Maternal nutrition affects the composition of follicular fluid and transcript content in gilt oocytes

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ABSTRACT: Metabolomics focused on reproduction have been the subject of special interest in the past decade. Metabolite profiling provides detailed information on the status of follicular fluid and follicular cells which accompany the growing oocyte. Although nutrients present in the diet reach oocytes via the follicular fluid, it is not evident whether oocyte/embryo quality can be predicted based on the follicular fluid composition. Since this phenomenon has not yet been investigated in the pig, the aim of the present study was to investigate associations between diet composition and (1) selected markers related to developmental potential of oocytes (brilliant cresyl blue – BCB test, relative transcript abundance of *EEF1A1* and *ATP5A1* marker genes) and (2) fatty acid profile in the follicular fluid. Gilts were fed control and experimental diets which differed in composition and fatty acid profiles. The experimental diet used in this study comprised mainly locally grown grains (barley and rye) traditionally used for fattening pigs in Poland. Cumulus-oocyte complexes were aspirated from individual pairs of ovaries after animal slaughter, evaluated morphologically and subjected to the BCB test. Relative transcript abundance for the two marker genes was monitored by real-time PCR in oocytes of both categories (BCB^+ and BCB^-). Fatty acid profile in follicular fluid was analyzed by gas chromatography. We show that the experimental diet rich in n-3 fatty acid significantly influences fatty acid composition of the follicular fluid. The fatty acid profile of the follicular fluid of gilts fed the experimental diet differed from that of the control females. The content of saturated fatty acids was higher in the experimental group, whereas unsaturated and polyunsaturated fatty acids were more abundant in the control group ($P < 0.05$). With regard to individual fatty acids, only C16:0 (palmitic acid), C18:2n-6 (linoleic acid) and C22:6 (docosahexaenoic acid) differed significantly. The abundance of *ATP5A1* mRNA was influenced neither by diet composition nor by oocyte category (BCB^+/BCB^-) whereas that of the *EEF1A1* was affected by both factors. Since higher mRNA level of the *EEF1A1* gene was noted in BCB^+ oocytes this may be considered as a marker of oocyte quality in the pig.

Keywords: fatty acids; BCB test; pig; *EEF1A1*; *ATP5A1*

List of abbreviations

ATP5A1 = gene controlling ATP synthesis, **BCB** = brilliant cresyl blue test, **BCB^-** = BCB negative, **BCB^+** = BCB positive, **CD** = control diet, **COC** = the cumulus-oocyte complex, **DHA** = docosahexaenoic acid, **ED** = experimental diet, ***EEF1A1*** = elongation factor 1a, **FA** = fatty acids, **FC** = follicular cells, **FF** = follicular fluid, **MUFA** = monounsaturated fatty acids, **PUFA** = polyunsaturated fatty acids, **RA** = transcript abundance, **SFA** = saturated fatty acids, **UFA** = unsaturated fatty acids

Maternal nutrition is one of the main factors affecting developmental competence of mammalian oocytes and embryos. Diet composition may alter the maternal environment of the oocyte and embryo growth through metabolic and endocrine modifications. Zak et al. (1997) showed that feed intake influenced the meiotic competence of porcine oocytes. They claimed that maternal under-nutrition may reduce the follicle's ability to support maturation of oocytes and thus their quality. Follicular fluid (FF) as an endogenous medium participates in transmitting nutrition signals to the

cumulus-oocyte complex (COC). Many reports support an important role for FF components in determining oocyte quality (Sinclair et al., 2008; Revelli et al., 2009). Metabolite profiling provides detailed information on the status of FF and follicular cells (FC) which accompany a growing oocyte. Metabolomic studies focused mainly on ruminant reproduction have been the subject of much interest in the past decade. The main area of focus has been the fatty acids (Zeron et al., 2001; Sinclair et al., 2008), amino acids (Booth et al., 2005; Sinclair et al., 2008) and sugars (Preis et al., 2005). Due to some inconsistency in data concerning ruminants it is not evident whether oocyte/embryo quality can be predicted based on the composition of follicular fluid. This phenomenon has not yet been investigated in the pig. It is evident that unlike ruminants, monogastric animals are not able to modify fatty acids provided in the diet, thus dietary fatty acids (FA) are directly incorporated into tissues.

Developmental competence (quality) of oocytes is defined as their ability to resume meiosis, to be fertilized, to transform into a blastocyst and finally to develop into viable offspring (Krisher, 2004; Sirard et al., 2006). Therefore, oocyte quality is a complex trait and as such needs a multifactorial assessment. The only non-invasive method used to date is morphology evaluation which is considered unreliable. Since the majority of other approaches involve cell injury or damage, a non-invasive protocol would be of great use. The brilliant cresyl blue test (BCB) is a method with several advantages. It is based on the ability of G6PDH to reduce BCB dye to a colourless form in the ooplasm of less competent, growing oocytes (BCB^-) whereas fully grown, more competent oocytes remain blue (BCB^+). Several advantages of the BCB^+ oocytes over their BCB^- counterparts have been documented in the pig (Roca et al., 1998; Wongsrikeao et al., 2006; Ishizaki et al., 2009), cattle (Alm et al., 2005; Torner et al., 2008) and goat (Rodriguez-Gonzalez et al., 2003).

Torner et al. (2008) demonstrated higher quality of bovine BCB^+ oocytes by evaluating relative transcript abundance (RA) of two marker genes *EEF1A1* (mRNA for elongation factor 1a,) and *ATP5A1* (gene controlling ATP synthesis). *ATP5A1* is involved in regulation of energetic status of the cell (Yotov and St-Arnaud., 1993). Higher expression of this gene contributes to more intensive metabolism whereas mutations cause embryonic death (Pedersen, 1994). Elevated levels of the *ATP5A1* transcript have been noted in bovine BCB^- oocytes (Torner et al., 2008), which was related to increased metabolic activity of growing gametes. The *EEF1A1* gene regulates protein synthesis (Moldave, 1985) and stimulates blastocyst development in cattle (Goossens et al., 2007). *In vitro*-produced bovine blastocysts contained significantly less transcripts of this gene when compared to their *in vivo*-derived counterparts, which usually display better quality. A positive correlation between *EEF1A1* transcript level and oocyte quality was also demonstrated in bovine BCB^+ oocytes, which were characterized by a higher RA compared to BCB^- oocytes (Torner et al., 2008).

The present study was focused on analysing the fatty acid profile in FF collected from gilt ovaries. Experimental animals were fed a diet rich in components of local origin that usually predominate in a traditional porcine diet of the Wielkopolska region (Greater Poland). The concentrate used for gilt fattening mostly composed of barley (64%) and rye (25%). Additionally, the experimental diet was characterized by elevated levels of n-3 fatty acids which are known to affect reproductive function in cattle (Fouladi-Nashta et al., 2007), mice (Wakefield et al., 2008) and humans (Abayasekara and Wathes, 1999; Wathes et al., 2007).

The aim of the present study was to investigate whether diet composition may be correlated with: (1) selected markers related to developmental potential of oocytes (BCB test and relative transcript abundance of the *EEF1A1*, *ATP5A1* genes) and (2) the fatty acid profile in the follicular fluid. We demonstrated a positive influence of the experimental diet based on local components on the fatty acid profile in follicular fluid as well as gene expression in oocytes. Moreover, higher levels of the *EEF1A1* transcript in BCB^+ oocytes may be considered a marker of oocyte quality in the pig.

MATERIAL AND METHODS

Animals

The animals used in the present study were crossbred gilts of the following genotypes: the paternal line – Camborough 22 and the maternal lines – Norsvin Landrace, 337PIC, Polish Landrace and White Pietrain. Moreover, all the animals were genotyped at the *RYR1* locus and showed no stress syndrome (data not shown). Gilts were randomly

allocated into two groups fed control (CD) and experimental (ED) diets. Animals were housed in typical indoor pens and had *ad libitum* access to food and fresh water.

Diets

The diets were pelleted and formulated to meet the requirements of the finishing pigs (Table 1). The two diets were introduced by the average animal age of 124 days (70 kg body mass) whereas the fattening period was finished by the average age of 192 days (120 kg body mass). The main part of the ED diet comprised locally produced grains, barley (64%) and rye (25%; Table 1). Feed samples were analyzed in three replicates for the dry matter, crude proteins (N × 6.25) and fat, according to the Association of Official Analytical Chemists (2006). The gross energy content of diets was measured using an adiabatic bomb calorimeter (KL-12Mn, Poland) whereas fatty acid composition was analyzed according to the protocol described by Cieslak et al. (2009).

Both diets used in this experiment were isoenergetic (16 MJ/kg), isonitrogenous (around 14.5% crude protein) and isolipidic (about 2.25%, Table 1).

Table 1. Composition of the control and experimental diets (%)

Item	Control	Experimental
Wheat	30.0	–
Barley	25.0	64.0
Molassed sugar beet pulp	11.5	–
Maize grain	20.0	–
Soybean meal	4.5	–
Rapeseed meal	6.0	–
Rye	–	25.0
Dried alfalfa	–	1.0
Rice protein	–	3.5
Potato protein	–	3.5
Premix	3.0	3.0
Analyses		
Dry matter (%)	90.1	90.5
Protein content (%)	14.8	14.4
Fat (%)	2.0	2.5
Crude energy (MJ/kg)	16.2	16.1

The ED diet showed a 2-fold decrease in the content of several fatty acids (C12:0, total C18:1, C18:1 *cis*-9, total C18:1 *cis* as well as total MUFA) compared to the CD diet. The experimental diet also contained higher levels of C14:0, C16:0, C18:3n-3, C20:5n-3 and total n-3, which resulted in a decrease in the n-6/n-3 ratio. With respect to the remaining fatty acids, both diets showed similar levels. A significant increase in total n-3 polyunsaturated fatty acids (PUFA) resulted mainly from a higher proportion of C18:3n-3 in ED containing 20% rye (8.5 g/100 g FA) as well as reduced content of molassed sugar beet when compared to CD. Molassed sugar beet contains a small percentage of ether extract (0.8%), a known source of fatty acids. A higher proportion of PUFA in the ED (Table 2) was due to a lack of components rich in C18:1 *cis*-9 like maize grain (27 g/100 g FA), soya bean meal (14.5 g/100 g FA) and rapeseed meal (52 g/100 g FA) present in the control diet.

Collection of ovarian follicular fluid and cumulus-oocyte complexes

Gilts were slaughtered at 7–8 months of age at approximately 120 kg in a commercial abattoir. Pairs of ovaries per gilt were collected and transported to the laboratory within 4–6 h after slaughter. Follicular fluid was aspirated from healthy, antral follicles (2–5 mm in diameter) from each pair of ovaries and left for 10 min at room temperature for COCs to sediment. Afterwards the pellet was aspirated with a Pasteur pipette and transferred to a Petri dish with Hepes-TALP medium at room temperature. The first step of COC analysis was morphology evaluation. Only COCs with proper morphology (> 3 layers of compact cumulus cells and even ooplasm granulation, no shrinkage) were subjected to a second step of quality assessment by the brilliant cresyl blue (BCB) test. The remaining FF aspirated from follicles of each ovary pair was centrifuged (30 min, 12 000 × g) and the clear supernatant was frozen at –80 °C for fatty acid analysis.

Evaluation of COCs by the brilliant cresyl blue test

Selected COCs with a proper morphology were initially washed twice in BCB solution (concentra-

Table 2. The fatty acid composition (percentage of total fatty acids) of control and experimental diets

Fatty acids	Control	Experimental
C12:0	0.32 ± 0.10	0.18 ± 0.07
C14:0	0.29 ± 0.09	0.53 ± 0.06
C16:0	15.67 ± 0.65	20.20 ± 0.58
C18:0	1.70 ± 0.52	1.37 ± 0.33
Total C18:1	19.89 ± 1.15	9.83 ± 0.32
C18:1 *cis*-9	17.74 ± 0.91	8.73 ± 0.50
Total C18:1 *trans*	0.34 ± 0.15	0.45 ± 0.14
Total C18:1 *cis*	19.55 ± 1.06	9.59 ± 0.42
C18:2n-6	56.82 ± 1.49	58.09 ± 1.38
C18:3n-3	4.54 ± 0.73	8.84 ± 0.90
C20:5n-3	0.26 ± 0.06	0.41 ± 0.14
SFA	18.22 ± 1.25	22.59 ± 0.78
UFA	81.78 ± 1.25	77.41 ± 0.78
MUFA	20.16 ± 1.11	10.07 ± 0.35
PUFA	61.62 ± 0.84	67.34 ± 0.83
n-3	4.80 ± 0.76	9.24 ± 1.02
n-6	56.82 ± 1.49	58.09 ± 1.38
n-6/n-3 ratio	11.8	6.3

SFA = saturated fatty acids (C12:0, C14:0, C15:0, C16:0, C18:0, C20:0, C22:0)

UFA = unsaturated fatty acids (C16:1, C18:1 *trans*-9, C18:1 *trans*-10–11, C18:1 *trans*-13–14, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-11, C20:1 *cis*-9, C18:2n-6, C18:3n-3, C20:3n-3, C20:5n-3)

MUFA = monounsaturated fatty acids (C16:1, C18:1 *trans*-9, C18:1 *trans*-10–11, C18:1 *trans*-13–14, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-11, C20:1 *cis*-9)

PUFA = polyunsaturated fatty acids (C18:2n-6, C18:3n-3, C20:5n-3); n-6 (C18:2n-6), n-3 (C18:3n-3, C20:5n-3)

tion 13μM) and then incubated for 90 min (39 °C, humidified 5% CO_2 atmosphere) (El Shourbagy et al., 2006). Following exposure to BCB, COCs were transferred to PBS supplemented with 0.4% BSA and washed twice. After washing, COCs were examined under a stereomicroscope and divided into two groups: COCs with ooplasm stained blue were classified as BCB positive (BCB^+) whereas COCs with a colourless ooplasm were classified as BCB negative (BCB^-). Afterwards cumulus cells were removed after 3–5 min incubation in 0.025% hyaluronidase (0.01 IU/100 ml) followed by vigorous pipetting.

At this point of the experiment it was decided to pool oocytes collected from different gilts within the experimental and control groups due to a variable number of oocytes derived from the ovarian pairs (ranged from 3 to 156 COCs) in order to have an equal number of oocytes per sample. A uniform number of cells per experimental sample was necessary to minimize the variation observed during mRNA extraction, reverse transcription and PCR amplification. Denuded oocytes were subsequently pooled (50 COCs per sample) and frozen in liquid nitrogen. Oocytes were pooled in groups according to two factors: (1) their BCB category (BCB^+ and BCB^-) and (2) diet composition (ED, CD). Four groups of oocytes were subjected to transcript analysis: ED BCB^+, ED BCB^-, CD BCB^+ and CD BCB^-.

Relative transcript abundance of the two marker genes (*ATP5A1*, *EEF1A1*)

A modified protocol described by Madeja et al. (2009) was applied. RNA extraction was carried out using the GenEluteTM Mammalian Total RNA kit (Sigma) according to the manufacturer's instructions. During sample incubation in the lysis solution, 1 μl (0.8 μg) of MS2 RNA was added (external standard sequence, Roche Diagnostics). Reverse transcription of 8 μl of mRNA was done using Transcriptor High Fidelity kit (Roche) according to the manufacturer's instructions. The final volume of each cDNA sample was 20 μl. The quantification of two gene transcripts was carried out by real-time semiquantitative RT-PCR assay. Transcripts of each gene were analyzed in 3–5 replicates (replicate = cDNA derived from one pool of 50 oocytes). In order to limit the variation caused by manual errors, each replicate was amplified twice. Real time PCR was performed using the LightCycler 2.0 Instrument (Roche Diagnostics GmbH, Germany) and hybridization probes. All primers and probes were designed and synthesized by TIB MolBiol (Germany; Table 3). The PCR reaction mixture (20 μl) consisted of $MgCl_2$ (3mM), primers (0.5μM each), probes (0.3μM each), LightCycler® FastStart DNA Master HybProbe (1×), cDNA (2 μl) and water. The cDNA used as a template in each PCR reaction was derived from approximately five oocytes. The PCR protocol included an initial step of cDNA denaturation at 95 °C (10 min), followed by 45 cycles of 95 °C (10 s), 55 °C (12 s) and 72 °C(12 s).

Table 3. Sequences of primers and probes used for real time PCR

Primers	Sequences	GenBank accession No.	Product size (bp)
*ATP5A1*_F	AGTTGCTGAAGCAAGGACAGTAT	this study	147
*ATP5A1*_R	GTGTTGGCTGATAACGTGAGAC		
*EEF1A1*_F	TGGCTTTACAGCTCAGGTGATTA	this study	144
*EEF1A1*_R	CTTTTTCCCAGAACGACGA		
MS2_F	CGGAGTGTTTACAGTTCCGAA	NC_001417	209
MS2_R	GACGATAAGTCTATCGTCGCAA		
Probes			
ATP5A1	5'-TCCAGCTTATCAAGATAGCCCCTG-FL 5'-CACCCGCATAGATAACAGCTACTTGCT-PH	this study	–
EEF1A1	5'-AATGTGAGCTGTGTGGCAATCC-FL 5'-CACAGGTGCGTAACCAGCACTG-PH	this study	–
MS2	5'-CCGACCCCTTTCTGGAGGTACATA-FL 5'-TCATATCAGGCTCCTTACAGGCAGC-PH	NC_001417	–

The method used for transcript quantification was the relative standard curve method. Standard curves were constructed for each individual gene, using ten-fold serial dilutions of corresponding PCR products of known concentration. Transcript abundance for each gene was calculated by software based on the standard curve normalized according to the relative concentration of the external MS2 standard.

Fatty acid profile in follicular fluid (gas chromatography)

Fatty acid composition was analyzed according to the protocol published by Cieslak et al. (2009) with some modifications. Sample hydrolysis was carried out in a closed system using screw-cap Tefl on-stoppered tubes (Pyrex, 15 ml). To 300–400 mg of follicular fluid 3 ml of 2M NaOH was added followed by incubation in a block heater at 90 °C for 40 min. Afterwards samples were cooled to room temperature and 2 ml of 4M HCl were added to bring the pH below 2. After hydrolysis, 1 ml of freshly prepared internal standard (IS, Pentadecanoic acid; C15:0, Fluka) and 2 ml of distilled diethyl ether were added. Closed tubes were vortexed vigorously for 10 min and centrifuged at 20 °C for 1 min (6160 g). The upper organic phase was transferred with a Pasteur pipette into a fresh Pyrex tube. The extraction procedure was repeated 3× per sample and the supernatant was evaporated at 30 °C for 10 min under a flux of nitrogen. The remaining residue was used for derivatization. The extracted fatty acids were esterified and converted into fatty acid methyl esters (FAME). Two ml of 0.5M NaOH in methanol was added to each sample and boiled for 3 min. Then 3 ml of borontrifluoride (Fluka) was added to the sample and boiled for 4 min. After cooling, 7 ml of 0.34M NaCl solution and 1 ml of hexane were added, vortexed and centrifuged (6160 g). A visible organic phase containing the FAME was transferred into glass vials and used for gas chromatographic analyses in a Varian Chromapack, CP-3380 gas chromatograph equipped with a flame ionisation detector and Chrompac CP-Sil 88 column (100 m, 0.25 mm, 0.2 μm film thickness, Varian). Helium was used as the carrier gas at a constant flow of 30.0 ml/min. The oven temperature was programmed as follows: initially 175 °C for 25 min, then increasing at 5 °C/min to 235 °C. 2 μl samples were injected in a splitless mode. Hexan was injected prior to each measurement to prevent carry-over contamination. Observed peaks were identified by comparison of retention times with appropriate fatty acid methyl ester standards (37 FAME Mix, Supelco, Poole, England) using the Varian Workstation (Version 5.31).

Statistical analysis

All the data were pooled and subjected to statistical analysis. The following parameters were compared:

(1) Association between diet composition and distribution of BCB^+ oocytes as well as the amount

of COCs with proper morphology was tested with the chi-square test,

(2) Fatty acid composition of follicular fluid in relation to diet composition - data were tested with the GLM procedure of SAS, according to the following model:

$$Yi = \mu + Di + ei$$

where:

Yi = response variable

μ = overall mean

Di = fixed effect of dietary treatment

ei = random residual error

Multiple comparisons of least squares means among dietary treatments were conducted using Scheff's test.

(3) Transcript abundance of analyzed genes in relation to both experimental factors (BCB test and diet composition) – Mann-Whitney Rank Sum Test.

Differences of $P < 0.05$ were considered significant. All statistical analyses were carried out using the SAS (9.1.) software package.

RESULTS

Diet composition and morphology of cumulus-oocyte complexes

Altogether 71 gilts were included in the experiment (CD 35, ED 36). The average number of COCs with proper morphology collected from a pair of ovaries equalled 50, whereas that of BCB^+ COCs was almost 40. Moreover, the majority (80.8%) of all analyzed COCs (CD + ED) were BCB positive. No effect of diet composition on the mean number of COCs with proper morphology and BCB^+ oocytes was observed (Table 4).

Fatty acid composition of follicular fluid in relation to diet composition

The FA composition of FF derived from ovaries of each gilt was analyzed in two replicates. The total fatty acid content was similar in FF samples from CD gilts (500.14 μg/ml ± 194.6) and ED gilts (501.51 μg per ml ± 160.6). Analysis of FF by gas chromatography revealed a significant influence of diet on fatty acid content. Saturated fatty acid (SFA) content was higher in the ED group, whereas unsaturated fatty acids (UFA) and PUFA were more abundant in the CD group ($P < 0.05$). When individual fatty acids were considered, only C16:0 (palmitic acid), C18:2n-6 (linoleic acid) and C22:6 (docosahexaenoic acid, DHA) differed significantly. Palmitic acid and DHA were more abundant in the ED group whereas there was more linoleic acid in the CD group. The content of the remaining fatty acids was similar in both groups of gilts (Table 5).

Relative transcript abundance of the *ATP5A1* and *EEF1A1* genes

RNA isolation was performed on 1653 oocytes that had been frozen in liquid nitrogen. Some of the cDNA samples were used for procedure optimization, whereas 16 samples were successfully analyzed: eight pools of oocytes originating from ED and CD gilts (5 BCB^+ and 3 BCB^-, respectively). Transcript levels of the external control sequence MS2 were found to be constant in all samples (data not shown).

The mRNA levels of the *ATP5A1* gene were influenced neither by diet composition nor by oocyte category (BCB^+ /BCB^-) whereas that of the *EEF1A1* was affected by both factors. BCB^+ oocytes contained more mRNA for the *EEF1A1* gene than BCB^- gametes ($P < 0.05$). Oocytes from ED gilts

Table 4. Distribution of gilts and COCs in relation to diet composition

Trait	CD		ED	
	n	%	*n*	%
Number of gilts	35	–	36	–
Total number of healthy COCs	1800	100	1675	100
Mean number of healthy COCs	54.5 ± 6.87	–	46.5 ± 5.79	–
BCB^+ COCs	1440	80	1366	81.6
Mean number of BCB^+ COCs	37.9 ± 4.35	–	43.6 ± 5.54	–

CD = control diet, ED = experimental diet, healthy COC = with a proper morphology

Table 5. Fatty acid composition (percentage of total fatty acids) of follicular fluid collected from the ovaries of peri-pubertal gilts fed control (CD) and experimental (ED) diets

	CD	ED
C16:0	**24.73^{b} ± 0.257**	**25.50^{a} ± 0.278**
C16:1	1.28 ± 0.030	1.36 ± 0.032
C18:0	15.50 ± 0.289	15.56 ± 0.313
SFA	**44.82^{b} ± 0.495**	**46.59^{a} ± 0.536**
C18:1 *cis*-9	17.81 ± 0.414	18.00 ± 0.449
Total C18:1 *trans*	1.04 ± 0.059	1.13 ± 0.064
Total C18:1 *cis*	20.06 ± 0.441	20.13 ± 0.471
Total C18:1	21.10 ± 0.435	21.26 ± 0.471
MUFA	23.97 ± 0.445	24.38 ± 0.482
UFA	**55.18^{a} ± 0.495**	**53.41^{b} ± 0.535**
C18:2 *cis*-9 *cis*-12	**16.77^{a} ± 0.354**	**14.10^{b} ± 0.383**
C18:3n-6	0.27 ± 0.008	0.26 ± 0.008
C18:3n-3	0.95 ± 0.110	1.19 ± 0.119
C20:4n-6	10.80 ± 0.254	10.29 ± 0.276
C20:5n-3	0.12 ± 0.024	0.16 ± 0.026
C22:6n-3	**0.85^{b} ± 0.048**	**1.08^{a} ± 0.052**
PUFA	**31.28^{a} ± 0.355**	**29.11^{b} ± 0.388**
n-6	23.89 ± 0.750	21.74 ± 0.812
n-3	7.09 ± 0.647	6.62 ± 0.700

SFA = saturated fatty acids (C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0)
MUFA = monounsaturated fatty acids (C16:1, C18:1 *cis*-9, C18:1 *cis*-11)
UFA = unsaturated fatty acids (C16:1, C18:1 *trans*, C18:1 *cis*-9, C18:1 *cis*-11, C18:2n-6, C18:3n-3, C20:3n-3, C20:4n-6, C20:5n-3, C22:6n-3)
PUFA = polyunsaturated fatty acids (C18:2n-6, C18:3n-3, C20:3n-3, C20:4n-6, C20:5n-3, C22:6n-3); n-6 (C18:2n-6, C20:4n-6), n-3 (C18:3n-3, C20:3n-3, C20:5n-3, C20:5n-3, C22:6n-3)
Data shown as LSmean ± SEM; statistical significance: $P < 0.05$

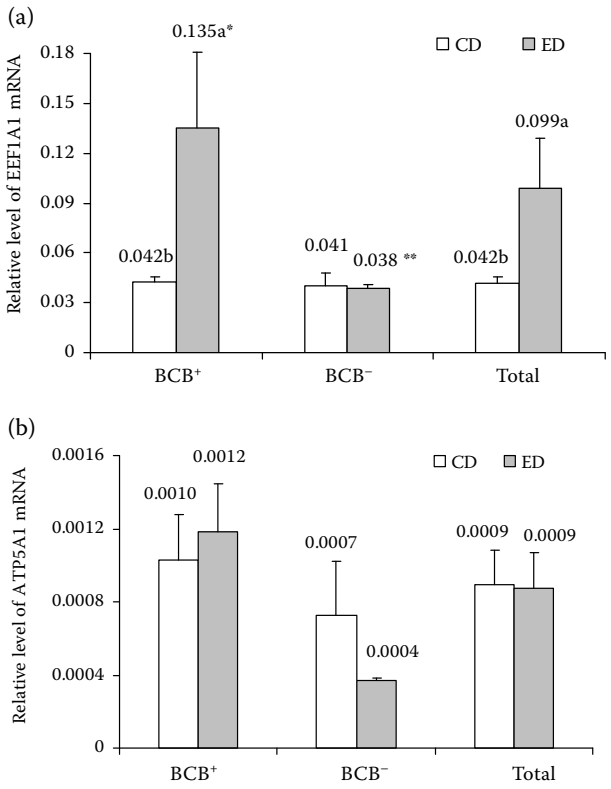

Figure 1. Relative abundance of *ATP5A1* and *EEF1A1* mRNA in pig oocytes (data shown as LSmean ± SEM; control diet = white columns, experimental diet = gray columns; statistical significance $P < 0.05$; RA values for *EEF1A1* gene differ significantly within BCB^{+} or total groups (marked with a, b) as well as ED samples from BCB^{+} and BCB^{-} groups (marked with stars)

also showed higher RA values for this gene compared with CD gilts ($P < 0.05$). When both factors (BCB test and diet) were considered, higher levels of the *EEF1A1* transcript were observed in oocytes from the experimental BCB^{+} group versus the control BCB^{+} group ($P < 0.05$). As far as BCB^{-} oocytes are concerned, the RA of the *EEF1A1* gene was not influenced by diet. All categories of CD oocytes (BCB^{+}, BCB^{-}, total) had similar RAs for the *EEF1A1* gene, whereas the RA value for ED oocytes differed significantly with the highest level in BCB^{+} cells (Figure 1a, b).

DISCUSSION

The present study was focused on the interactions between diet composition and some parameters related to the quality of porcine oocytes. Several articles have been published on the effect of nutrition on superovulation in pigs (Kojima et al., 1997) and cows (Boland et al., 2001; Fouladi-Nashta et al., 2007; Cerri et al., 2009). However, only a few studies, which have reported contradictory results, were focused on the evaluation of oocyte quality in relation to diet composition. The experimental diet (ED) used in this study was mainly comprised of locally produced grains (barley and rye)

traditionally used to fatten pigs in this region. We have shown that ED rich in n-3 fatty acid (FA) significantly influenced fatty acid composition of the follicular fluid (FF) and transcriptional abundance of the *EEF1A1* marker gene. The FA profile of follicular fluid from ED gilts differed from that of the control females. Moreover, oocytes from ED gilts had higher mRNA levels of the *EEF1A1* gene, previously described as a marker of oocyte competency in cattle.

Fatty acid composition of the diets

A growing body of evidence suggests that diet supplementation with particular fatty acids may influence reproductive functions (e.g. Abayasekara et al., 1999; Fouladi-Nashta et al., 2007; Wathes et al., 2007). It is worth mentioning that the FA profile of the ED used in this study was modified by a change in its composition and not by the addition of commercial supplements. Significant differences in the amount of selected fatty acids between ED and CD were observed. The most interesting finding is a 2-fold elevation of total n-3 FA in the ED, which caused a 2-fold reduction in the n-6/n-3 proportion. It has been shown that n-3 PUFAs (particularly C20:5n-3 and C22:6n-3) are involved in the regulation of reproductive processes through a variety of mechanisms linked to oocyte and embryo quality (Wakefield et al., 2008). In this experiment elevation of n-3 PUFA in ED reflected higher levels of C20:5n-3 and C18:3n-3 (α-linolenic acid). It is questionable whether α-linolenic acid exerts a positive effect on reproduction via conversion to C20:5n-3 or C22:6n-3 since these two FAs are known prostaglandin precursors. This hypothesis may be supported by Marei et al. (2009) who showed that the addition of C18:3n-3 stimulated meiotic maturation of bovine oocytes and improved subsequent embryo development. This effect was mediated directly through the MAPK pathway and indirectly through PGE2 synthesis. The elevation in C22:6n-3 noted in this study could have resulted from higher levels of α-linolenic acid in ED.

Another interesting point is the analysis of total n-3 and n-6 content in the diets. Pike and Barlow (2000) suggested that monitoring the n-3 level is not as important as the n-6/n-3 ratio. Our experimental diet contained 2-times more n-3 FA and a constant level of n-6 in relation to CD which caused a decrease in the n-6/n-3 proportion. This suggests a positive effect of ED on oocyte quality.

FA composition of FF in relation to the diet and its influence on oocyte quality

The evidence concerning a possible correlation between FA profile in blood plasma, follicular fluid or follicular cells remains controversial. There exists a hypothesis describing an axis "diet – serum – FF – oocyte" which suggests a flow of nutrients from the digestive tract, through serum to follicular fluid and further via cumulus cells to the oocyte. However uptake of follicular PUFAs by the oocyte may be selective and therefore may limit the impact of diet on FA profile in oocytes (Santos et al., 2008). Wakefield et al. (2008) observed an increase in PUFAs in plasma and ovaries in response to elevated n-3 PUFA concentration in diet. On the other hand, some reports show a lack of correlation between FA content in plasma and FF of dairy cows (Leroy et al., 2005; Renaville et al., 2010) and ewes (Wonnacott et al., 2010).

In the present study the fatty acid profile in follicular fluid was affected by the diet. Differences were found in total SFA, UFA and PUFA as well as in particular fatty acids (C16:0, C18:2n-6 and C22:6n-6). The most interesting data in our opinion concerns C16:0, which was more abundant in the FF of the ED group. According to many authors, C16:0 is the most abundant FA in pig oocytes (Sturmey et al., 2009) which suggests a special function during oocyte growth and maturation. The published data are, however, quite inconsistent and mostly focused on ruminants. Renaville et al. (2010) observed a positive correlation between developmental potential of bovine oocytes and higher content of C16:0 in the FF of active follicles (oestrogen to progesterone ratio – E2/P4 > 1). Homa and Brown (1992) reported no significant differences (except for C18:2) in FA composition between small (1–3 mm diameter) and large (7–13 mm) follicles. In contrast, the higher level of C16:0 in FF has been attributed to reduced fertility (Zeron et al., 2001), lower COC quality (Sinclair et al., 2008) and decreased cryotolerance of embryos (Shehab-El-Deen et al., 2009). Also, a negative effect of supplementation of IVM medium with C16:0 on oocyte maturation, fertilization, cleavage and blastocyst rates was reported (Leroy et al., 2005). To our knowledge there is no published data dealing with the effect of C16:0 in

the FF on oocyte/embryo quality in the pig. Our experiment showed higher concentrations of C16:0 in the FF of ED gilts. Since other analyzed parameters demonstrated an advantage of the experimental diet over the control, it may indirectly support a hypothesis regarding the positive effect of C16:0 in FF on the quality of porcine oocytes. Although this suggestion contradicts evidence published for ruminants, species-related differences cannot be excluded (e.g., total FA content in porcine oocytes is over 2.5 times higher than that in bovine gametes; McEvoy et al., 2000).

Follicular fluid from ED gilts was also characterized by a lower content of PUFAs. Nonogaki et al. (1994) observed an arrest in embryo development after exposure of mouse zygotes to high concentrations of PUFAs (linoleic and α-linolenic acid). Also, oocyte exposure to a maternal environment enriched with n-3 PUFA (diet supplemented with n-3) resulted in altered mitochondrial distribution and calcium levels and increased ROS production (Wakefield et al., 2008). Therefore, lower levels of PUFAs in the follicular fluid collected from ED gilts may provide evidence of the positive influence of the experimental diet on ovarian functions.

Oocyte quality in relation to diet composition

It has been documented that porcine BCB^+ oocytes are larger, more often reach the MII stage and show higher rate of monospermic fertilization (Roca et al., 1998; Wongsrikeao et al., 2006; Ishizaki et al., 2009). Published information on their developmental potential after fertilization is however contradictory (Wongsrikeao et al., 2006; Ishizaki et al., 2009). To our knowledge there is no published data on the interactions between maternal nutrition and the incidence of BCB^+ oocytes. Our experiments showed no effect of diet on the number of competent BCB^+ oocytes. Bilby et al. (2006) also did not observe any effect of a diet supplemented with C18 on the quality of bovine oocytes. In contrast, cows fed a high energy diet were reported to produce more oocytes of proper morphology than did cows fed a low energy diet (Kendrick et al., 1999). Considering this data it is hard to conclude whether dietary modifications are more connected to metabolic pathways in oocytes or to their morphology.

Transcript content of genes related to oocyte quality

Accumulating evidence points to important changes in gene expression pattern in oocytes and embryos in response to nutrition (Wrenzycki et al., 2000, cattle; Pisani et al., 2008, sheep; Wakefield et al., 2008, mouse). In our opinion the intrinsic features of oocytes including elements related to maternal nutrition, can be analyzed only directly after COC recovery from ovarian follicles. It is well documented that *in vitro* maturation significantly affects the profile and quality (e.g., adenylation status) of transcripts in mammalian oocytes (Brevini-Gandolfi et al., 1999; Racedo et al., 2009). Thus, by analyzing the transcripts of *in vitro*-matured oocytes it would not be possible to distinguish between changes induced by maternal nutrition and by the IVM process itself. Therefore, we decided to investigate the relative transcript abundance (RA) of two genes (*ATP5A1* and *EEF1A1*) in immature oocytes. Both genes have previously been described as markers of oocyte quality in cattle. Bovine BCB^- oocytes with reduced quality displayed an elevated RA of the *ATP5A1* gene whereas more competent BCB^+ oocytes were characterized by a higher transcript level of the *EEF1A1* gene (Torner et al., 2008). In our experiments the RA of the *ATP5A1* gene was neither related to BCB category nor to the nutritional regime. According to Torner et al. (2008) this may reflect either the lack of difference in energetic status of oocytes in both groups or some species-specific effects. It is also possible that expression of the *ATP5A1* gene which regulates energy production is affected by environmental and genetic factors.

Similarly to bovine oocytes analyzed by Torner et al. (2008), porcine BCB^+ oocytes contained more *EEF1A1* mRNA than the BCB^- oocytes. Transcript abundance of this gene was moreover influenced by the diet. Control BCB^+ and BCB^- oocytes as well as experimental BCB^- oocytes showed similar RA values whereas experimental BCB^+ oocytes contained significantly more *EEF1A1* mRNA. It has been shown previously that murine BCB^+ oocytes exhibit a variety of sizes and that their quality is affected by puberty of the female and by gonadotropin stimulation (Wu et al., 2007). Our results indicated that porcine BCB^+ oocyte sub-population was not homogenous and differed in transcript levels of the *EEF1A1* gene and that this was affected by composition of the diet. Oocytes of ED females

were also characterized by a higher RA of this gene. This phenomenon might result from a direct modification of the *EEF1A1* transcript level by the diet or its influence on G6PDH activity.

In summary, the present study showed that feeding the experimental diet characterized by higher levels of n-3 (particularly C18:3n-3) as well as a decreased n-6/n-3 ratio resulted in elevated SFA, UFA, PUFA and some particular FAs (e.g. C16:0) in the follicular fluid of gilts. These factors had further impact on oocyte quality as revealed by the higher RA of *EEF1A1* mRNA. Moreover, the C16:0 concentration in FF may have a beneficiary effect on oocyte quality, which is in contrast to previous data for bovine oocytes. We suggest that diets composed of locally produced grains (barley and rye) may positively influence some reproductive parameters of fattening gilts.

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