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N. G. J. Costermans[1,2], K. J. Teerds[1], J. Keijer[1], E. F. Knol[3] , R. E. Koopmanschap[2], B. Kemp[2], N. M. Soede[2]

[1] *Human and Animal Physiology, Wageningen University and Research, De Elst 1, 6708WD, Wageningen, The Netherlands*

[2] *Adaptation Physiology Group, Wageningen University and Research, De Elst 1, 6708WD, Wageningen, The Netherlands*

[3] *Topigs Norsvin Research Center B. V.,Schoenaker 6, 6641 SZ, Beuningen, The Netherlands*

Corresponding author: Natasja Costermans. Email: natasja.costermans@wur.nl

Short title: Follicular development and birth weight variation

Abstract

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

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

Implications

The metabolic state of lactating sows was monitored to assess phenotypic relations with follicular development at weaning. Lactational backfat loss was related to a higher backfat thickness at parturition and to a higher average follicle size at weaning. These results could implicate that sows with more backfat are able to mobilise more energy from backfat during lactation and could thereby spare protein reserves to support follicular development. A better understanding of the relation between energy mobilisation of different substrates during lactation in relation to follicular development could eventually be used for optimal breeding and feeding strategies for lactating sows.

Introduction

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

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

6.3±17.1 (-41.9 to 28.0). A total of 15 sows were selected with a high EBV variation in birth weight (High-EBV; 15.8±1.6; 5.4 to 28.0) and 15 sows were selected with a low EBV (Low-EBV; -24.7±1.5; -14.9 to -31.1). Average parity was 3.7±0.3 for High-EBV sows and 3.9±0.2 for Low-EBV sows. The sows were fed a standard lactation diet (ca. 12.5MJ NE/kg, 154 g/kg CP, 9.3 g/kg lysine; Lacto Excellent, Agrifirm, Apeldoorn, The Netherlands) and feed intake was assessed daily. Within 24 hours after parturition, piglets were cross-fostered to ensure 13 suckling piglets per sow. The sows had a lactation period of 26.1±0.2 (25 to 27) days and the experiment took place over a period of 10 weeks. Each week, 1 or 2 sows of each group (High-EBV and Low-EBV; so a total of 2 to 4 sows per week) entered the experiment. The sows were slaughtered at the slaughterhouse by stunning and exsanguination within 2 hours after weaning.

Body weight and backfat thickness

The sows were weighed approximately 1 week before parturition and immediately after weaning. Weight after parturition was estimated according to Bergsma *et al.* (2009) by correcting the body weight of the sows as measured 1 week before parturition for weight of the foetuses, placenta and intra-uterine fluid. Backfat thickness was measured 6.5 cm from the midline over the last rib both on the left and the right side using A-mode ultrasonography (Renco Lean-Meater, Renco Corporation, Golden Valley, MN, USA) 1 week before parturition, within 12 hours after parturition and immediately after weaning. Weight loss during lactation was calculated by subtracting body weight at weaning from the estimated body weight at parturition. A correction according to Bergsma *et al.* (2009) was used for water content of mammary glands to prevent underestimation of body weight loss, since

mammary glands contain more water at the end of lactation compared to the start of lactation. Body weight loss was expressed as a percentage of bodyweight at parturition. Piglets were weighed within 24 hours after parturition and at weaning (26.1±0.2 days postpartum). Litter growth during lactation, as a measure for sow milk production, was calculated and corrected for mortality weight of the piglets that died during lactation, according to Bergsma *et al.* (2009).

Collection of ovaries and blood samples

The left ovary was stored in a thermo-container and covered with the uterus to keep the ovaries at a temperature above 30 ºC for subsequent follicle aspiration. The right ovary was cut in 2 halves, immediately frozen in liquid nitrogen and stored at -80 ºC until further analysis. Blood was collected from the jugular vein at slaughter in 9 ml serum clot activator collection tubes and in 9 ml EDTA coated tubes (Greiner Bio-One, Monroe, NC, USA), to obtain serum and plasma samples, respectively. The serum collection tubes and the EDTA coated tubes were stored on ice and transported to the laboratory. In the lab, the EDTA tubes were immediately centrifuged at 500 x g for 10 min at 4^0C to collect plasma. The serum tubes were first incubated overnight at 4^0C and subsequently centrifuged to collect serum. Both plasma and serum samples were stored in -20^0C until further analysis.

Measurements

Left ovary (fresh)

The left ovary was, after transportation to the lab, placed in phosphate buffered saline pH 7.4 (**PBS**) in a water bath at 37^0C. Within 5 hours after slaughter, the ovaries were used for follicle aspiration. The 15 largest follicles were aspirated as

these are assumed to represent approximately half of the ovulatory follicle pool, as ovulation rates in modern sows are around 25-30 (da Silva *et al.*, 2016). The contents were collected in a tube and allowed to settle for 5 min. The supernatant was removed and centrifuged at 1900 x g at 4ºC for 30 min to collect the follicular fluid and assess the amount of follicular fluid. The recovered cumulus-oocyte complexes (**COCs**) were morphologically classified under a dissection microscope as normal (intact cumulus and normal-shaped oocyte) or atretic (degraded cumulus or degenerated oocyte), according to Alvarez *et al.* (2009).

Right ovary (frozen)

The 2 halves of the right ovary were used to measure follicle size. Of each half of the ovary, 3 cutting planes were made in a cryostat in order to study (almost) all follicles on the surface of the ovary. Of each of these cutting planes, photographs were taken; the ovaries were held against a ruler to measure the size of the follicles. Follicle size was determined as the largest macroscopically visible diameter of the follicle.

To determine if the follicles were healthy or atretic, cryo-sections of the right ovary were made and immunohistochemical staining for the presence of cleaved-Caspase 3 was performed (Supplemental Figure S1), a marker for cells in apoptosis similar to Slot *et al.* (2006). In short, cryo-sections were mounted on superfrost plus glass slides (Menzel- Gläser, Braunschweig, Germany). Sections were fixed in 4% buffered formalin for 10 min, washed in H_2O, microwaved for 3 X 5 min in 0.1 M sodium citrate buffer (pH 6) for epitope antigen retrieval, cooled down to room temperature and subsequently rinsed PBS pH 7.4. Endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide in methanol solution for 30 min and aldehyde residues were blocked with 0.3% glycine in PBS for 10 min. After rinsing with PBS, sections 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^{o}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

and Creatinine PAP FS, DiaSys Diagnostic Systems GmbH, Holzheim, Germany, respectively).

Plasma insulin growth factor 1 (**IGF1**) was measured with an immunoradiometric assay according to the manufacturer's protocol (A15729, Beckman Coulter, Woerden, The Netherlands) supplemented with additional acid-ethanol extraction (87.5 %v/v EtOH and 2.9 % v/v 12N HCl).

For serum non-esterified fatty acid (**NEFA**) analysis, a calorimetric detection method was used (NEFA-HR(2) kit, Wako Chemicals, Neuss, Germany). Different from the manufacturer's protocol, we added 5 µl serum to the plate and 100 µl of reagent 1 was added to the wells and incubated for 10 min at 37^0C. Subsequently, 50 µl of reagent 2 was added and another incubation step of 10 min at 37^0C followed.

Statistical analyses

One of the animals of the High-EBV group ovulated during lactation and was excluded from further analyses. Body weight of one animal was not recorded before farrowing and 2 FGF21 values were removed because the CV values were ≥ 15%. Distributions of the means and residuals were examined to verify model assumptions of normality and homogeneity of variance. The presence of outliers was tested by calculating the studentized residuals using proc REG and 2 outliers (1 NEFA and 1 urea value) were removed from further analyses. Follicular and metabolic differences between EBV classes (High-EBV, N=14 and Low-EBV, N=15), follicle size classes (FS: large: >5.1mm average follicle size of the 15 largest follicles of the right ovary (N=14) and small: <5.0mm average follicle size (N=15)), variation in follicle size classes (VARFS: large: >0.09mm SD in follicle size of the 15 largest follicles of the right ovary (N=15) and small: <0.09mm (N=14)) were analysed using proc GLM in

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

Results

Follicular parameters

Right ovary (frozen)

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

Left ovary (fresh)

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

EBV class for within-litter variation in piglet birth weight

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

nor did they differ in any of the piglet parameters (average birth weight, variation in birth weight (SD), litter growth during lactation; Table 1).

In addition, follicular parameters at weaning did not differ between sows with High-EBV and Low-EBV; neither average follicle size or variation in follicle size of the 15 largest follicles (Figure1), nor percentage healthy COCs or percentage healthy follicles (Figure 2; all Supplemental Table S1). Interactions between EBV class and PAR were never significant.

Follicle size class (FS)

Large-FS sows (average follicle size of the 15 largest follicles >5.1mm) had a higher backfat thickness at parturition (17.9 vs. 16.1; $p = 0.02$), higher backfat loss during lactation (4.0 vs. 2.6; $p < 0.01$) and lower creatinine levels at weaning (2.13 vs. 2.52; $p = 0.03$) compared to Small-FS (<5mm) sows (Table 2). Large-FS sows did not differ in any of the follicular parameters except for follicle size (Supplemental Table S2). Interactions between FS and PAR were only significant for bodyweight at parturition (Small-FS*PAR3 = 228, Small-FS*PAR4+5 = 259, Large-FS*PAR3 = 252, Large-FS*PAR4+5 = 239; $p < 0.01$) and plasma insulin levels at weaning (Small-FS*PAR3 = 8.5, Small-FS*PAR4+5 = 11.3, Large-FS*PAR3 = 17.2, Large-FS*PAR4+5 =7.3; $p = 0.03$).

Variation in follicle size class (VARFS)

Large-VARFS sows (variation (SD) in follicle size of the 15 largest follicles >0.09mm) vs. Small-VARFS sows (<0.09mm) did not differ in any of the metabolic (Table 3) or follicular parameters, except for variation in follicle size (Supplemental Table S3). Interactions between VARFS and PAR were only significant for urea levels at weaning (Small-VARFS*PAR3 = 4.1, Small-VARFS*PAR4+5 = 5.1, Large-VARFS*PAR3 = 4.3, Large-VARFS*PAR4+5 = 3.7; $p = 0.04$).

Parity class

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

Weight loss during lactation

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

Backfat loss during lactation

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

more variation in birth weight of the subsequent litter, which indicates that variation in birth weight could also originate from follicular development during lactation.

These studies both indicate that sow metabolic status during and immediately after lactation can affect follicular development. Indeed, we observed phenotypic relations between the metabolic state and follicular development at weaning.

More body weight loss during lactation was related to lower plasma IGF1 levels and higher creatinine levels at weaning. This is expected since insulin and IGF1 are usually suppressed in catabolic states (Quesnel *et al.*, 1998b; van den Brand *et al.*, 2001), while creatinine levels are higher when sows lose more weight due to restricted feeding (Baidoo *et al.*, 1992) or when they receive less lysine in their diet (Yang *et al.*, 2009). Unexpectedly, we did not find any relations between weight loss during lactation and insulin levels at weaning.

We also find that a larger body weight loss during lactation is related to a lower percentage healthy COCs. This corroborates findings by Zak *et al.* (1997), who reported decreased maturation rates of oocytes isolated 38 hours before the anticipated onset of oestrus in primiparous sows that were feed restricted from day 21-28 of lactation compared to sows that were fed ad libitum from day 21-28.

We do not observe relations between body weight loss and follicle size. This is in contrast to a study by Quesnel *et al.* (1998a) who found that feed restriction during a 28-day lactation period (50% ad lib vs. ad lib) resulted in smaller follicles at weaning in primiparous sows. Similar to our study, these investigators did not observe effects of feed restriction on follicular atresia.

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

In our study, the amount of fat loss during lactation was estimated by measuring backfat thickness after parturition and at weaning. Higher backfat loss during lactation is related to a higher backfat thickness at parturition. In addition, higher backfat loss is related to higher serum NEFA levels at weaning which, when measured in a fasted state as has been done in our study, is a marker for lipid mobilization (Lafontan and Langin, 2009). Together these findings suggest that the sows, which had more backfat at parturition, mobilised more lipid during lactation. Unexpectedly, in our study, no relations between the amount of backfat and leptin levels at weaning are found, in contrast to the findings of a study by De Rensis *et al.,* *(*2005).

In order to further elucidate relations between backfat loss during lactation, NEFA levels and follicular development at weaning, we measured FGF21, a hormone-like circulating protein recently identified as a metabolic regulator of glucose and lipid metabolism (reviewed by: Fisher and Maratos-Flier, 2016). We observe a tendency towards higher FGF21 levels in Low-EBV sows compared to High-EBV ($p = 0.06$). However no relations between FGF21 and any of the other measured metabolic or follicular measurements can be found. More studies need to be performed to elucidate possible relations between FGF21 and follicular development.

As mentioned previously, it is not known to which extent negative effects of energy mobilization during lactation on follicular development, can be attributed to either protein or fat loss, since studies investigating effects of feed restriction on follicular development report a simultaneous loss of fat mass and lean mass. Most studies find that weight loss and backfat loss of sows during lactation, so the simultaneous loss of 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

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

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Declaration of interest

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

Ethics statement

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

Software and data repository resources

None of the data were deposited in an official repository.

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Table 1 *Effects of estimated breeding value classes for within-litter variation in piglet birth weight (EBV; High (N=14) vs. Low (N=15)) and parity classes (PAR; 3 (N=14) vs. 4+5 (N=15)) on gestation and lactation parameters, body condition and metabolic 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

EBV = estimated breeding value, LVR = within-litter variation in piglet birth weight (standard deviation), NEFA = non-esterified fatty acid, IGF1= insulin-like growth factor 1, FGF = fibroblast growth factor.

[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

Figure captions

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

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

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

Figure 4 - Regression equations (β) for the relation between backfat loss during lactation and A average follicle size of the 15 largest follicles, B average follicle size 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.