 seminiferous tubules per animal. Because differentiation between preleptotene
229 spermatocyte and type B spermatogonia is difficult after immunolabeling staining and
230 both of them are placed in the same topographic position in the basal compartment,

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231 proliferation activity of germ cells in 8 month old males was determined as the number of
232 all labeled cells close to the basal membrane.
233
234 ***Hormonal assay***
235 Five-mL blood samples were withdrawn from the jugular vein at orchiectomy
236 (through venipuncture) in all 8 days and 8 months old boars for analysis of plasma
237 testosterone concentrations. Blood samples were collected into heparinized tubes,
238 centrifuged at 1,500 x g for 15 minutes, and plasma stored at -20° C until analysis.
239 Testosterone concentrations were quantified in duplicate, through
240 electrochemiluminescence immunoassay "ECLIA" commercial kit (Roche Diagnostics
241 USA, Indianapolis, USA) and used in COBAS E 411 immunoassay analyzers(Roche
242 Diagnostics USA, Indianapolis, USA). The antibody used for the testosterone assay has
243 less than 1% cross reactivity to other androgens. The sensitivity, estimated as 96.2% of
244 total binding, was 1.0 ng/mL, and intra- and inter-assay CV were 15.0% and 6.3%,
245 respectively.
246
247 ***Total RNA extraction and cDNA synthesis***
248 In order to evaluate possible birth weight effects on sexual maturation, the
249 expression of the steroidogenic enzyme 17alpha-hydroxylase (17a-OH), one of the
250 enzymes in the steroidogenesis process which is responsible for the conversion of
251 pregnenolone to testosterone, was measured by quantitative polymerase chain reaction
252 (qPCR).

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253 RNA was extracted from 20 - 50µg of tissue from each testis as previously
254 described by Hernandez *et al.* (2013). The RNA was quantified and the quality assessed
255 spectrophotometrically using a Nanodrop ND-1000 (Labtech International Ltd., East
256 Sussex, United Kingdom) and electrophoretically using a Tapestation 2200 (Agilent
257 Technologies LDA UK Limited, Cheshire, United Kingdom). The mean A_{260}/A_{280} was
258 2.10 (range 2.06-2.16) and the mean RNA Integrity Number Equivalent (RIN^e) was 7.5
259 (range was 6.4 – 8.7). Extracted RNA was stored at -80ºC.
260 Complementary DNA (cDNA) was prepared from 1µg of each RNA with
261 SuperScript III reverse transcriptase (Life Technologies, Paisley, United Kingdom)
262 following the manufacturer's instructions. Each reaction contained 250 ng random
263 primers (Promega, Southampton, United Kingdom) and 40 units RNaseIn (Promega,
264 Southampton, United Kingdom). Negative controls without reverse transcriptase were
265 included in order to check for genomic contamination. Complimentary DNA was stored
266 at -20ºC.
267
268 ***Relative expression of 17a-OH in testis***
269 Quantitative PCR was performed on a Stratagene MX3000 instrument using
270 Platinum SYBR Green SuperMix UTG (Life Technologies, Paisley, United Kingdom).
271 The final concentrations of magnesium, ROX reference dye and each primer were 3mM,
272 50nM and 400nM, respectively. The reaction volume was 25µl. All qPCRs were carried
273 out at an annealing temperature of 60ºC and dissociation curves consisting of single
274 peaks were generated. Three reference genes were used: hydroxymethylbilane (HMBS),
275 succinate dehydrogenase (SDHA) and tyrosine 3-monooxygenase/tryptophan 5-

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276 monooxygenase activation protein zeta polypeptide (YWHAZ). These had previously
277 been selected (Ashworth *et al.* 2011) as stably expressed genes in pig testes tissue from a
278 panel of nine candidates identified by Nygard *et al.* (2007) using geNORM V3.5 (Ghent
279 University Hospital, Center for Medical Genetics). The primers sequences of the four
280 genes used are shown in Table 1.
281 Serial dilutions of pooled cDNA ranging from 1:4 to 1:512 in nuclease-free water
282 were used as standards. Sample cDNA was diluted 1:20 and 5µl of sample, standard or
283 control were added per well. Each plate contained duplicate wells of a no template
284 control (NTC), standards, one of two sets of sample cDNA and reverse transcriptase
285 blanks (RTBs). Data was analyzed using qbase$^+$ softwareV3.0 (Biogazelle, Zwijnaarde,
286 Belgium). A target and run specific strategy was employed and the results, normalized to
287 the three reference genes, are scaled to a representative sample. The mean slope,
288 intercept, PCR efficiency and R^2 values are shown in Table 2.
289
290 ***Statistical analysis***
291 All variables measured were tested for normality prior to analyses, using the
292 univariate procedure of the Statistical Analysis System (SAS Institute, 2001). Data were
293 analyzed as a randomized complete block design, each block consisting of two
294 littermates. The statistical model included birth weight class and block as fixed factors
295 and boar as random factor. Treatment effects on biometrical parameters,
296 histomorphometrical analysis, immunohistochemical analysis, testosterone
297 concentrations, and the relative expression of 17a-OH in the testis were analyzed using
298 the general linear model (GLM) procedure of SAS. Least square means were compared

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299 using the Student's t-test with $P < 0.05$ being considered significant. In the tables and
300 figures, data are reported as least square means and the pooled SEM. Potentially relevant
301 associations among characteristics measured were examined across treatment groups
302 using correlation analysis (INSIGHT procedure of SAS).

303

304 **RESULTS**

305 ***Body weight changes and testicular measurements***

306 Body weight changes in both sub-sets (8 days and 8 months) from birth to the
307 time of orchiectomy are shown in Table 3. Body weight differences observed at birth
308 were maintained until the time of orchiectomy, as LW animals had lower body weights
309 compared to their HW littermates in both sub-sets ($P < 0.05$).

310 Testicular weight and volume were also affected by birth weight, which was
311 shown by the lower values in LW compared to HW boars (Table 3) at both ages
312 evaluated. Interestingly, testis weight relative to body weight (gonadossomatic index –
313 GSI: testis weight divided by body weight X 100) was similar between both experimental
314 treatments at the ages studied, demonstrating a proportional relationship between body
315 weight and testicular weight.

316 A litter of origin effect was evident for testicular weight, GSI and testicular
317 volume at 8 days ($P < 0.05$), which revealed the importance of the use of littermates when
318 designing experiments of this kind to account for the differences due to family.
319 Moreover, at 8 days of age, testicular weight was highly correlated with birth weight ($r =$
320 0.59, $P <0.01$) and body weight at castration ($r = 0.73$, $P < 0.01$) and Sertoli cell number

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321 per testis (r = 0.65; P = 0.012); body weight was also positively correlated with Sertoli
322 cell number per testis (r = 0.56; P = 0.04).
323
324 ***Histomorphometrical analysis and spermatogenic parameters***
325 Seminiferous cord/tubule diameter and the number of Sertoli cells per cross
326 section of seminiferous cord/tubule were not affected by birth weight class in both ages
327 studied. However, LW animals presented a significant reduction in seminiferous
328 epithelium height compared to their HW counterparts at 8 months of age (P < 0.05; Table
329 3).
330 The volumetric density of the testicular parenchyma components in both ages
331 studied is shown in Table 4.The percentage of interstitium, seminiferous cord/tubule were
332 similar between groups at both ages, as well as the percentage of seminiferous tubule
333 components in 8 month-old boars. Additionally, the volumetric density of somatic
334 (Sertoli and Leydig) and germ (gonocytes at 8 days and type A spermatogonia at 8
335 months of age) cells were similar between groups at both ages. However, the numbers of
336 Sertoli, Leydig and germ cells (gonocytes at 8 days)per testis were lower at 8 days old
337 LW boars (P < 0.05), which was not observed in LW males at 8 months of age. Despite
338 the differences in total cell numbers per testis at 8 days-old, when these parameters were
339 calculated per gram of testis the results were similar between both experimental groups
340 (Table 4).
341 Additionally, low birth weight was not associated with depletion in
342 spermatogenesis efficiency, represented by mitotic, meiotic and Sertoli cell efficiency
343 indexes (Fig.1). Further evidence of normal spermatogenesis efficiency was established

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344 by counting type A spermatogonia (HW: 1.0 ± 0.3; LW: 0.8 ± 0.2) and preleptotene
345 spermatocyte (HW: 23.4 ± 2.2; LW: 20.7 ± 4.1) per seminiferous tubule cross section,
346 which were similar between both experimental groups. Notwithstanding the lack of
347 treatment effect on spermatogonia A and preleptotene spermatocyte, a significant
348 reduction in the number of pachytene spermatocyte and round spermatid was observed in
349 LW boars ($P < 0.05$ – Table 5), that caused a decrease in the total number of round
350 spermatids, the most mature spermatogenic cells at stage I of the seminiferous epithelium
351 cycle.
352
353 ***Plasma testosterone concentration and relative expression of 17a-OH in the testes***
354 Birth weight did not affect plasma testosterone concentrations or the relative
355 expression of 17a-OH in the testes, as represented by similar values for both parameters
356 evaluated between experimental groups at both ages (Table 3).
357
358 ***Sperm counts and daily sperm production***
359 Sperm counts, determined after tissue sonication, and daily sperm production
360 were affected by birth weight as LW boars presented lower total spermatids number.
361 However, when this number was adjusted for testicular weight (spermatid number per
362 gram of testis), both experimental groups showed similar values.
363 Interestingly, lower daily sperm production was observed in the LW males
364 compared to HW littermates ($P < 0.05$; Fig. 2). Moreover, a litter of origin effect was also
365 observed for spermatids number and daily sperm production ($P < 0.05$).
366

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367 ***Cellular proliferation activity***

368 Cellular proliferation activity, measured by the percentage of MCM7-stained cells
369 relative to the total number of cells, was not affected by birth weight. Both treatment
370 groups showed similar proliferation activity for Sertoli cells (89.8 ± 2.6 vs 94.8 ± 2.6 %),
371 Leydig cells (24.4 ± 3.0 vs 26.1 ± 3.0 %), and gonocytes (63.0 ± 6.0 vs 62.4 ± 6.0 %;),
372 respectively for HW and LW 8-day old boars (Fig.3A).

373 As Sertoli cells do not proliferate in post-pubertal boars, proliferation activity was
374 measured in Leydig cells, and type A spermatogonia in the 8 month-old subset. Again,
375 the proliferation activity at this age was not affected by birth weight, as observed by the
376 similar values obtained for Leydig (6.3 ± 1.2 vs 7.8 ± 1.2 %) and type A spermatogonia
377 (98.5 ± 0.6 vs 98.5 ± 0.6 %) cellular proliferation, respectively in HW and LW boars
378 (Fig.3B).

379

380 **DISCUSSION**

381 Many studies have investigated the effects of birth weight on postnatal growth
382 performance, and yet there is a lack of information on subsequent reproductive
383 performance of low birth weight males. As birth weight may be an important parameter
384 to include in sire line breeding programs, a better understanding of the impact of birth
385 weight on fertility seems critical. Hence, the present study investigated the effects of
386 birth weight on testicular development and spermatogenesis in littermate boars. In
387 particular, it was shown that birth weight affects spermatogenesis leading to a decrease in
388 sperm production. This is believed to be the first report showing that low birth weight
389 alters the spermatogenic process in male pigs.

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390 Similar to previous studies (Beaulieu *et al*. 2010; Alvarenga *et al*. 2013; Lin *et al*.
391 2015) where growth rate of different birth weight pigs was investigated, LW boars
392 showed lower body weight at castration in both ages studied. Moreover, testicular weight
393 and volume were also affected by birth weight, which was also reported by Almeida *et al*.
394 (2009) and Smit *et al*. (2013) in neonatal males and by Lin *et al*. (2015) in adult boars.
395 Despite the differences in body and testes absolute weights, the gonadossomatic index,
396 which is an indicator of the testicular relative weight, was similar between both
397 experimental groups, suggesting that testes size is proportional to body size (Table 3).
398 The correlation between birth weight and body weight at castration ($r = 0.73$, $P < 0.01$)
399 provides strong evidence of their dependence. Furthermore, the results of proliferation
400 activity obtained for somatic and germ cells suggest that testis growth was progressing at
401 similar intensity in both experimental groups at either 8 days or 8 months of age.

402 On the other hand, the reduction of testicular weight and volume in LW boars may
403 not be related to the structural organization of the testicular parenchyma. Since there is a
404 proportion between testicular tissue components and organ size, as shown by the
405 similarities in volumetric density and number of cells per gram of testis between the
406 experimental groups, birth weight may not be associated with impaired testicular
407 organogenesis. Hence, the components and cells of the testicular parenchyma in LW
408 animals are proportional to their smaller size.

409 Studies considering different breeds have shown that testis weight and volume are
410 highly correlated to the number of Sertoli cells and this to sperm production in post-
411 pubertal boars (Okwun *et al*. 1996; Ren *et al*. 2009). In fact, LW pigs presented a reduced
412 number of Sertoli and Leydig cells and gonocytes compared to HW group at 8 days of

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413 age, which was also shown in the study of Smit *et al*. (2013). Some studies demonstrated
414 that Sertoli cells can support a relatively fixed number of germ cells depending on the
415 species, for instance rabbits, rats, and monkeys (Russell and Peterson 1984; Orth *et al*.
416 1988). Therefore, the number of Sertoli cells established during testis development until
417 puberty may be a limiting factor for sperm production in adulthood (Orth *et al*. 1988).
418 Nevertheless, at 8 months, the differences in testis weight and volume could not be
419 explained by the number of somatic and germ cells present in the testis, which were
420 similar in both experimental groups.

421 Compromised fetal growth did not affect spermatogenic efficiency, as also
422 described by Melo *et al*. (2014) in rats submitted to protein deficiency *in utero*. However,
423 in contrast to the present study, Melo *et al*. (2014) observed a reduction in Sertoli cell
424 support capacity. Our results also show that germ cell death and proliferation activity,
425 which are important to the regulation of spermatogenic cell population (França *et al*.
426 2005), were not affected by compromised fetal growth.

427 Another important parameter for evaluating spermatogenic efficiency is
428 seminiferous tubular diameter, which is also related to the number of Sertoli cells per
429 cross section of seminiferous tubule and epithelium height (França and Russell 1998). In
430 the present study, LW boars did not show changes in tubular diameter and the number of
431 Sertoli cell per cross section, which is in agreement with the findings reported by Lin *et*
432 *al*. (2015) in adult boars. However, a reduction in epithelium height was observed in LW
433 boars. Despite the similarities in tubular diameter, the differences in epithelium height
434 can be associated with a decrease in germ cell number present in seminiferous tubule at
435 stage I of the epithelium cycle. In fact, a reduction in the total number of pachytene

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436 spermatocyte and round spermatids per cross section of seminiferous tubule was
437 demonstrated in the present study, which did not alter tubular diameter but affected
438 epithelium height in LW males. We believe that the decrease in the number of
439 spermatids, present in seminiferous tubule cross sections, may be related to a further
440 decrease in sperm production as germ cell division follows a geometric progression. Even
441 though a small numeric difference was observed in the early germ cells stages it became
442 more pronounced overtime, reaching statistical significance in the later stages. This
443 difference still remained for elongated spermatid number and daily sperm production in
444 8-month old boars. Assuming that sperm concentration in the ejaculate would be
445 proportional to the daily sperm production in the testis, HW boars would produce
446 approximately 34% more semen doses, based on the data presented herein (daily sperm
447 production: HW – 122 x 10^6 vs LW – 80 x 10^6 per testis per day). Actually, Lin *et al.*
448 (2015) provided evidence of deleterious effects of prenatal programming on sperm
449 production in intra-uterine growth restricted boars, whereas the present results
450 demonstrated negative effects of birth weight on germ and somatic cells population in small,
451 but perfectly formed piglets.

452 Similar testis expression of 17a-OH and plasma testosterone concentrations in LW
453 and HW boars at 8-days and 8-months of age suggest that sexual maturation may not be
454 compromised by altered fetal growth, as this enzyme, which catalyses the production of
455 precursors for glucocorticoid, estrogen and androgen synthesis, is involved in sexual
456 development during fetal life and at puberty (Majdic *et al.* 1996). The absence of birth
457 weight effects on circulating testosterone levels were also reported in 10 months old
458 boars (Lin *et al*. 2015).

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459 Taken together, our results suggest that low birth weight is associated with a
460 decrease in testicular somatic and germ cell numbers in the neonatal period. During the
461 post-pubertal period, low birth weight affected sperm production. The reductions in
462 biometrical measures and somatic and germ cell numbers shown in the present study did
463 not seem to originate from compromised organogenesis and function, but were
464 proportional to the smaller size of the animal. However, HW boars produce more sperm
465 and consequently more semen doses per ejaculate. As semen from elite boars is in huge
466 demand around the world, elite boars that produce more sperm per ejaculate would be
467 very valuable to an industry that relies on AI. Hence, the selection of potential AI boars
468 of high birth weight would be predictive of better lifetime productivity in the boar stud.

469 Assuming that these results will be confirmed at the multiplication level in sire-
470 line selection programs, the implications of birth weight for lifetime sperm production
471 seem real. This suggests that prenatal programming of testis development will
472 predetermine the reported relationship between adult testis size and lifetime semen
473 production. Therefore, additional studies are necessary to better understand the effects of
474 birth weight on other reproductive parameters related to semen quality and fertility.

475

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639
640 **Tables**

641 **Table 1**. Porcine-specific primer sequences for qPCR

Primer names	Sequence(5'– 3')	Ampliconsize (bp)	Tm (ºC)	Accession numbers
17α-OH		202	60	M63507
Forward	CTGTGGGCAAGGAAATTTTG			
Reverse	ACTTTCTGCGTTCGTCTTGG			
HMBS2		83	60	DQ845174
Forward	AGGATGGGCAACTCTACCTG			
Reverse	GATGGTGGCCTGCATAGTCT			
SDHA		141	60	DQ845177
Forward	CTACAAGGGGCAGGTTCTGA			
Reverse	AAGACAACGAGGTCCAGGAG			
YWHAZ		203	60	DQ845179
Forward	TGATGATAAGAAAGGGATTGTGG			
Reverse	GTTCAGCAATGGCTTCATCA			

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651 **Table 2.** qPCR calibration curve data

Gene	Slope	Intercept	Efficiency	R^2
17αOHase	-3.33	17.33	100.6	0.995
HMBS	-3.51	28.02	92.7	0.998
SDHA	-3.218	29.71	104.6	0.992
YWHAZ	-3.245	21.172	103.5	0.996

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668 **Table 3.** Body and testicular biometry, tubular, hormonal and molecular parameters of 8
669 day and 8 month high (HW) and low (LW) birth weight littermate boars

670

PARAMETERS	**8 days**		**8 months**	
	HW	**LW**	**HW**	**LW**
Body weight at birth (kg)	1.8 ± 0.1[a*]	1.0 ± 0.1[b]	1.9 ± 0.1[a]	1.0 ± 0.1[b]
Body weight at castration (kg)	3.6 ± 0.1[a]	2.3 ± 0.1[b]	176 ± 3.6[a]	158 ±3.6[b]
Testisweight (g)	2.4 ± 0.1[a]	1.4 ± 0.1[b]	413 ± 15[a]	355 ± 16[b]
Gonadossomatic index	0.07 ± 0.003[a]	0.06 ± 0.003[a]	0.23 ± 0.01[a]	0.23 ± 0.01[a]
Testis volume (cm^3)	3.9 ± 0.2[a]	2.3 ± 0.2[b]	676± 30[a]	575 ± 32[b]
Cord/tubulediameter (µm)	48 ± 1.2[a]	51 ± 1.2[a]	248 ± 5.3[a]	246 ± 5.3[a]
Seminiferousepitheliumheight (µm)	-	-	92 ±5.3[a]	81 ± 6.2[b]
Testosterone (ng/mL)	2.4 ± 0.6[a]	2.4 ± 0.6[a]	3.9 ± 0.9[a]	3.4 ± 0.9[a]
17α- hydroxylase mRNA expression	9.0 ± 2.2[a]	7.8 ± 1.8[a]	1.8 ± 0.6[a]	1.6 ± 0.5[a]

671 [a,b]Within a row and age sub-set, lsmeans without a common superscript differ ($P < 0.05$).

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679 **Table 4.** Volume density (Vv%) of testicular parenchyma components and number of
680 somatic and germ cells at 8 days and 8 months of age in high (HW) and low (LW) birth
681 weight boars

	8days		**8months**	
	HW	**LW**	**HW**	**LW**
Testicular parameters (%)				
Interstitium	76.4 ± 5.7[a*]	78.6 ± 4.8[a]	28.8 ± 4.7[a]	28.9 ± 6.7[a]
Seminiferouscord/tubule	23.6 ± 5.3[a]	21.4 ± 4.8[a]	71.2 ± 5.0[a]	71.1 ± 6.0[a]
Seminiferostubule (%)				
Epithelium	-	-	54 ± 10[a]	53.4 ± 6.0[a]
Tunica propria	-	-	6.2 ± 0.6[a]	6.2 ± 0.9[a]
Lumen	-	-	11 ± 2.0[a]	11.5 ± 2.0[a]
Sertolicells				
Volume density (%)	9.4 ± 2.2[a]	8.5 ± 2.0[a]	1.4 ± 0.3[a]	1.5 ± 0.3[a]
Number (10^9)/testis	1.2 ± 0.1[a]	0.6 ± 0.1[b]	86 ± 12[a]	98 ± 12[a]
Number (10^6)/g of testis	500 ± 64[a]	429 ± 64[a]	208 ± 26[a]	276 ± 26[a]
Leydig cells				
Volume density (%)	6.3 ± 1.2[a]	7.0 ± 1.1[a]	2.1 ± 0.7[a]	2.2 ± 0.6[a]
Number (10^9)/testis	0.6 ± 0.1[a]	0.3 ± 0.1[b]	4.3 ± 0.4[a]	4.3 ± 0.4[a]
Number (10^6)/ g of testis	250 ± 42[a]	214 ± 42[a]	10.4 ± 1.8[a]	12.1 ± 1.8[a]
Gonocyte/A spermatogonia*				
Volume density (%)	0.7 ± 0.2[a]	0.6 ± 0.2[a]	0.5 ± 0.2[a]	0.6 ± 0.2[a]
Number (10^8)/ testis	0.1 ± 0.1[a]	0.06 ± 0.1[b]	41 ± 11[a]	30 ± 10[a]
Number (10^6)/ g of testis	4.2 ± 0.3[a]	4.3 ± 0.3[a]	10 ± 1.8[a]	8.5 ± 1.8[a]

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682 [a,b] Within a row and age sub-set, lsmeans without a common superscript differ ($P < 0.05$).

683 *Gonocytes and type A spermatogonia were scored at 8 days and 8 months of age, respectively.

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688 **Table 5.**Germ cell numbers per cross section in high (HW) and low (LW) birth weight

689 boars, present at stage I of the seminiferous epithelium cycle, at 8 months of age

690

PARAMETERS	HW	LW
A spermatogonia	1.0 ± 0.1[a]	0.9 ± 0.1[a]
A spermatogonia/g of testis	0.002 ± 0.001 [a]	0.003 ± 0.001 [a]
Preleptotene spermatocyte	23.3 ± 2.1[a]	20.6 ± 4.1[a]
Preleptotene spermatocyte/g of testis	0.06 ± 0.01[a]	0.06± 0.02[a]
Pachytene spermatocyte	25.6± 3.9 [a]	20.0± 2.9[b]
Pachytene spermatocyte/g of testis	0.06 ± 0.002 [a]	0.06± 0.001[a]
Round spermatid	72.4 ± 14.0[a]	59.7 ± 7.3[b]
Round spermatid/g of testis	0.18 ± 0.03[a]	0.18 ± 0.04[a]

691 [a,b] Within a row, lsmeans without a common superscript differ ($P < 0.05$).
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698 **Figure Legends**

699 **Fig.1**- Spermatogenesis efficiency in high (HW) and low (LW) birth weight boars,
700 calculated based on germ cell counts present at stage I of the seminiferous epithelium
701 cycle at 8 months of age.

702

703 **Fig. 2**- Sperm count and daily sperm production (DSP) of high (HW) and low (LW) birth
704 weight boars at 8 months of age (^{a,b} $P < 0.05$).

705

706 **Fig. 3** - Immunostaining of MCM7-positive cells in 8 days (A) and 8 months (B) boars.
707 Ai and Bi correspond to negative control. G, gonocyte; Se, Sertoli cell; S, spermatogonia;
708 L, Leydig cell. Bars: 10 µm.

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