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1 **Follicular development of sows at weaning in relation to estimated breeding**

2 **value for within-litter variation in piglet birth weight**

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17 Short title: Follicular development and birth weight variation

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26

27 **Abstract**

28 In this study we aimed to identify possible causes of within-litter variation in piglet
29 birth weight (birth weight variation) by studying follicular development of sows at
30 weaning in relation to their estimated breeding value (**EBV**) for birth weight variation.
31 Twenty-nine multiparous sows (parity 3 to 5) were selected on their EBV for birth
32 weight variation (SD in grams; High-EBV: 15.8±1.6, N=14 and Low-EBV: -24.7±1.5,
33 N=15). The two groups of sows had similar litter sizes (15.7 vs. 16.9). Within 24
34 hours after parturition, piglets were cross fostered to ensure 13 suckling piglets per
35 sow. Sows weaned 12.8±1.0 and 12.7±1.0 piglets, respectively, at day 26.1±0.2 of
36 lactation. Blood and ovaries were collected within two hours after weaning. The right
37 ovary was immediately frozen to assess average follicle size and percentage healthy
38 follicles of the 15 largest follicles. The left ovary was used to assess the percentage
39 morphologically healthy cumulus-oocyte complexes (**COCs**) of the 15 largest
40 follicles. To assess the metabolic state of the sows, body condition and the
41 circulating metabolic markers insulin, insulin-like growth factor 1, non-esterified fatty
42 acid, creatinine, leptin, urea and fibroblast growth factor 21 were analysed at
43 weaning. No significant differences were found in any of the measured follicular or
44 metabolic parameters between High-EBV and Low-EBV. A higher weight loss during
45 lactation was related to a lower percentage healthy COCs (β= -0.65, p = 0.02).
46 Serum creatinine, a marker for protein breakdown, was negatively related to average
47 follicle size (β = -0.60, p = 0.05). Backfat loss during lactation was related to a higher
48 backfat thickness at parturition and to a higher average follicle size (β = 0.36, p <
49 0.001) at weaning. In conclusion, we hypothesise that modern hybrid sows with more
50 backfat at the start of lactation are able to mobilise more energy from backfat during
51 lactation and could thereby spare protein reserves to support follicular development.

52

53 **Keywords:** Sows, litter uniformity, reproduction, lactation, metabolism

54

55 **Implications**

56 The metabolic state of lactating sows was monitored to assess phenotypic relations

57 with follicular development at weaning. Lactational backfat loss was related to a

58 higher backfat thickness at parturition and to a higher average follicle size at

59 weaning. These results could implicate that sows with more backfat are able to

60 mobilise more energy from backfat during lactation and could thereby spare protein

61 reserves to support follicular development. A better understanding of the relation

62 between energy mobilisation of different substrates during lactation in relation to

63 follicular development could eventually be used for optimal breeding and feeding

64 strategies for lactating sows.

65

66 **Introduction**

67 Over the last decades, pigs have been genetically selected to produce larger litters.
68 Sows with larger litter sizes usually have lower average piglet birth weights
69 (Tuchscherer *et al.*, 2000) and higher birth weight variation (Tuchscherer *et al.*, 2000;
70 Milligan *et al.*, 2002; Wientjes *et al.*, 2012) which are related to higher piglet mortality
71 during subsequent lactation (Milligan et al., 2002). Lower piglet birth weights and
72 higher birth weight variation therefore strongly impact pig welfare and profitability (Fix
73 *et al.*, 2010). The cause and underlying mechanism of highly variable piglet birth
74 weights and consequently higher birth weight variation are not completely clear. One
75 factor that has been identified to increase birth weight variation is a negative energy
76 balance (**NEB**) during lactation (Wientjes *et al.*, 2013). This NEB negatively
77 influences, during and after lactation, the development of follicles that will give rise to
78 the next litter (reviewed by: Prunier and Quesnel, 2000). Therefore, we hypothesise
79 that decreased piglet birth weights and increased variation in birth weight could result
80 from impaired and more variable follicular development during the previous lactation.
81

82 Evidence for this hypothesis comes from studies in which the pre-mating metabolic
83 state of sows affects embryonic development and uniformity (e.g. Ferguson *et al.*,
84 2006; Patterson *et al.*, 2011) and subsequent piglet birth weights and uniformity (van
85 den Brand *et al.*, 2006). In addition, a study by Wientjes *et al.* (2013) showed that
86 body condition loss during lactation was related to increased variation in birth weight
87 of the next litter. The influence of the metabolic state during lactation on variation in
88 birth weight of the next litter may be explained by metabolic influences on follicular
89 development during lactation. For instance, feed restriction during lactation has been
90 shown to result in a decreased LH pulse frequency during lactation and around

91 weaning (Quesnel *et al.*, 1998a; van den Brand *et al.*, 2000), a smaller follicle size at
92 weaning and 48 hours later (Quesnel *et al.*, 1998a) and decreased oocyte maturation
93 rates when isolated 38 hours before the anticipated onset of oestrus (Zak *et al.*,
94 1997).
95 Furthermore, follicular heterogeneity has been related to embryonic heterogeneity
96 (Pope *et al.*, 1988 and 1989; Xie *et al.*, 1990) which, in turn, is an important
97 determinant of variation in birth weights (Van der Lende *et al.*, 1990).
98 These observations together indicate the importance of a more detailed analysis of
99 follicular development during lactation in order to identify possible causes for
100 variation in birth weight. Therefore, the aim of the current study is to identify possible
101 causes of variation in birth weight by studying follicular development and oocyte
102 quality of sows directly after weaning. To investigate this, we compared sows with a
103 high vs. low estimated breeding value (**EBV**) for variation in birth weight. In addition,
104 the metabolic state of the sows is monitored to assess phenotypic relations between
105 the metabolic state and follicular development at weaning.
106
107 **Material and methods**
108 *Animals*
109 A total of 30 multiparous Dutch Landrace sows (parity 3 to 5; Topigs Norsvin, Vught,
110 the Netherlands) housed at a local farm were selected based on EBV for variation in
111 birth weight. Breeders can produce an EBV for each individual sow and it is regarded
112 as the best estimate of the genetic potential for heritable traits such as variation in
113 birth weight. The EBV is expressed as the standard deviation of the piglet birth
114 weights within one litter in grams and is calculated based on genetic background and
115 previous performance. Average EBV of all parity 3 to 5 animals on the farm was -

116 6.3±17.1 (-41.9 to 28.0). A total of 15 sows were selected with a high EBV variation
117 in birth weight (High-EBV; 15.8±1.6; 5.4 to 28.0) and 15 sows were selected with a
118 low EBV (Low-EBV; -24.7±1.5; -14.9 to -31.1). Average parity was 3.7±0.3 for High-
119 EBV sows and 3.9±0.2 for Low-EBV sows. The sows were fed a standard lactation
120 diet (ca. 12.5MJ NE/kg, 154 g/kg CP, 9.3 g/kg lysine; Lacto Excellent, Agrifirm,
121 Apeldoorn, The Netherlands) and feed intake was assessed daily. Within 24 hours
122 after parturition, piglets were cross-fostered to ensure 13 suckling piglets per sow.
123 The sows had a lactation period of 26.1±0.2 (25 to 27) days and the experiment took
124 place over a period of 10 weeks. Each week, 1 or 2 sows of each group (High-EBV
125 and Low-EBV; so a total of 2 to 4 sows per week) entered the experiment. The sows
126 were slaughtered at the slaughterhouse by stunning and exsanguination within 2
127 hours after weaning.
128
129 *Body weight and backfat thickness*
130 The sows were weighed approximately 1 week before parturition and immediately
131 after weaning. Weight after parturition was estimated according to Bergsma *et al.*
132 (2009) by correcting the body weight of the sows as measured 1 week before
133 parturition for weight of the foetuses, placenta and intra-uterine fluid. Backfat
134 thickness was measured 6.5 cm from the midline over the last rib both on the left and
135 the right side using A-mode ultrasonography (Renco Lean-Meater, Renco
136 Corporation, Golden Valley, MN, USA) 1 week before parturition, within 12 hours
137 after parturition and immediately after weaning. Weight loss during lactation was
138 calculated by subtracting body weight at weaning from the estimated body weight at
139 parturition. A correction according to Bergsma *et al.* (2009) was used for water
140 content of mammary glands to prevent underestimation of body weight loss, since

141 mammary glands contain more water at the end of lactation compared to the start of
142 lactation. Body weight loss was expressed as a percentage of bodyweight at
143 parturition. Piglets were weighed within 24 hours after parturition and at weaning
144 (26.1±0.2 days postpartum). Litter growth during lactation, as a measure for sow milk
145 production, was calculated and corrected for mortality weight of the piglets that died
146 during lactation, according to Bergsma *et al.* (2009).
147
148 *Collection of ovaries and blood samples*
149 The left ovary was stored in a thermo-container and covered with the uterus to keep
150 the ovaries at a temperature above 30 ºC for subsequent follicle aspiration. The right
151 ovary was cut in 2 halves, immediately frozen in liquid nitrogen and stored at -80 ºC
152 until further analysis. Blood was collected from the jugular vein at slaughter in 9 ml
153 serum clot activator collection tubes and in 9 ml EDTA coated tubes (Greiner Bio-
154 One, Monroe, NC, USA), to obtain serum and plasma samples, respectively. The
155 serum collection tubes and the EDTA coated tubes were stored on ice and
156 transported to the laboratory. In the lab, the EDTA tubes were immediately
157 centrifuged at 500 x g for 10 min at 4^0C to collect plasma. The serum tubes were first
158 incubated overnight at 4^0C and subsequently centrifuged to collect serum. Both
159 plasma and serum samples were stored in -20^0C until further analysis.
160
161 *Measurements*
162 *Left ovary (fresh)*
163 The left ovary was, after transportation to the lab, placed in phosphate buffered
164 saline pH 7.4 (**PBS**) in a water bath at 37^0C. Within 5 hours after slaughter, the
165 ovaries were used for follicle aspiration. The 15 largest follicles were aspirated as

166 these are assumed to represent approximately half of the ovulatory follicle pool, as
167 ovulation rates in modern sows are around 25-30 (da Silva *et al.*, 2016). The
168 contents were collected in a tube and allowed to settle for 5 min. The supernatant
169 was removed and centrifuged at 1900 x g at 4ºC for 30 min to collect the follicular
170 fluid and assess the amount of follicular fluid. The recovered cumulus-oocyte
171 complexes (**COCs**) were morphologically classified under a dissection microscope as
172 normal (intact cumulus and normal-shaped oocyte) or atretic (degraded cumulus or
173 degenerated oocyte), according to Alvarez *et al.* (2009).
174 *Right ovary (frozen)*
175 The 2 halves of the right ovary were used to measure follicle size. Of each half of the
176 ovary, 3 cutting planes were made in a cryostat in order to study (almost) all follicles
177 on the surface of the ovary. Of each of these cutting planes, photographs were taken;
178 the ovaries were held against a ruler to measure the size of the follicles. Follicle size
179 was determined as the largest macroscopically visible diameter of the follicle.
180 To determine if the follicles were healthy or atretic, cryo-sections of the right ovary
181 were made and immunohistochemical staining for the presence of cleaved-Caspase
182 3 was performed (Supplemental Figure S1), a marker for cells in apoptosis similar to
183 Slot *et al.* (2006). In short, cryo-sections were mounted on superfrost plus glass
184 slides (Menzel- Gläser, Braunschweig, Germany). Sections were fixed in 4% buffered
185 formalin for 10 min, washed in H_2O, microwaved for 3 X 5 min in 0.1 M sodium citrate
186 buffer (pH 6) for epitope antigen retrieval, cooled down to room temperature and
187 subsequently rinsed PBS pH 7.4. Endogenous peroxidase activity was blocked with
188 3% (v/v) hydrogen peroxide in methanol solution for 30 min and aldehyde residues
189 were blocked with 0.3% glycine in PBS for 10 min. After rinsing with PBS, sections
190 were pre-incubated with 5% (wt/v) normal goat serum in PBS for 60 min at room

191 temperature. Subsequently, the sections were incubated overnight at 4°C in a humid
192 chamber with primary polyclonal rabbit anti-cleaved-Caspase 3 antibody (9661S, Cell
193 Signalling Technology, Danvers, MA, USA) diluted 1:1000 (v/v) in PBS-BSA-c
194 (Aurion, Wageningen, The Netherlands). Next, sections were rinsed with PBS and
195 treated with a secondary biotin labelled goat-anti-rabbit antibody (Vector
196 Laboratories, Burlingame, CA, USA) diluted 1:400 (v/v) in PBS-BSAc for 1 hour at
197 room temperature. After a wash with PBS and incubation with avidin-biotin complex
198 (ABC) diluted 1:1500 (v/v) in PBS-BSAc (Vector stain kit Elite, Vector Laboratories)
199 for 60 min at room temperature, sections were rinsed with PBS and bound antibody
200 was visualized using the Immpact DAB kit (stock solution diluted 1:400 (v/v); Vector
201 Laboratories). Sections were briefly counterstained with Mayer's haematoxylin
202 (Klinipath, Duiven, The Netherlands), visualised using light microscopy (Axioskop 2,
203 Carl Zeiss Microscopy, Thornwood, NY, US) and imaged using imaging software
204 (Axiovision 4.8, Carl Zeiss Microscopy).
205
206 *Assay procedures*
207 All assay procedures were performed according to manufacturer's instructions,
208 unless stated otherwise. All analyses were performed in duplo and only samples with
209 an intra-assay CV ≤ 15% were included.
210 Plasma insulin and leptin concentrations were measured using a radioimmunoassay
211 kit (Porcine Insulin PI-12K and Multi-Species Leptin XL-85K, respectively, EMD
212 Millipore corporation, Billerica, MA, US), fibroblast growth factor 21 (**FGF21**) was
213 measured using an ELISA kit (Abbexa, Cambridge, UK) and plasma urea and
214 creatinine were measured using an enzymatic colorimetric test (Urea liquicolor,
215 Human Gesselschaft fur Biochemica und Diganostica mbH, Wiesbaden, Germany

216 and Creatinine PAP FS, DiaSys Diagnostic Systems GmbH, Holzheim, Germany,
217 respectively).
218 Plasma insulin growth factor 1 (**IGF1**) was measured with an immunoradiometric
219 assay according to the manufacturer's protocol (A15729, Beckman Coulter,
220 Woerden, The Netherlands) supplemented with additional acid-ethanol extraction
221 (87.5 %v/v EtOH and 2.9 % v/v 12N HCl).
222 For serum non-esterified fatty acid (**NEFA**) analysis, a calorimetric detection method
223 was used (NEFA-HR(2) kit, Wako Chemicals, Neuss, Germany). Different from the
224 manufacturer's protocol, we added 5 µl serum to the plate and 100 µl of reagent 1
225 was added to the wells and incubated for 10 min at 37^0C. Subsequently, 50 µl of
226 reagent 2 was added and another incubation step of 10 min at 37^0C followed.
227
228 *Statistical analyses*
229 One of the animals of the High-EBV group ovulated during lactation and was
230 excluded from further analyses. Body weight of one animal was not recorded before
231 farrowing and 2 FGF21 values were removed because the CV values were ≥ 15%.
232 Distributions of the means and residuals were examined to verify model assumptions
233 of normality and homogeneity of variance. The presence of outliers was tested by
234 calculating the studentized residuals using proc REG and 2 outliers (1 NEFA and 1
235 urea value) were removed from further analyses. Follicular and metabolic differences
236 between EBV classes (High-EBV, N=14 and Low-EBV, N=15), follicle size classes
237 (FS: large: >5.1mm average follicle size of the 15 largest follicles of the right ovary
238 (N=14) and small: <5.0mm average follicle size (N=15)), variation in follicle size
239 classes (VARFS: large: >0.09mm SD in follicle size of the 15 largest follicles of the
240 right ovary (N=15) and small: <0.09mm (N=14)) were analysed using proc GLM in

241 SAS 9.4 (Cary, NC) in models that also contained the factor PAR (PAR3 (parity 3,
242 N=14) and PAR4+5 (parity 4 and 5, N=15)) and the interaction with PAR. Interactions
243 were excluded from the models when not significant. All values are presented as LS
244 means. Additionally, relations between metabolic parameters and between metabolic
245 and follicular parameters were estimated using the model: $Y_{ijk} = \mu + EBV + PAR +$
246 $\beta X_{ijk} + EBV*PAR + \beta X*PAR + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable and either a
247 metabolic or follicular parameter, β is the regression coefficient and X_{ijk} is one of the
248 metabolic parameters. The interactions were excluded from the models when not
249 significant.
250

251 **Results**

252 *Follicular parameters*

253 *Right ovary (frozen)*

254 Average follicle size of the 15 largest follicles was 5.04±0.74 mm while average
255 follicle size of the 10 largest healthy follicles was 5.11±0.82 mm. Of the 15 largest
256 follicles, 67.1±17.3% was classified as healthy based on cleaved-Caspase 3 staining.

257 *Left ovary (fresh)*

258 72.1±21.1% of the cumulus-oocytes complexes (COCs) isolated from the 15 largest
259 follicles was classified as healthy. The total amount of follicular fluid of the 15 largest
260 follicles was 369 ±153 µl.

261 *EBV class for within-litter variation in piglet birth weight*

262 High-EBV sows had an average EBV for variation in birth weight of 15.8±1.6 and
263 Low-EBV had an average EBV of -24.7±1.5 ($p < 0.001$). High-EBV and Low-EBV
264 sows did not differ in body condition or any of the measured metabolic parameters

265 nor did they differ in any of the piglet parameters (average birth weight, variation in
266 birth weight (SD), litter growth during lactation; Table 1).
267 In addition, follicular parameters at weaning did not differ between sows with High-
268 EBV and Low-EBV; neither average follicle size or variation in follicle size of the 15
269 largest follicles (Figure1), nor percentage healthy COCs or percentage healthy
270 follicles (Figure 2; all Supplemental Table S1). Interactions between EBV class and
271 PAR were never significant.
272 *Follicle size class (FS)*
273 Large-FS sows (average follicle size of the 15 largest follicles >5.1mm) had a higher
274 backfat thickness at parturition (17.9 vs. 16.1; $p = 0.02$), higher backfat loss during
275 lactation (4.0 vs. 2.6; $p < 0.01$) and lower creatinine levels at weaning (2.13 vs. 2.52;
276 $p = 0.03$) compared to Small-FS (<5mm) sows (Table 2). Large-FS sows did not
277 differ in any of the follicular parameters except for follicle size (Supplemental Table
278 S2). Interactions between FS and PAR were only significant for bodyweight at
279 parturition (Small-FS*PAR3 = 228, Small-FS*PAR4+5 = 259, Large-FS*PAR3 = 252,
280 Large-FS*PAR4+5 = 239; $p < 0.01$) and plasma insulin levels at weaning (Small-
281 FS*PAR3 = 8.5, Small-FS*PAR4+5 = 11.3, Large-FS*PAR3 = 17.2, Large-
282 FS*PAR4+5 =7.3; $p = 0.03$).
283 *Variation in follicle size class (VARFS)*
284 Large-VARFS sows (variation (SD) in follicle size of the 15 largest follicles >0.09mm)
285 vs. Small-VARFS sows (<0.09mm) did not differ in any of the metabolic (Table 3) or
286 follicular parameters, except for variation in follicle size (Supplemental Table S3).
287 Interactions between VARFS and PAR were only significant for urea levels at
288 weaning (Small-VARFS*PAR3 = 4.1, Small-VARFS*PAR4+5 = 5.1, Large-
289 VARFS*PAR3 = 4.3, Large-VARFS*PAR4+5 = 3.7; $p = 0.04$).

290 *Parity class*

291 PAR4+5 sows had a higher body weight at weaning (239 vs. 225; $p = 0.02$) and
292 higher creatinine levels at weaning (2.51 vs. 2.15; $p = 0.05$) and lost more backfat
293 during lactation (3.5 vs. 2.3; $p = 0.02$) compared to PAR3 sows (Table 1). Sows with
294 a different parity class did not differ in any of the measured follicular parameters
295 (Supplemental Table S1).

296 *Weight loss during lactation*

297 More body weight loss during lactation was related to lower plasma IGF1 levels at
298 weaning ($\beta = -6.43$ ng/ml per %, $p < 0.01$), higher serum creatinine levels at weaning
299 ($\beta = 0.01$ mg/dl per %, $p = 0.05$) and to a smaller percentage healthy COCs ($\beta =$ -
300 0.65 %/%, $p = 0.02$; all Figure 3). Furthermore, higher IGF1 levels tended to be
301 related to a higher percentage healthy COCs ($\beta = 0.001$ % per ng/ml, $p = 0.10$), while
302 higher creatinine levels were related to a smaller average follicle size ($\beta = -0.60$ mm
303 per mg/dl, $p = 0.05$; Fig 3) and serum urea levels were not related to any of the
304 measured metabolic or follicular parameters.

305 *Backfat loss during lactation*

306 A higher backfat thickness at parturition was related to a higher backfat loss during
307 lactation ($\beta = 0.92$ mm/mm, $p < 0.01$, Supplemental Figure S2). In addition, a higher
308 backfat loss during lactation was related to higher serum NEFA levels at weaning (β
309 $= 0.15$ mmol/L per mm, $p = 0.03$; Figure 4) and lower creatinine levels ($\beta = -0.14$
310 mg/dl per mm, $p = 0.05$; Figure 4). A higher backfat thickness at parturition and a
311 higher backfat loss during lactation were both related to a higher average follicle size
312 of the 15 largest follicles at weaning ($\beta = 0.19$ mm/mm, $p = 0.01$ and $\beta = 0.36$
313 mm/mm, $p < 0.001$; Figure 4, respectively). A higher backfat loss during lactation was
314 also related to a higher average follicle size of the 10 largest healthy follicles ($\beta =$

315 0.38 mm/mm, p = 0.01; Fig 4).

316

317 **Discussion**

318 We hypothesised that variation in the follicle pool may be a cause for variation in birth
319 weight. In order to test this, sows were selected based on their EBV for variation in
320 birth weight to apply a contrast in expected phenotypical variation in birth weight and
321 to correlate this to variation in follicular development. We therefore have explored
322 variation in follicular development in the follicle pool at weaning as from this antral
323 follicle pool follicles will be recruited for ovulation to give rise to the next litter. This
324 recruitment is due to the weaning associated change in pulsatile gonadotropin
325 releasing hormone and LH patterns: these patterns change from a low frequency and
326 high amplitude pattern to a high frequency and low amplitude pattern while FSH
327 levels increase (Shaw and Foxcroft, 1985). Since it has been reported that the antral
328 follicle pool is very heterogeneous regarding size and biochemical status in sows at
329 48 hours after weaning (Foxcroft *et al.*, 1987), it is very well possible that variation in
330 birth weight is caused by variation in the follicle pool at weaning.

331

332 The results of the present study do not support this assumption, as no relations could
333 be found between (variation in) follicular development at weaning and EBV for
334 variation in birth weight. One explanation for this unexpected finding may be that the
335 contrast in EBV for variation in birth weight that we were able to obtain in this study
336 or the heritability of the trait variation in birth weight (mean h2 = 0.08; Bidanel, 2011)
337 might be too small to detect phenotypic differences with the present sample size
338 (N=14 and N=15 for High-EBV and Low-EBV, respectively). In addition, the
339 repeatability of the trait variation in birth weight might be too low to relate the sows'

340 previous performance in variation in birth weight to variation in follicular development
341 of the follicle pool that will give rise to the next litter. Generally low repeatability's for
342 variation in piglet birth weight are found in literature (0.14, Quesnel *et al.*, 2008),
343 although for the genetics used higher repeatability's (0.19) are seen (E.G.Knol,
344 personal communication). Furthermore, we were able to obtain a difference between
345 High-EBV and Low-EBV of 40.5 gram (15.8±1.6 vs.-24.7±1.5) while the average EBV
346 of all the sows on the nucleus farm was -6.3±17.1. In addition, no linear relations
347 between EBV for variation in birth weight and follicular and metabolic parameters
348 could be found which confirms that EBV for variation in birth weight was not related
349 with any of the measured parameters.
350
351 Another explanation could be that variation in birth weight is not related to variation in
352 the follicle pool at weaning but is related with variation in follicular development later
353 in the follicular phase after recruitment.
354 Following recruitment, follicles are either selected to ovulate or degenerate; indeed
355 several studies have shown that many small and medium-sized antral follicles
356 become atretic (reviewed by: Guthrie, 2005). It is therefore likely that some of the
357 follicles that we have studied at weaning will become atretic in a later stage, will not
358 ovulate and will therefore not be related to the EBV for variation in birth weights.
359 Support for the assumption that follicular development after weaning may be an
360 important phase in determining variation in birth weights comes from a study by van
361 den Brand *et al.* (2006) which show that feeding insulin-stimulating diets in the post-
362 weaning period can reduce variation in birth weights. On the other hand, Wientjes *et*
363 *al*. (2013) show that more body weight and backfat loss during lactation is related to

364 more variation in birth weight of the subsequent litter, which indicates that variation in
365 birth weight could also originate from follicular development during lactation.
366 These studies both indicate that sow metabolic status during and immediately after
367 lactation can affect follicular development. Indeed, we observed phenotypic relations
368 between the metabolic state and follicular development at weaning.
369
370 More body weight loss during lactation was related to lower plasma IGF1 levels and
371 higher creatinine levels at weaning. This is expected since insulin and IGF1 are
372 usually suppressed in catabolic states (Quesnel *et al.*, 1998b; van den Brand *et al.*,
373 2001), while creatinine levels are higher when sows lose more weight due to
374 restricted feeding (Baidoo *et al.*, 1992) or when they receive less lysine in their diet
375 (Yang *et al.*, 2009). Unexpectedly, we did not find any relations between weight loss
376 during lactation and insulin levels at weaning.
377 We also find that a larger body weight loss during lactation is related to a lower
378 percentage healthy COCs. This corroborates findings by Zak *et al.* (1997), who
379 reported decreased maturation rates of oocytes isolated 38 hours before the
380 anticipated onset of oestrus in primiparous sows that were feed restricted from day
381 21-28 of lactation compared to sows that were fed ad libitum from day 21-28.
382 We do not observe relations between body weight loss and follicle size. This is in
383 contrast to a study by Quesnel *et al.* (1998a) who found that feed restriction during a
384 28-day lactation period (50% ad lib vs. ad lib) resulted in smaller follicles at weaning
385 in primiparous sows. Similar to our study, these investigators did not observe effects
386 of feed restriction on follicular atresia.
387

388 Body weight loss consist of loss of fat mass or loss of lean mass. In general, sows
389 lose around 5-fold more kilograms of fat during lactation compared to protein
390 (Bergsma *et al.*, 2009). As sows lose both fat and protein simultaneously, it is difficult
391 to establish which of the two is responsible for the negative effects of weight loss
392 during lactation on follicular development. Some studies suggest that especially
393 protein loss during lactation is detrimental for follicular development. In a study by
394 Clowes *et al.* (2003a) first-parity sows were fed either 50, 35 or 24 g of lysine/day
395 during a 23-day lactation period. The sows lost approximately 7, 9, and 16% of the
396 calculated body protein mass while no differences in backfat loss were found. When
397 the 8 largest follicles of both ovaries were analysed, it was found that sows which lost
398 more protein during lactation had a lower percentage follicles larger than 4 mm at
399 weaning (23.6% vs. 55.4% for high vs. low protein loss) and follicles contained less
400 follicular fluid (32 µl vs. 68 µl, respectively) with lower concentrations of estradiol and
401 IGF1. In addition, the follicular fluid of protein restricted sows reduced oocyte
402 maturation *in vitro*. Yang *et al.* (2000) found similar results to Clowes *et al.* (2003a)
403 when analysing the 15 largest pre-ovulatory follicles at pro-oestrus in primiparous
404 sows. Moreover, the severity of the effects of lysine restriction on follicle size and
405 follicular fluid content is larger for sows with a lower calculated protein mass at
406 parturition (Clowes *et al.*, 2003b).In line with these studies, we find that increased
407 creatinine levels, which can be considered a marker for protein loss (Yang *et al.*,
408 2009), are related to a lower average follicle size of the 15 largest follicles at
409 weaning. This indicates that increased protein loss during lactation has a negative
410 effect on follicle size at weaning. We found no relation between urea levels, which is
411 a marker for protein turnover, and follicle size.
412

413 In our study, the amount of fat loss during lactation was estimated by measuring
414 backfat thickness after parturition and at weaning. Higher backfat loss during
415 lactation is related to a higher backfat thickness at parturition. In addition, higher
416 backfat loss is related to higher serum NEFA levels at weaning which, when
417 measured in a fasted state as has been done in our study, is a marker for lipid
418 mobilization (Lafontan and Langin, 2009). Together these findings suggest that the
419 sows, which had more backfat at parturition, mobilised more lipid during lactation.
420 Unexpectedly, in our study, no relations between the amount of backfat and leptin
421 levels at weaning are found, in contrast to the findings of a study by De Rensis *et al.,*
422 *(*2005).
423 In order to further elucidate relations between backfat loss during lactation, NEFA
424 levels and follicular development at weaning, we measured FGF21, a hormone-like
425 circulating protein recently identified as a metabolic regulator of glucose and lipid
426 metabolism (reviewed by: Fisher and Maratos-Flier, 2016). We observe a tendency
427 towards higher FGF21 levels in Low-EBV sows compared to High-EBV ($p = 0.06$).
428 However no relations between FGF21 and any of the other measured metabolic or
429 follicular measurements can be found. More studies need to be performed to
430 elucidate possible relations between FGF21 and follicular development.
431
432 As mentioned previously, it is not known to which extent negative effects of energy
433 mobilization during lactation on follicular development, can be attributed to either
434 protein or fat loss, since studies investigating effects of feed restriction on follicular
435 development report a simultaneous loss of fat mass and lean mass. Most studies find
436 that weight loss and backfat loss of sows during lactation, so the simultaneous loss of
437 lean and fat mass, is related to a smaller follicle size (Quesnel *et al.*, 1998a and Zak

438 *et al.*, 1997, respectively). Surprisingly, in our study, more backfat loss during
439 lactation is related to a higher average size of the 15 largest follicles and higher
440 average size of the 10 largest healthy follicles at weaning. In addition, higher serum
441 NEFA levels at weaning tended to be related to a higher average follicle size at
442 weaning. Together, these findings suggest that increased lipid mobilization during
443 lactation is related to an increased follicle size.
444
445 One hypothesis for these surprising findings could be that sows which have low
446 levels of backfat at parturition mobilise less backfat during lactation to fulfil the energy
447 requirements of milk production and therefore have to use their protein reserves,
448 which might have a detrimental effect on follicular development. Indeed, in our study,
449 lower backfat loss during lactation was related to higher creatinine levels at weaning
450 and high creatinine levels were related to a smaller follicle size at weaning. So the
451 relation between increased backfat loss during lactation and a larger average follicle
452 size at weaning might be explained by protein sparing effects. It may be worthwhile
453 to study relations between energy mobilization of different energy substrates during
454 lactation and follicular development using reliable measurements of lean mass and
455 fat mobilization, such as balance trials and body composition measurements.
456
457 To conclude, in this study, follicular development at weaning appeared to be similar
458 for sows with a High vs. Low-EBV for variation in birth weight. It is possible that
459 variation in birth weights is (partly) explained by variation in the follicle pool at
460 weaning, but this is not reflected in EBV. Another possibility is that variation in birth
461 weight is explained by follicle development at a later time point during the follicular
462 phase or by other factors which play a role after ovulation. Our study does show that

463 energy mobilization from different sources during lactation, adipose tissues or muscle
464 reflecting fat or lean mass, respectively, could have divergent effects on follicular
465 development at weaning. These relations need to be further elucidated.
466

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471

472 **Declaration of interest**

473 The authors declare that there is no conflict of interest that could be perceived as
474 prejudicing the impartiality of the results reported.
475

476 **Ethics statement**

477 The experiment was approved by the Animal Care and Use Committee of
478 Wageningen University (DEC2016036) and performed according to national and EU
479 guidelines.
480

481 **Software and data repository resources**

482 None of the data were deposited in an official repository.
483

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590

591

592 **Table 1** *Effects of estimated breeding value classes for within-litter variation in piglet*

593 *birth weight (EBV; High (N=14) vs. Low (N=15)) and parity classes (PAR; 3 (N=14)*

594 *vs. 4+5 (N=15)) on gestation and lactation parameters, body condition and metabolic*

595 *parameters at weaning in sows. All values are presented as LS means.*

	EBV		PAR		RMSE	P-values[1]	
Parameter	High	Low	3	4 + 5		EBV	PAR
EBV LVR (g)	15.8	-24.7	-6.3	-2.6	5.7	<0.001	0.11
Gestation + parturition							
Gestation (days)	115.2	114.9	114.8	115.3	1.1	0.51	0.27
Total number born	15.7	16.9	17.2	15.4	3.6	0.43	0.19
Average piglet birth weight (g)	1 476	1 371	1 390	1 457	258	0.31	0.51
Variation (SD) (g)	267	291	313	275	69	0.84	0.18
Total litter weight start (kg)	19.7	18.0	18.6	19.2	3.0	0.17	0.63
Lactation							
Lactation (days)	26.1	26.2	26.3	26.0	0.89	0.80	0.89
Total litter weight weaning (kg)	91.1	90.2	87.5	93.8	9.9	0.83	0.12
Total litter growth (kg)	71.8	72.5	69.4	75.0	9.1	0.84	0.13
N of piglets weaned	12.8	12.7	12.7	12.7	0.6	0.87	0.87
Feed intake sow (kg/day)	6.0	5.9	6.0	5.9	0.5	0.45	0.71
Body condition							
Weight parturition (kg)	248	248	248	251	20	0.91	0.47
Weight weaning (kg)	232	232	225	239	15	0.99	0.02
Weight loss lactation (%)	10.6	10.3	11.1	9.8	6.3	0.91	0.60
Backfat parturition (mm)	17.0	17.1	16.6	17.5	2.2	0.91	0.30
Backfat weaning (mm)	13.9	14.4	14.3	13.9	1.9	0.48	0.61
Backfat loss lactation (mm)	3.1	2.7	2.3[a]	3.5[b]	1.3	0.42	0.02
Metabolic parameters							
Insulin (uU/ml)	11.8	11.9	14.1	9.6	7.7	0.96	0.15
IGF1 (ng/ml)	154	136	139	151	63	0.47	0.64
FGF21 (pg/ml)	5 813	8 861	7 978	6 697	3 143	0.06	0.40
Urea (mmol/l)	4.46	4.33	4.23	4.56	1.00	0.74	0.43
Creatinine (mg/dl)	2.38	2.31	2.15[a]	2.54[b]	0.48	0.69	0.05
NEFA (mmol/L)	0.98	0.97	1.01	0.94	0.45	0.92	0.73
Leptin (ng/ml)	13.6	13.0	11.4	10.5	2.3	0.67	0.33

596 EBV = estimated breeding value, LVR = within-litter variation in piglet birth weight (standard deviation),

597 NEFA = non-esterified fatty acid, IGF1= insulin-like growth factor 1, FGF = fibroblast growth factor.

598 [1]Interactions between EBV and PAR were never significant.

599 **Table 2** *Effects of average sow follicle size classes (FS; Small <5.0 mm (N=15) vs.*

600 *Large>5.1 mm (N=14)) and parity classes (PAR; 3 (N=14) vs. 4+5 (N=15)) on*

601 *gestation and lactation parameters, body condition and metabolic parameters at*

602 *weaning in sows. All values are presented as LS means.*

	FS		PAR		RMSE	P-values	
Parameter	Small	Large	3	4 + 5		FS	PAR
EBV LVR (g)	-9.6	-0.3	-1.8	-8.4	20.5	0.24	0.41
Gestation + parturition							
Gestation (days)	114.8	115.3	114.8	115.3	1.1	0.22	0.22
Total number born	15.4	17.2	16.8	15.7	3.5	0.20	0.42
Average piglet birth weight (g)	1 473	1 375	1 418	1 430	258	0.33	0.91
Variation (SD) (g)	293	295	313	275	69	0.93	0.16
Total litter weight start (kg)	19.3	18.5	18.9	18.5	3.1	0.51	0.91
Lactation							
Lactation (days)	26.0	26.2	26.3	26.0	0.9	0.54	0.50
Total litter weight weaning (kg)	90.0	91.3	87.5	93.8	9.9	0.73	0.11
Total litter growth (kg)	70.8	73.4	68.9	75.3	9.0	0.46	0.08
N of piglets weaned	12.8	12.7	12.8	12.7	0.6	0.76	0.76
Feed intake sow (kg/day)	5.8	6.0	6.0	5.9	0.5	0.34	0.70
Body condition							
Weight parturition (kg)[1]	244	246	240	249	20	0.76	0.20
Weight weaning (kg)	234	230	225	239	15	0.53	0.03
Weight loss lactation (%)	10.6	10.3	11.2	9.7	6.3	0.68	0.61
Backfat parturition (mm)	16.1	17.9	16.3	17.7	2.0	0.02	0.08
Backfat weaning (mm)	13.9	14.3	13.9	14.3	1.9	0.61	0.86
Backfat loss lactation (mm)	2.6	4.0	2.7	3.9	1.1	<0.01	<0.01
Metabolic parameters							
Insulin (uU/ml)[2]	9.9	12.3	12.8	9.3	7.6	0.40	0.21
IGF1 (ng/ml)	153	138	144	147	63	0.56	0.88
FGF21 (pg/ml)	7 296	7 758	7 303	7 751	3 420	0.75	0.76
Urea (mmol/l)	4.6	4.3	4.3	4.5	1.0	0.49	0.53
Creatinine (mg/dl)	2.52	2.13	2.22	2.43	0 44	0.03	0.21
NEFA (mmol/L)	0.91	1.02	0.98	0.95	0.45	0.54	0.82
Leptin (ng/ml)	10.7	11.2	11.4	10.5	2.28	0.61	0.31

603 EBV = estimated breeding value, LVR = within-litter variation in piglet birth weight (standard deviation),

604 NEFA = non-esterified fatty acid, IGF1 = insulin-like growth factor 1, FGF = fibroblast growth factor.

605 [1]LS means estimates for the interaction FS*PAR (p < 0.01): Small-FS*PAR3 = 228, Small-FS*PAR4+5

606 = 259, Large-FS*PAR3 = 252, Large-FS*PAR4+5 = 239.

607 [2]LS means estimates for the interaction FS*PAR (p = 0.03): Small-FS*PAR3 = 8.5, Small-FS*PAR4+5

608 = 11.3, Large-FS*PAR3 = 17.2, Large-FS*PAR4+5 =7.3.

609 **Table 3** *Effects of average sow variation (SD) in follicle size classes (VARFS; Small*

610 *<0.09mm (N=14) vs. Large>0.09 mm (N=15)) and parity classes (PAR; 3 (N=14) vs.*

611 *4+5 (N=15)) on gestation and lactation parameters, body condition and metabolic*

	VARFS		PAR		RMSE	P-values	
Parameter	Small	Large	3	4 + 5		VARFS	PAR
EBV LVR (g)	-1.7	-7.8	0.4	-9.9	20.9	0.45	0.21
Gestation + parturition							
Gestation (days)	114.9	115.2	114.8	115.3	1.1	0.58	0.30
Total number born	16.1	16.5	17.0	15.6	3.6	0.74	0.31
Average piglet birth weight (g)	1 409	1 434	1 401	1 442	263	0.81	0.69
Variation (SD) (g)	282	305	310	277	68	0.38	0.21
Total litter weight start (kg)	18.8	18.9	18.8	18.9	3.1	0.92	0.94
Lactation							
Lactation (days)	26.0	26.2	26.3	26.0	0.9	0.54	0.50
Total litter weight weaning (kg)	90.4	90.9	87.6	93.7	9.9	0.90	0.12
Total litter growth (kg)	71.7	72.6	69.2	75.2	9.2	0.82	0.10
N of piglets weaned	12.8	12.7	12.8	12.7	0.6	0.76	0.76
Feed intake sow (kg/day)	5.9	5.9	6.0	5.8	0.5	0.97	0.55
Body condition							
Weight parturition (kg)	248	248	245	251	19.7	0.99	0.42
Weight weaning (kg)	231	233	224	239	15	0.71	0.02
Weight loss lactation (%)	11.0	9.9	11.3	9.6	6.3	0.67	0.49
Backfat parturition (mm)	16.4	17.6	16.4	17.6	2.1	0.14	0.14
Backfat weaning (mm)	13.9	14.4	14.2	14.1	1.9	0.54	0.88
Backfat loss lactation (mm)	3.2	3.5	2.9	3.8	1.3	0.54	0.08
Metabolic parameters							
Insulin (uU/ml)	12.08	11.7	14.1	9.6	7.7	0.89	0.14
IGF1 (ng/ml)	152	139	144	147	63	0.59	0.88
FGF21 (pg/ml)	7 658	7 468	7 413	7 712	3 426	0.89	0.83
Urea (mmol/l)[1]	4.6	4.0	4.2	4.4	1.0	0.12	0.64
Creatinine (mg/dl)	2.32	2.30	2.16	2.46	0.48	0.92	0.13
NEFA (mmol/L)	1.12	0.86	1.06	0.92	0.43	0.15	0.43
Leptin (ng/ml)	11.0	11.0	11.5	10.5	2.3	0.92	0.27

612 *parameters at weaning in sows. All values are presented as LS means.*

613

614 EBV = estimated breeding value, LVR = within-litter variation in piglet birth weight (standard deviation),

615 NEFA = non-esterified fatty acid, IGF1 = insulin-like growth factor 1, FGF= fibroblast growth factor.

616 [1]LS means estimates for the interaction VARFS*PAR (p = 0.04): Small-VARFS*PAR3 = 4.1, Small-

617 VARFS*PAR4+5 = 5.1, Large-VARFS*PAR3 = 4.3, Large-VARFS*PAR4+5 = 3.7.

618

619 **Figure captions**

620 **Figure 1** - A Average follicle size and variation (SD) in follicle size of the 15 largest
621 follicles of the right ovary for estimated breeding value (EBV) class for within-litter
622 variation in piglet birth weight (High-EBV (N=14) and Low-EBV (N=15)) B Average
623 size (cm) of the 15 largest follicles (1=largest, 15=smallest) of the right ovary for
624 High–EBV and Low-EBV sows.

625

626 **Figure 2** - Percentage healthy cumulus-oocyte complexes (COC) of the left ovary
627 and percentage healthy follicles of the right ovary for estimated breeding value (EBV)
628 class for within-litter variation in piglet birth weight (High-EBV (N=14) and Low-EBV
629 (N=15)) in sows. The COCs were morphologically classified as healthy or atretic
630 according to Alvarez *et al*. (2009) and follicles were classified as healthy or atretic
631 using the cleaved-Caspase 3 staining as a marker for cells in apoptosis.

632

633 **Figure 3** - Regression equations (β) for the relations between A creatinine levels at
634 weaning (mg/dl) and follicle size of the 15 largest follicles at weaning and weight loss
635 during lactation (%) and B percentage healthy cumulus-oocyte complexes (COCs), C
636 insulin-like growth factor 1 (IGF1) (ng/ml) levels at weaning and D creatinine levels
637 (mg/dl) at weaning in sows. The COCs were morphologically classified as healthy or
638 atretic according to (Alvarez *et al*., 2009). No interactions with parity class (PAR)
639 have been found.

640

641 **Figure 4** - Regression equations (β) for the relation between backfat loss during
642 lactation and A average follicle size of the 15 largest follicles, B average follicle size
643 of the 10 largest healthy follicles, C serum non-esterified fatty acid (NEFA) levels

644 (mmol/L) at weaning and D serum creatinine (mg/dl) levels at weaning in sows. No

645 interactions with parity class (PAR) have been found.