Animal 16 (2022) 100540

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

# Effect of dietary protein and energy intake on embryonic survival and gene expression in the uterine endometrium of early pregnant gilts

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ARTICLE INFO

*Article history:*
Received 17 December 2021
Revised 13 April 2022
Accepted 14 April 2022

*Keywords:*
Embryo mortality
Nutrient transporter
Nutrition level
Pigs
Progesterone receptor

ABSTRACT

Porcine embryonic loss during early gestation is a serious problem in swine production. Improving embryonic survival can be achieved by maternal manipulation. Protein and energy are two major components of the diet, which play decisive roles in embryonic survival. This study was performed to evaluate the effects of enhancing maternal protein or energy intake on embryonic survival during early gestation in gilts and to explore the underlying mechanism. From day (**d**) 0 to 30 of gestation, 40 gilts (Landrace × York) were randomly allocated to 5 diets according to daily intake of low (**L**, National Research Council (**NRC**) recommendation for gestation gilts), medium (**M**, 20% higher than NRC) or high (**H**, 40% higher than NRC) CP or metabolisable energy (**ME**) ($L_{CP}L_{ME}$, $M_{CP}L_{ME}$, $H_{CP}L_{ME}$, $L_{CP}H_{ME}$, $H_{CP}H_{ME}$). Gilts were sacrificed on d 30 of gestation, and number of foetuses and corpora lutea, embryonic survival rate, uterine weight, and total volume of allantoic fluid were recorded or calculated. Gene expression was determined by Quantitative Real-time PCR (**qPCR**), western blot or immunohistochemistry. Results showed that increasing protein or ME intake significantly increased embryonic survival rate. Compared with diet $L_{CP}L_{ME}$, plasma progesterone (**P4**) concentration in diet $L_{CP}H_{ME}$ increased at d 14 and d 30 of gestation. Progesterone receptor (**PGR**) was found not to be expressed in the epithelia but was strongly expressed in the stroma of the endometrium. Increasing protein or ME intake did not alter PGR expression in the endometrium. There was also no change in the amount of P4, hepatocyte growth factor, and fibroblast growth factor-7 in the endometrium. The mRNA abundance of cationic amino acid transporter 1 in the endometrium in diet $L_{CP}H_{ME}$ and $H_{CP}H_{ME}$ was significantly lower than in diet $L_{CP}L_{ME}$. Diet $H_{CP}L_{ME}$ showed a tendency to increase neutral amino acid transporter 1 mRNA expression in the endometrium compared to diet $L_{CP}L_{ME}$ ($P = 0.087$). In conclusion, increasing maternal protein or ME intake had a positive effect on the embryonic survival. Increased protein intake by 20 or 40% did not alter plasma P4 level, but increasing ME intake by 40% improved plasma P4 concentration at d 14 and 30 of gestation. Increasing maternal protein or ME intake did not induce PGR expression in the endometrium. Maternal protein and energy intake likely mediate transportation of cationic and neutral amino acids from mother to foetus to affect embryonic survival and development.



## Implications

The plane of nutrition can have an effect on embryonic survival. We found that increasing maternal protein intake by 20 or 40%, or metabolisable energy intake by 40%, has a positive effect on embryonic survival rate, however, maternal progesterone concentration in plasma did not decrease. These results challenged the dogma that high feeding levels increase embryonic loss in early pregnant sows. Both maternal protein and energy intake play roles in regulating embryonic survival. This research provides the basis for developing new feeding strategies for early gestation sows to improve embryonic survival and sow's welfare.

## Introduction

During the first month of gestation, the porcine conceptus undergoes dramatic morphological changes in the process of implantation and secrets oestrogen to signal the establishment of pregnancy (Geisert and Schmitt, 2002). Failure of conceptus implantation and pregnancy establishment leads to early embryonic loss. It was estimated that 20–30% of the embryonic losses occurred at this stage of pregnancy causing a major challenge for

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https://doi.org/10.1016/j.animal.2022.100540
1751-7311/

modern highly prolific sows (Spencer, 2013). Early studies reported adverse effects of increased feeding level on embryonic survival (Jindal et al., 1996), resulting in restricted feeding as a common practice for sows at early pregnancy. However, this feeding practice erodes sow's welfare and reproductive longevity. Thus, increasing numbers of studies have challenged this feeding practice, finding that an increased feeding level has no or even a positive effect on embryonic survival (Hoving et al., 2011; 2012). Hence, the knowledge on the effect of feeding level on embryonic survival remains inconsistent.

Progesterone (**P4**) is a critical steroid hormone for the establishment and maintenance of pregnancy that can act as a mediator of nutritional effects on embryonic survival (Wu et al., 2009). P4 mediates a series of biological processes via the progesterone receptor (**PGR**). Previous studies indicated that increasing the feed level reduced systemic concentration of P4 in sows (Jindal et al., 1996), suggesting that P4 signalling may mediate the effects of feeding levels on embryonic survival. P4 stimulates the synthesis and secretion of the histotroph (a mixture of amino acid, mitogens, cytokines, enzymes and growth factors) in the endometrium, which can directly have effects on embryonic survival, and development and growth of conceptus throughout the pregnancy (Spencer and Bazer, 2004). Among the different components of the histotroph, glucose and amino acids are the major nutrients essential for foetuses. Glucose can enhance the growth and proliferation of the trophoblast cell by activating the mTOR signalling pathway (Wen et al., 2005). Amino acids are necessary for the intrauterine growth of the conceptus (embryo/foetus and placenta) as building blocks of proteins in cells. Glucose and amino acids are transported into the uterine lumen through specific transmembrane transporters. Therefore, the expression of glucose and amino acid transporters in the endometrium is critical for the viability of conception. It has been reported that the expression of nutrient transporters can be affected by PGR (Bazer et al. 2012a), and that PGR abundance can be affected by maternal nutrition (Grazul-Bilska et al., 2018). Protein and energy are two major components in the diet; however, it is still not known if they play a role in P4 function and embryonic survival.

To investigate the roles of dietary levels of protein and energy on embryonic survival and the underlying mechanism, gilts in our study were fed with enhanced protein or metabolisable energy (**ME**) in comparison to the National Research Council (**NRC**) recommendation to assess the effects on embryonic survival, plasma P4 levels, as well as the expression and localisation of PGR and nutrient receptors in the endometrium of gilts during early pregnancy.

## Material and methods

### *Animals and experimental design*

Forty gilts (Landrace × York crossed) of similar age (8-month-old) were used. Before breeding, gilts were group-housed in a breeding facility with a totally controlled environment. Gilts had ad libitum access to a corn-and soybean meal-based gilt diet containing 3100 kcal/kg of ME and 14% CP, and had free access to water. Gilts were checked twice a day for oestrus using a mature boar. At 12 and 24 hours after the onset of 2nd oestrus, all gilts were artificially inseminated twice with fresh-pooled semen. The day of the first insemination was considered day (**d**) 0 of gestation. The BW of gilts was 150 ± 4.52 kg on d 0. Two gilts were not pregnant during the trial, one was in diet $M_{CP}L_{ME}$ and the other in diet $H_{CP}H_{ME}$. Immediately after breeding, gilts were assigned randomly to five dietary regimes according to daily intake of low (**L**), medium (**M**) or high (**H**) CP and metabolisable energy (ME). The control group was defined as $L_{CP}L_{ME}$, in which the protein intake per day (280 g/d) was based on the NRC recommendations (2012) and ME intake per day (6450 kcal/d) is 1.5 fold of the ME necessary for maintenance (**MEm**). The value of MEm used in the gestation period is 100 kcal ME/kg $BW^{0.75}$. The gilts in this group were fed 2.0 kg/d of diet A. The gilts in other groups were fed with increased protein intake by 20 and 40% or increased ME by 40% through adjusting diet CP levels and feeding levels as follows: $M_{CP}L_{ME}$ – middle protein (340 g/d), low energy (6450 kcal/d), fed 2.0 kg/d of diet B; $H_{CP}L_{ME}$ – high protein (400 g/d), low energy (6450 kcal/d), fed 2.0 kg/d of diet C; $L_{CP}H_{ME}$ – low protein (280 g/d), high energy (9030 kcal/d), fed 2.8 kg/d of diet D; $H_{CP}H_{ME}$ – high protein (392 g/d), high energy (9030 kcal/d), fed 2.8 kg/d of diet A. The composition and nutrient levels of the diets are shown in Table 1. The levels of standardised ileal digestible lysine, methionine, threonine, and tryptophan in the control diet (diet A) were met NRC (2012) recommended amino acid requirements for gestating gilts. Gilts were housed in individual feeding stalls in a breeding facility with a controlled environment and had free access to drinking water.

### *Sample collection*

On d 3, 14, and 30 of gestation, blood samples were collected from the ear vein of gilts into heparin sodium vacutainer tubes and centrifuged at 4 000*g* for 20 min. Plasma was stored at −20 °C until analysis. On d 30 of gestation, gilts were slaughtered at a local abattoir to obtain the uterus and the corpora lutea (**CL**) from the ovaria. Uterine weight, number of CL, foetuses, and viable foetuses, and volume of allantoic fluid (**ALF**) were measured and recorded. The embryonic survival rate was calculated as the number of total viable foetuses divided by the number of total CL as used in previous studies (Jindal et al., 1997; Quesnel et al., 2010). Embryos were considered as being viable or dead on the basis of their appearance within the amnion. The bloody embryo within the amnion was considered as a dead foetus (Jindal et al., 1996; Virolainen et al., 2004). Allantoic fluid (10 mL) from the first foetus of the left uterine was collected to measure the amount of fructose and glucose. The endometrium was separated from the myometrium (**MYO**) using curved scissors and samples of the endometrium from the first foetus of the left uterine were collected and either snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde for 36 h and further stored in 70% ethanol. All snap-frozen samples were stored at −80 °C until further analysis.

### *Hormone analysis*

The plasma concentrations of P4 and estradiol (**E2**) were measured by competitive radioimmunoassay (**RIA**) by the Beijing North Biotechnology Company. Tissues (100 mg) were treated with 1.0 mL ethanol and exposed to ultrasonic vibrations for 20 min. The mixture was centrifuged at 3 000 g for 20 min, and the supernatant solution was collected. In short, radiolabelled and non-labelled target antigen are simultaneously applied with a restricted amount of specific antibodies. By separating the unbound labelled antigen, the radioactive count of the labelled antigen–antibody complex is determined. The target molecule is quantified by use of a standard curve and RIA mathematical model. The coefficient of variation was lower than 10% intra-assay and lower than 15% inter-assay for both P4 and E2.

### *Fructose, glucose and growth factors*

Fructose and glucose in ALF were measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The concentration of fructose and glucose is measured by the UV-method and glucose oxidase

**Table 1**
Composition of experimental diets for gilts (as fed basis).

| Items | A | B | C | D |
|---|---|---|---|---|
| Ingredients, % | | | | |
| Corn | 67.71 | 59.00 | 54.30 | 88.70 |
| Soybean meal | 15.00 | 23.60 | 23.60 | 6.20 |
| Soy protein concentrate | 0.00 | 0.00 | 5.10 | 0.00 |
| Wheat bran | 5.00 | 5.00 | 5.00 | 0.00 |
| Rice bran | 6.00 | 6.00 | 6.00 | 0.00 |
| Limestone | 1.10 | 1.05 | 1.00 | 1.10 |
| Calcium phosphate | 0.60 | 0.55 | 0.50 | 0.80 |
| Soybean oil | 1.50 | 1.80 | 1.50 | 0.00 |
| L-lysine sulphate | 0.05 | 0.00 | 0.00 | 0.18 |
| DL-methionine | 0.03 | 0.00 | 0.00 | 0.02 |
| Threonine | 0.01 | 0.00 | 0.00 | 0.00 |
| Vitamin and mineral premix[1] | 3.00 | 3.00 | 3.00 | 3.00 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 |
| Analysed nutrient content, % | | | | |
| DM | 95.71 | 94.91 | 95.16 | 94.79 |
| CP | 13.38 | 16.89 | 19.69 | 11.00 |
| Calcium | 0.68 | 0.66 | 0.73 | 0.77 |
| Phosphorus | 0.52 | 0.54 | 0.57 | 0.42 |
| Lysine | 0.88 | 0.90 | 1.15 | 0.57 |
| Methionine | 0.25 | 0.19 | 0.29 | 0.19 |
| Threonine | 0.76 | 0.70 | 0.93 | 0.47 |
| Calculated nutrient content, % | | | | |
| CP | 14.01 | 17.02 | 20.00 | 10.09 |
| Calcium | 0.63 | 0.63 | 0.63 | 0.63 |
| Phosphorus | 0.54 | 0.56 | 0.59 | 0.41 |
| [2]STTD phosphorus | 0.23 | 0.23 | 0.23 | 0.23 |
| [3]SID Lysine | 0.62 | 0.81 | 1.00 | 0.45 |
| SID Methionine | 0.18 | 0.18 | 0.22 | 0.14 |
| SID Threonine | 0.51 | 0.64 | 0.75 | 0.34 |
| SID Methionine + Cysteine | 0.42 | 0.48 | 0.56 | 0.30 |
| SID Tryptophan | 0.15 | 0.19 | 0.23 | 0.09 |
| Metabolisable energy, Mcal/kg | 3.21 | 3.23 | 3.25 | 3.22 |

[1] Premix supplied per kg of diet: retinyl acetate, 8.07 mg; cholecalciferol, 0.05 mg; D-a-tocopheryl acetate, 63.6 mg; menadione sodium bisulphate, 1.76 mg; choline, 1 106 mg; riboflavin, 8.57 mg; niacin, 65.2 mg; pantothenic acid, 34.6 mg; vitamin B12, 0.04 mg; biotin, 0.23 mg; vitamin B6, 7.93 mg; and thiamine, 4.51 mg; iron, 228 mg; manganese, 45.2 mg; copper, 22.2 mg; selenium, 0.39 mg; cobalt, 0.15 mg; iodine, 0.61 mg.
[2] STTD = Standardised total tract digestibility.
[3] SID = Standardised ileal digestibility.

method, respectively. The optical density (**OD**) value was read at a wavelength of 285 nm for fructose and 505 nm for glucose. Variation coefficients in intra-assay (%) and inter-assay (%) are less than 5% for fructose and glucose. Hepatocyte growth factor (**HGF**) and fibroblast growth factor-7 (**FGF-7**) in the endometrium were measured by an ELISA method including a standard curve using commercial kits (Shanghai MLBIO Biotechnology Co. Ltd, Shanghai, China) according to the manufacturer's instructions. A stop solution was used after the colorimetric reaction, and the OD value was read at a wavelength of 450 nm using a microtiter plate reader within 15 min. Variation coefficients in intra-assay (%) are less than 10% and inter-assay (%) are less than 15% for HGF and FGF-7.

*Quantitative Real-time PCR*

Total RNA from the endometrium was extracted using Trizol reagent, according to the manufacturer's instructions. The Epoch microplate spectrophotometer (Agilent) was used to determine the concentration and quality of RNA. Extracted RNA (1 μg) was converted to single-stranded cDNA using a first-strand synthesis kit following the manufacturer's instructions. The analysis of gene expression was done on an ABI 6 flex real-time PCR instrument using SYBR Green. Primers and their references are shown in Supplementary Table S1. The qPCR reaction system and reaction stages are shown in Supplementary Tables S2 and S3. The relative mRNA expression levels of target genes were calculated using the $2^{-\Delta\Delta CT}$. The expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) was used as an internal control to normalise gene expression.

*Western blotting*

Endometrial tissue (20 mg) was treated with 200 μL RIPA buffer and 2 μL protease inhibitor for 30 min on ice. The cell debris was removed by centrifugation at 12 000*g* for 15 min at 4 °C. Protein concentration was determined by a bicinchoninic acid kit according to the manufacturer's instructions. A mixture of 30 μg of protein and loading buffer was heated at 95 °C for 10 min, with the exception of the protein sodium glucose cotransporter 1 (**SGLT1**) that was heated at 40 °C for 15 min. Total protein was separated on a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis using the Bio-Rad Mini-PROTEAN Tetra electrophoresis system and then transferred onto a polyvinylidene difluoride membrane for 2 h at 0.2 A. After blocking in 5% non-fat milk with Tween 20 buffer for 3 h, the membranes were incubated with primary antibodies at 4 °C for about ten hours. Tris-buffered saline with Tween 20 was used to wash the membranes three times, before incubation at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h. The enhanced chemiluminescence prime reagents were applied to the blot following the manufacturer's recommendations. Protein signals were detected by the ChemiDocMP Imaging System. Protein expression levels were normalised to GAPDH using image analysis

software (J1.v8.0). All antibodies are listed in Supplementary Table S4.

*Immunohistochemistry*

Dewaxed and hydrated tissue sections (5 μm thick) were placed in 0.01 Mol/L citrate buffer (pH 6.0) and boiled in a microwave oven (70% power for 10 minutes) to restore antigenicity. After cooling for more than 30 min, the slides were immersed in 3% $H_2O_2$ hydrogen peroxide methanol for 10 min in a dark place to quench endogenous peroxidase activity. Blocking of sections was done with 10% goat serum at room temperature for 1 h to reduce non-specific binding. Then, sections were incubated overnight at 4 °C with the primary antibody in a humid chamber. See Supplementary Table S4 for an overview of the antibodies used. After washing three times with phosphate-buffered saline (**PBS**), the second antibody was added to samples for 1 h at room temperature. After washing with PBS, DAB was used for colorimetric reaction and haematoxylin was done as counterstaining. The negative control sample underwent the same process, but PBS was used instead of the primary antibody. The images of each slide (10 images) were saved from the digital slides in TIF (Tagged Image File) format using the Olympus U-LHEAD SN 4K80006 and digital camera DP73 (Olympus, Tokyo, Japan). The immunohistochemical images were analysed, and integrated optical density (IOD/Area) was determined using Image J1.v8.0 (Wayne Rasband, National Institutes of Health; https://imagej.nih.gov/ij).

*Statistical analysis*

Data were analysed using GLM procedure of SAS v. 9.2. Diet comparisons were performed using Dunnett's t-test for reproductive performance and endometrium, corpora lutea and allantoic fluid parameters of gilts. The results of plasma parameters were analysed by repeated measures over time using the MIXED procedure. In addition, data on embryonic survival were analysed using the chi-square test. Results are expressed as means with standard error of the mean (**SEM**). Significance was considered when $P < 0.05$, and tendency was considered when $0.05 < P < 0.10$.

## Results

*Reproductive performance*

Compared with diet $L_{CP}L_{ME}$, embryonic survival rate in diet $M_{CP}L_{ME}$, $H_{CP}L_{ME}$, $L_{CP}H_{ME}$ and $H_{CP}H_{ME}$ was significantly increased (Table 2). No differences in uterine weight, number of CL, and ALF volume were detected between diet $L_{CP}L_{ME}$ and the other four diets ($P > 0.05$) (Table 2).

*Hormones, growth factors, fructose and glucose measurements*

Compared with diet $L_{CP}L_{ME}$, gilts in diet $L_{CP}H_{ME}$ tended to have a higher plasma P4 concentration ($P = 0.068$) on d 14 and a significantly higher concentration ($P = 0.047$) on d 30 of gestation (Table 3). Compared with diet $L_{CP}L_{ME}$, plasma E2 concentration in diet $M_{CP}L_{ME}$ showed a tendency to increase ($P = 0.094$) on d 14 and in diet $H_{CP}L_{ME,}$ the concentration was significantly improved on d 30 of gestation ($P = 0.048$) (Table 3). Maternal plasma glucose concentration in diet $H_{CP}L_{ME}$ was significantly lower than in diet $L_{CP}L_{ME}$ on d 30 of gestation ($P = 0.024$) (Table 4). Compared with diet $L_{CP}L_{ME}$, ALF fructose concentration in diet $M_{CP}L_{ME}$ showed a tendency to increase on d 30 of gestation ($P = 0.056$) (Table 4). No differences in total amounts of fructose and glucose in ALF were detected between diet $L_{CP}L_{ME}$ and the other four diets ($P > 0.05$) (Table 4).

*Changes on gene expression and protein levels in the endometrium of gilts on day 30 of pregnancy*

Compared with diet $L_{CP}L_{ME}$, endometrial *PGR* mRNA expression and protein abundance in diet $M_{CP}L_{ME}$, $H_{CP}L_{ME}$, $L_{CP}H_{ME}$ and $H_{CP}H_{ME}$ showed no differences ($P > 0.05$) (Fig. 1). Only progesterone receptor B (**PGRB**) isoform was detected in the endometrium on d 30 of pregnancy. *SGLT1*, $Na^+$-independent sugar transporter 1, 2, 3 (***GLUT1***, ***GLUT2***, ***GLUT3***) mRNA expression did not differ between diet $L_{CP}L_{ME}$ and the other four diets ($P > 0.05$) (Fig. 1). Western blotting showed that SGLT1 protein in the endometrium was not abundant and showed no differences between diet $L_{CP}L_{ME}$ and the other four diets ($P > 0.05$) (Fig. 2). Cationic amino acid transporter 1 (***CAT1***) mRNA expression in diet $L_{CP}H_{ME}$ and $H_{CP}H_{ME}$ was significantly lower than in diet $L_{CP}L_{ME}$ ($P < 0.001$) (Fig. 1). Compared with diet $L_{CP}L_{ME}$, CAT1 protein abundance in $L_{CP}H_{ME}$ showed a tendency to decrease ($P = 0.071$) (Fig. 2). Cationic amino acid transporter 2 (***CAT2***) and cationic amino acid transporter 3 (***CAT3***) mRNA expression and protein abundance did not differ between diet $L_{CP}L_{ME}$ and the other four diets ($P > 0.05$). Neutral amino acid transporter 1 (***SNAT1***) mRNA expression in diet $H_{CP}L_{ME}$ tended to be higher than in diet $L_{CP}L_{ME}$ ($P = 0.087$) (Fig. 1). Expression of SNAT1 and neutral amino acid transporter 2 (**SNAT2**) protein showed no significant differences between diet $L_{CP}L_{ME}$ and the other four diets ($P > 0.05$) (Fig. 2).

*Localisation of receptors and transporters in endometrium by immunohistochemistry of gilts of different nutritional groups*

The antibody used to detect PGR in the present study was generated against both progesterone receptor A (**PGRA**) and PGRB antigens, henceforth referred to as PGR. On d 30 of pregnancy,

**Table 2**
Effect of CP and metabolisable energy levels on reproductive performance of gilts.

| Items | Treatment | | | | | SEM | *P*-value | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $L_{CP}L_{ME}$ | $M_{CP}L_{ME}$ | $H_{CP}L_{ME}$ | $L_{CP}H_{ME}$ | $H_{CP}H_{ME}$ | | ANOVA | $M_{CP}L_{ME}$ vs $L_{CP}L_{ME}$ | $H_{CP}L_{ME}$ vs $L_{CP}L_{ME}$ | $L_{CP}H_{ME}$ vs $L_{CP}L_{ME}$ | $H_{CP}H_{ME}$ vs $L_{CP}L_{ME}$ |
| BW at breeding, kg | 150.6 | 150.5 | 152.0 | 152.0 | 151.1 | 4.78 | 0.999 | 1.000 | 0.999 | 0.999 | 1.000 |
| BW at d 30 of gestation, kg | 159.4 | 158.6 | 158.9 | 167.3 | 166.6 | 4.17 | 0.393 | 1.000 | 1.000 | 0.485 | 0.591 |
| ADG, g/d | 292 | 269 | 231 | 508 | 514 | 73.9 | 0.040 | 0.999 | 0.958 | 0.183 | 0.188 |
| Total ALF volume, L | 3 155 | 2 657 | 3 541 | 3 614 | 3 443 | 275.7 | 0.156 | 0.564 | 0.752 | 0.603 | 0.891 |
| Uterine weight, kg | 5.44 | 4.90 | 5.75 | 5.83 | 5.78 | 0.361 | 0.412 | 0.720 | 0.936 | 0.865 | 0.919 |
| Total foetus, n | 13.8 | 15.0 | 16.7 | 15.4 | 15.7 | 1.06 | 0.422 | 0.818 | 0.188 | 0.652 | 0.526 |
| Live foetus, n | 13.8 | 14.8 | 16.6 | 15.4 | 15.7 | 1.03 | 0.425 | 0.900 | 0.209 | 0.639 | 0.511 |
| CL, n | 20.1 | 18.8 | 20.4 | 17.8 | 18.9 | 1.11 | 0.442 | 0.789 | 0.999 | 0.365 | 0.847 |
| Embryonic survival rate[1], % | 68.32 | 78.67 | 81.12 | 86.62 | 83.33 | - | 0.001 | 0.039 | 0.011 | <0.001 | 0.003 |

Abbreviations: ADG = Average daily gain; ALF = Allantoic fluid; CL = Corpora lutea. ME = Metabolisable energy; L = Low; M = Medium; H = High.
[1] Chi-square test was used.

**Table 3**
Effect of different CP and metabolisable energy levels on plasma parameters of gilts.

| Items | Treatment (Tr) | | | | | Pooled SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|
| | $L_{CP}L_{ME}$ | $M_{CP}L_{ME}$ | $H_{CP}L_{ME}$ | $L_{CP}H_{ME}$ | $H_{CP}H_{ME}$ | | Tr | Time (t) | Tr × t |
| P4 content, ng/mL | | | | | | | | | |
| d3 | 0.68 | 0.44 | 0.41 | 0.52 | 0.33 | 0.33 | 0.114 | <0.0001 | 0.773 |
| d14 | 2.01$^{ab}$ | 2.24$^{ab}$ | 2.26$^{ab}$ | 2.82$^{a}$ | 1.69$^{b}$ | | | | |
| d30 | 1.94$^{b}$ | 2.10$^{ab}$ | 2.03$^{ab}$ | 2.83$^{a}$ | 2.05$^{ab}$ | | | | |
| E2 content, pg/mL | | | | | | | | | |
| d3 | 9.31 | 8.88 | 7.38 | 7.18 | 7.41 | 1.81 | 0.669 | <0.0001 | 0.588 |
| d14 | 6.14 | 6.02 | 9.05 | 6.75 | 5.35 | | | | |
| d30 | 10.49$^{b}$ | 14.55$^{ab}$ | 15.30$^{a}$ | 13.71$^{ab}$ | 14.28$^{ab}$ | | | | |
| Glucose, mg/mL | | | | | | | | | |
| d3 | 0.83 | 0.86 | 0.78 | 0.83 | 0.78 | 0.05 | 0.024 | <0.0001 | 0.344 |
| d14 | 0.82 | 0.86 | 0.77 | 0.82 | 0.78 | | | | |
| d30 | 1.06$^{ab}$ | 1.19$^{a}$ | 0.89$^{c}$ | 1.01$^{bc}$ | 1.11$^{ab}$ | | | | |

Abbreviations: P4 = Progesterone; E2 = Estradiol. d = Day of gestation. ME = Metabolisable energy; L = Low; M = Medium; H = High.
$^{a-c}$Values within a row with different superscripts differ significantly at $P < 0.05$.

**Table 4**
Effect of different CP and metabolisable energy levels on endometrium, corpora lutea and allantoic fluid parameters of gilts.

| Items | Treatment | | | | | SEM | *P*-value | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $L_{CP}L_{ME}$ | $M_{CP}L_{ME}$ | $H_{CP}L_{ME}$ | $L_{CP}H_{ME}$ | $H_{CP}H_{ME}$ | | ANOVA | $M_{CP}L_{ME}$ vs $L_{CP}L_{ME}$ | $H_{CP}L_{ME}$ vs $L_{CP}L_{ME}$ | $L_{CP}H_{ME}$ vs $L_{CP}L_{ME}$ | $H_{CP}H_{ME}$ vs $L_{CP}L_{ME}$ |
| Endometrium | | | | | | | | | | | |
| P4, ng/mg | 5.58 | 8.41 | 6.23 | 5.34 | 5.60 | 1.765 | 0.730 | 0.621 | 0.997 | 1.000 | 1.000 |
| HGF, pg/mg | 1.97 | 2.05 | 1.85 | 2.80 | 2.69 | 0.353 | 0.371 | 1.000 | 0.999 | 0.418 | 0.542 |
| FGF-7, pg/mg | 2.03 | 2.06 | 1.98 | 2.65 | 2.65 | 0.322 | 0.522 | 1.000 | 1.000 | 0.590 | 0.597 |
| CL | | | | | | | | | | | |
| P4, ng/mg | 6.97 | 6.67 | 7.01 | 7.15 | 7.53 | 0.393 | 0.681 | 0.955 | 1.000 | 0.994 | 0.758 |
| ALF | | | | | | | | | | | |
| Fructose, mg/mL | 1.45 | 2.22 | 1.34 | 1.71 | 1.76 | 0.206 | 0.069 | 0.056 | 0.995 | 0.810 | 0.727 |
| Glucose, mg/mL | 0.388 | 0.436 | 0.379 | 0.425 | 0.457 | 0.0330 | 0.422 | 0.696 | 0.996 | 0.847 | 0.433 |
| Total Fructose, mg | 4 391 | 5 520 | 4 795 | 6 356 | 6 062 | 802.9 | 0.393 | 0.737 | 0.991 | 0.258 | 0.423 |
| Total Glucose, mg | 1 210 | 1 087 | 1 332 | 1 541 | 1 596 | 151.1 | 0.129 | 0.947 | 0.949 | 0.345 | 0.248 |

Abbreviations: P4 = Progesterone; HGF = Hepatocyte growth factor; FGF-7 = Fibroblast growth factor-7; CL = Corpora lutea; ALF = Allantoic fluid.
ME = Metabolisable energy; L = Low; M = Medium; H = High.

PGR protein was not detected in the luminal epithelium (**LE**) and glandular epithelium (**GE**), but it was detected in the endometrial stroma (**ST**) (Fig. 3). The intensity of staining in the ST between diet $L_{CP}L_{ME}$ and the other four diets showed no differences ($P > 0.05$) (Supplementary Fig. S1). High expression levels of SGLT1 and CAT1 proteins were detected in the trophectoderm (**Tr**) of conceptus, with less expression in the LE and GE (Fig. 4). The intensity of SGLT1 and CAT1 staining did not differ between diet $L_{CP}L_{ME}$ and the other four diets ($P > 0.05$) (Supplementary Fig. S1).

## Discussion

The novel finding of the present study is that enhancing maternal protein intake by 20 or 40%, or ME intake by 40% in comparison to the NRC recommendation significantly increased the embryonic survival rates in early pregnancy of gilts. Our results not only indicated that increasing the feeding level did not cause a higher embryonic loss but also revealed that both dietary protein and energy play a role in regulating embryonic survival. The results were in line with those of a previous study in which first and second parity sows that were fed a 30% higher feeding level had a significantly higher litter size compared to the control sows (Hoving et al., 2011). These results challenged the dogma that enhanced feeding levels increase embryonic loss in early pregnant sows (Jindal et al., 1996). P4 is a master hormone for the establishment and maintenance of pregnancy. The CL is the main source of P4 during pregnancy in pigs. P4 was considered as a mediator of nutritional effects on embryonic survival in previous studies, in which increasing maternal feeding level led to a lower systemic concentration of P4 and lower embryonic survival rate (Jindal et al., 1996; 1997). Decreased plasma P4 may be caused by a higher P4 clearance rather than secretion as an increasing feed intake accelerated portal blood flow and therefore also the metabolism of P4 by the liver (Virolainen et al., 2004). Plasma P4 increased with the advance of gestation in the present study, reflecting the increasing secretion of P4 with the maturation of CL. Here, we also showed that enhancing maternal ME intake by 40% without changing protein intake increased the plasma levels of P4 on d 14 and 30, however, no change was observed on d 3 of gestation. Our results corroborated those of another study that showed that increasing maternal feed intake did not decrease plasma P4 concentration or embryonic survival in multiparous sows (Virolainen et al., 2005). Thus, ours' and others' results suggest that systemic P4 may not reflect the P4 reaching the uterus. P4 is secreted by the CL and transferred from the ovaries directly to the uterus. P4 in the uterine artery cannot be modulated by the hepatic metabolism, and its level can be 20–70% higher than the plasma P4 concentration (Stefańczyk-Krzymowska et al., 1998). Previously, it had been reported that higher feeding levels may have a positive effect on the utero-ovarian P4 level (Virolainen et al., 2004). The elevated plasma P4 level by enhancing maternal ME intake in present study may be the result of higher P4 secretion in the CL than P4 clearance

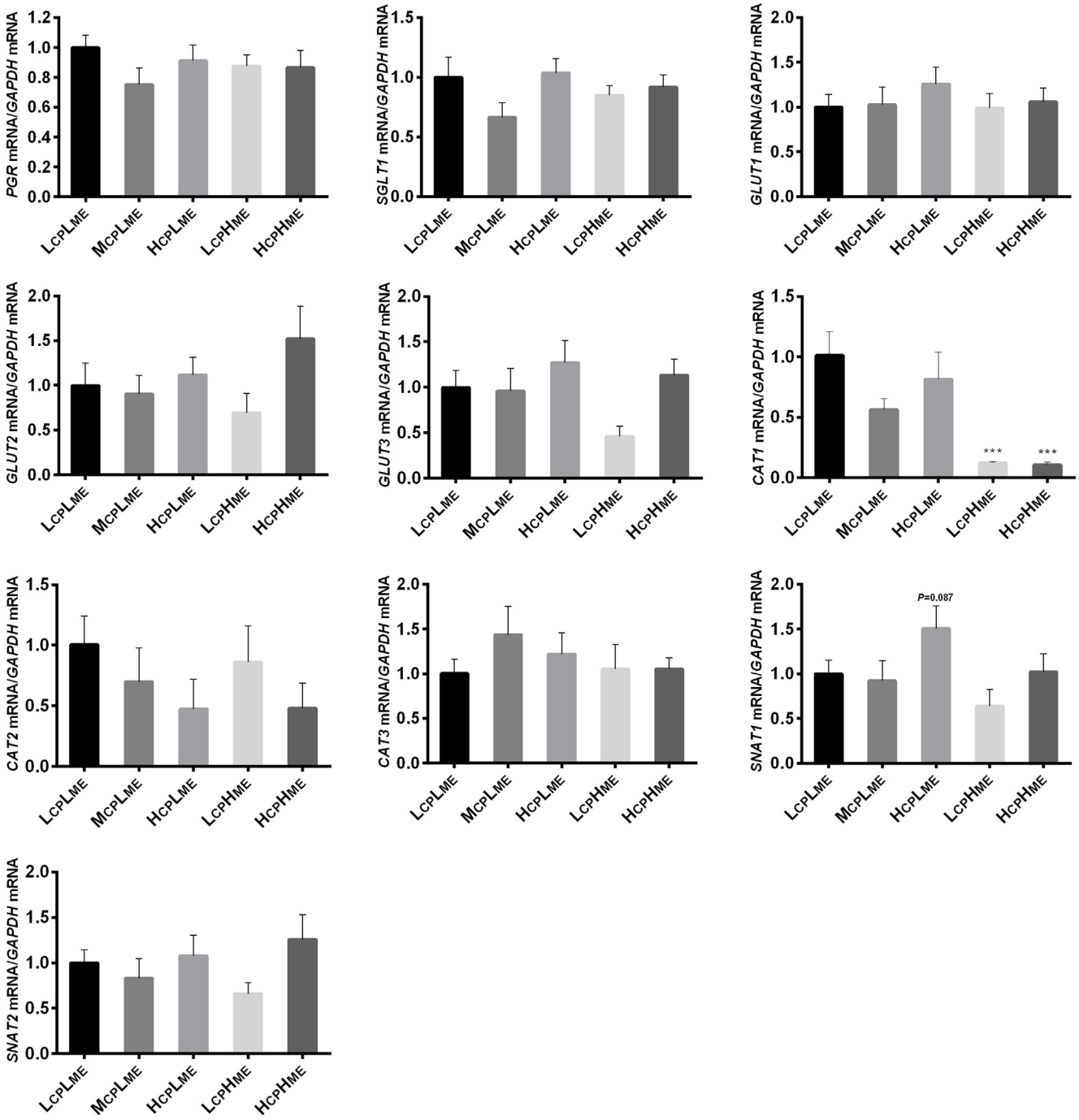


**Fig. 1.** Relative gene expression in endometrium on day 30 of gestation of gilts fed different CP and metabolisable energy levels. The relative expression levels of the target genes were measured by qPCR, calculated using the $2^{-\Delta\Delta CT}$ method and expressed as target gene mRNA/control gene mRNA. *GAPDH* was used as an endogenous control gene to normalise the data. ***Differs significantly from control ($P < 0.001$) using Dunnett's *t*-test. All of the data are expressed as the mean ± SEM. Abbreviations: $L_{CP}L_{ME}$ = Low protein, low energy; $M_{CP}L_{ME}$ = Middle protein, low energy; $H_{CP}L_{ME}$ = High protein, low energy; $L_{CP}H_{ME}$ = Low protein, high energy; $H_{CP}H_{ME}$ = High protein, high energy; qPCR = Quantitative real-time PCR; PGR = Progesterone receptor; SGLT = Sodium glucose cotransporter; GLUT = $Na^+$-independent sugar transporter; CAT = Cationic amino acid transporter; SNAT = Neutral amino acid transporter; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

in the liver. Surprisingly, the change of P4 concentration in the endometrium and CL was not found in our study. Whether the utero-ovarian P4 level altered by increased protein or ME needs further investigation in future studies.

The physiological effects of P4 are mediated via two intracellular P4 receptors, PGRA and PGRB. Previous studies have indicated that the *PGR* mRNA is expressed in the porcine uterus throughout gestation (Steinhauser et al., 2017a). However, there are no studies indicating the effects of different nutrition levels of sows on PGR expression during early pregnancy. Using western blot analysis, only the PGRB protein expression could be detected, which was localised strongly in the ST, but not in LE by immunohistochemistry. This is consistent with a previous study showing that the loss of PGR in the endometrial epithelia appears to be an essential prerequisite for implantation (Bazer et al., 2009). The present study showed that different nutrition levels did not affect *PGR* mRNA

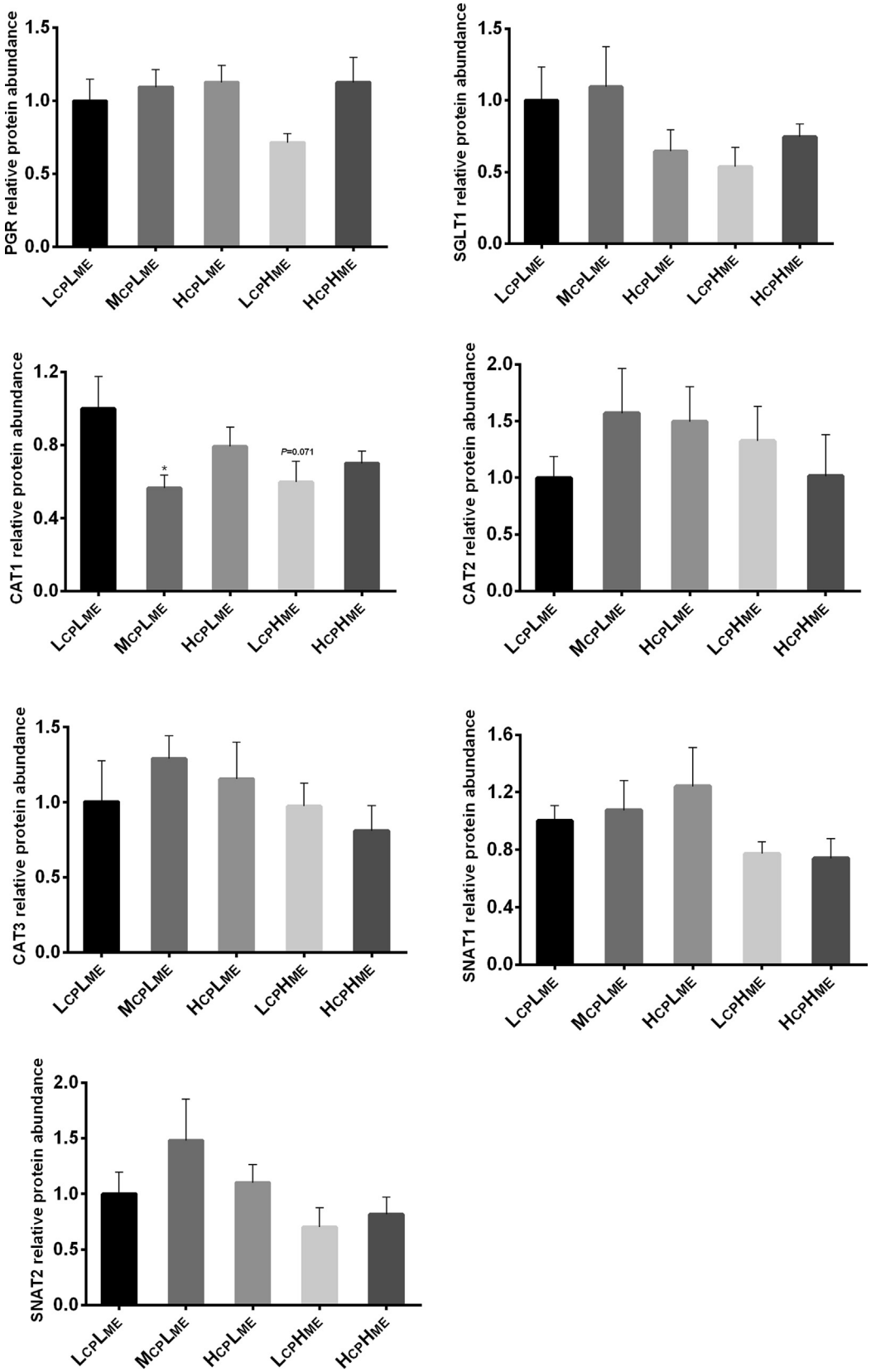


**Fig. 2.** PGR, SGLT1, cationic and neutral amino acid transporter protein abundance in endometrium on day 30 of pregnancy in gilts fed different CP and metabolisable energy levels by western blotting. *Differs significantly from control ($P < 0.05$) using Dunnett's t-test. All of the data are expressed as the mean ± SEM. The PGR antibody used in the current study has two forms (PGRA and PGRB). By western blotting, we only detected PGRB. Abbreviations: $L_{CP}L_{ME}$ = Low protein, low energy; $M_{CP}L_{ME}$ = Middle protein, low energy; $H_{CP}L_{ME}$ = High protein, low energy; $L_{CP}H_{ME}$ = Low protein, high energy; $H_{CP}H_{ME}$ = High protein, high energy; PGR = Progesterone receptor; SGLT = Sodium glucose cotransporter; CAT = Cationic amino acid transporter; SNAT = Neutral amino acid transporter.

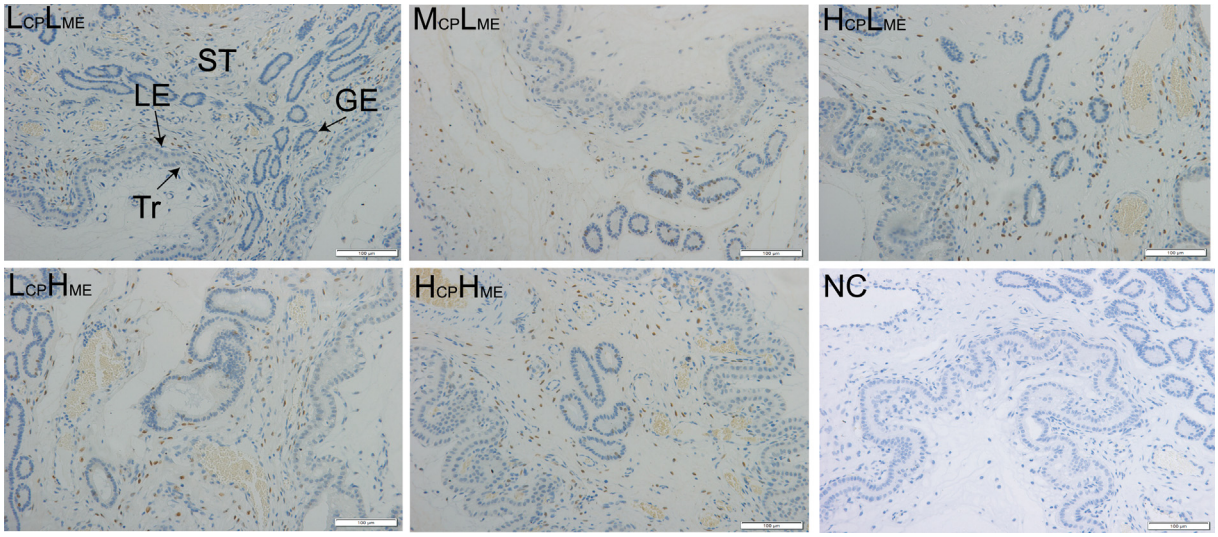


**Fig. 3.** Immunohistochemical localisation of PGR in the porcine endometrium (200×). A: representative images of PGR staining in porcine endometrial stroma. Abbreviations: $L_{CP}L_{ME}$ = Low protein, low energy; $M_{CP}L_{ME}$ = Middle protein, low energy; $H_{CP}L_{ME}$ = High protein, low energy; $L_{CP}H_{ME}$ = Low protein, high energy; $H_{CP}H_{ME}$ = High protein, high energy; NC = Negative control; PGR = Progesterone receptor; LE = Luminal epithelium; GE = Glandular epithelium; ST = Stroma; Tr = Trophectoderm.

and protein expression. The regulation of PGR expression by nutrients such as amino acids and glucose needs to be examined further.

Uterine receptivity to implantation requires silencing the expression of PGR in the uterine epithelia; however, PGR is still expressed in uterine stroma. It has been proposed that progestamedins such as FGF-7 and HGF induced by PGR in the uterine stroma exert paracrine effects on the uterine epithelia (Bazer et al., 2009). FGF-7 has been shown to stimulate cell proliferation, differentiation, migration and angiogenesis (Ka et al., 2007). HGF appears to be associated with endometrial morphogenesis and differentiated function required for the establishment and maintenance of gestation, conceptus implantation and placentation (Taylor et al., 2001). However, in the present study, endometrial concentrations of HGF and FGF-7 were not altered by enhancing protein or ME intake, which reiterated the results obtained on PGR expression in the endometrium that were not changed by enhanced protein or ME.

E2, another pregnancy hormone, is essential for the initiation and maintenance of gestation in mammals. E2 can maintain the function of the CL and promote the secretion of P4. A study had shown that E2 could affect embryonic survival, foetal-placental development and the P4 levels secreted by the CL (Koziorowski et al., 1984). In the current study, increasing dietary protein intake by 40% positively influenced plasma E2 concentration on d 30 of gestation. However, we did not observe the effects of increasing dietary protein intake on plasma, endometrial and corpora luteal P4 concentrations. This may be because the plasma E2 level is not high enough to increase P4 secretion of CL or we should measure the P4 in uterine artery.

Increasing dietary protein and/or energy intake had no effects on the total volume of ALF. Dyck and Strain (1979) reported a similar result by which the total volume of ALF was not influenced by different feeding levels in gilts. However, in our study, increasing dietary protein intake positively influenced the fructose concentration in ALF. This means that the conceptus may utilise more fructose, and its productions. Fructose-6 phosphate can be converted to hyaluronan, which is necessary for the formation of foetal-placental stroma and foetal-placental development (Bazer et al., 2012b). In porcine tissues, fructose cannot provide energy but it may regulate cell signalling pathways to regulate cell proliferation, migration, and mRNA translation in the placenta (Kim et al., 2012). Thus, the function of an increased fructose availability in the ALF warrants for further investigation.

Pigs have a diffuse epitheliochorial placentae, and thus, the histotroph from the uterine gland is important for conceptus survival and development (Roberts and Bazer, 1988). Glucose and amino acids are two major histotroph components. Glucose provides energy to support the elongation and implantation of conceptuses during the peri-implantation period; amino acids are essential for protein synthesis. Glucose must be transported into the uterine lumen by glucose transporters due to the incapacity of synthesising glucose in the endometrium (Vrhovac Madunić et al., 2021). Our study showed that GLUT1-3 and SGLT1 were expressed in the porcine endometrium, and that their expression levels were not influenced by maternal nutrition. GLUT1 and SGLT1 have been shown to be abundantly expressed in uterine LE and superficial GE in pregnant ewes (Gao et al., 2009), GLUT1, GLUT3 and SGLT1 have been shown to be expressed in human endometrium (von Wolff et al., 2003). To our knowledge, no study had reported the effects of maternal nutrition on glucose transporter expression in swine. However, SGLT1 expression in porcine endometrium was affected by long-term E2 treated (Steinhauser et al., 2017b). High glucose concentrations can be toxic to embryos (Diamond et al., 1991). So, we hypothesise that the mother herself can regulate the expression of glucose transporters and further control the transportation of glucose. In our study, E2 affected by maternal nutrition is within normal range and affected only for a short period of time, which would not influence the glucose transporters' expression. A study by Salker et al. found that the loss of endometrial SGLT1 was detrimental to embryonic survival in pregnant mice (Salker et al., 2017). The question remains whether the glucose transporters expressed in the endometrium will have an effect on the embryonic survival. Using immunohistochemistry, SGLT1 was strongly localised to the Tr, LE and GE. Maternal nutrition did not influence the intensity of SGLT1 staining in the different compartments. There is, to our knowledge, no study that shows the localisation of SGLT1 proteins by immunohistochemistry in the porcine endometrium. Moreover, SGLT1 proteins were detected by immunohistochemistry in ovine endometria, where it was detected in LE/GE and ST on d 9 and was more abundant in LE/GE of P4-treated ewes on d 12 (Satterfield et al., 2010).

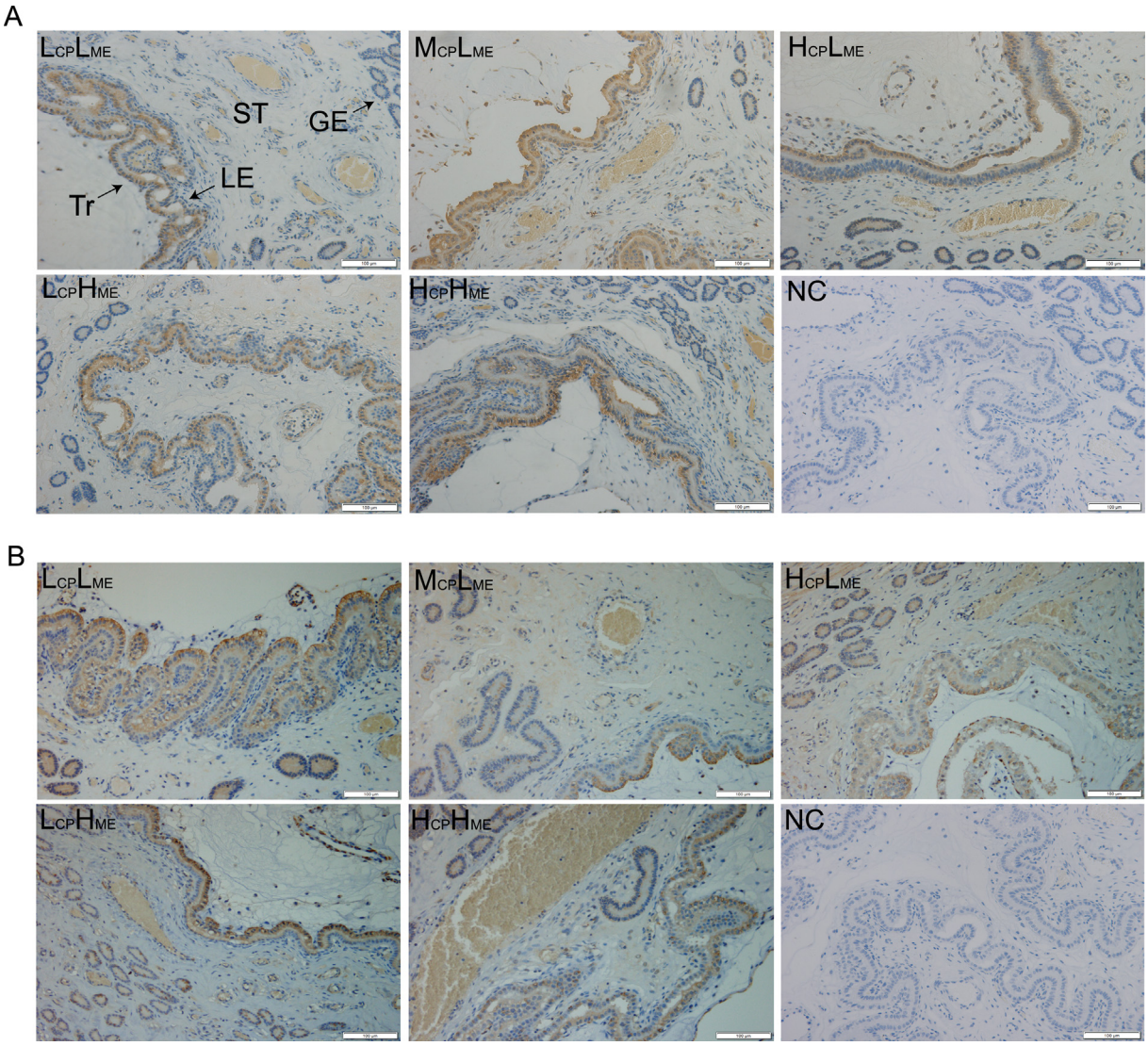


**Fig. 4.** Immunohistochemical localisation of SGLT1 and CAT1 in the porcine endometrium and conceptus trophectoderm. A: representative images of SGLT1 staining in porcine endometrial epithelium, stroma and conceptus trophectoderm; B: representative images of CAT1 staining in porcine endometrium epithelial, stroma and conceptus trophectoderm (×200). Abbreviations: $L_{CP}L_{ME}$ = Low protein, low energy; $M_{CP}L_{ME}$ = Middle protein, low energy; $H_{CP}L_{ME}$ = High protein, low energy; $L_{CP}H_{ME}$ = Low protein, high energy; $H_{CP}H_{ME}$ = High protein, high energy; NC = Negative control; SGLT = Sodium glucose cotransporter; CAT = Cationic amino acid transporter; LE = Luminal epithelium; GE = Glandular epithelium; ST = Stroma; Tr = Trophectoderm.

Next, we examined the expression of cationic amino acid transporters, CAT1, CAT2, and CAT3 in the endometrium. In ovine conceptus Tr, CAT1 was a preferential choice to transport arginine (Crouse et al., 2017), which is an essential amino acid for conceptus (embryo/foetus and trophoblast/placenta) growth and development. A study found that the expression of CAT1 and CAT2 in uterine epithelia and CAT1 in conceptuses increased during the periimplantation period of pregnant sheep, which coincides with a rapid elongation of ovine conceptuses (Wang et al., 2014). This illustrates that CAT1 is the key amino acid transporter for conceptus survival and development. Although we observed that high energy diets may decrease CAT1 expression in the endometrium, embryonic survival was positively influenced. Retarded growth and development were found when CAT1 was knocked out in ovine conceptus Tr (Wang et al., 2014). We speculated that the amino acids transported by CAT1 were enough for conceptuses to utilise in all diets, and that the absence of CAT1 expression in the endometrium will have an impact on embryonic survival and development. Of course, the effects of maternal nutrition on regulating cationic amino acid transporters need further investigation. By immunohistochemistry, CAT1 was strongly localised to the Tr, with less staining in the LE and ST, and maternal nutrition did not change the intensity of CAT1 staining in the different compartments.

SNAT1 and SNAT2 can transport glutamine and proline, and they are necessary for conceptus development. In our study, increasing protein intake showed a tendency to increase *SNAT1* mRNA expression in the endometrium. SNAT1 and SNAT2 also had a high protein abundance, despite not reaching statistical significance among different diets. This indicates that SNAT1 and SNAT2 are the principal factors in neutral amino acid transport across the endometrium to affect embryo implantation during early pregnancy. To our knowledge, no study reported the effect of maternal nutrition on endometrial neutral amino acid transporters expression during early pregnancy in pigs. However, a study found that SNAT1 expression was decreased in response to a low protein diet in the placenta of early pregnant rats (Rosario et al., 2011). SNAT2 protein expression was elevated 9fold in the

placenta when mice were fed high fat diets, conceptus was also overgrown (Jones et al., 2009). These results may indicate that maternal nutrition can influence the expression of neutral amino acid transporters which can further affect the conceptus.

## Conclusions

Increasing maternal protein intake by 20% or 40%, or energy intake by 40% has a positive effect on embryonic survival rate, and only increasing dietary energy intake by 40% improved maternal plasma P4 concentration in gilts during early pregnancy. Diets with higher protein levels may increase *SNAT1* mRNA expression, and high energy diets decreased CAT1 abundance in the porcine endometrium, which likely mediates transportation of cationic and neutral amino acid transporter from mother to foetus to affect embryonic survival and development.

## Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100540.

## Ethics approval

The animal experiment was approved by the Animal Care and Use Committee of the Institute of Feed Research of Chinese Academy of Agricultural Sciences (Approved number FRI-CAAS-20180728), and the trial was conducted at the Tianpeng husbandry located at Langfang, Hebei province, China.

## Data and model availability statement

No data were deposited in an official repository. The study findings are available on request.

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## Author contributions

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**X.R. Jiang:** data analysis, critically revising the manuscript.
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**M. Schroyen:** critically revising the manuscript.
**N. Everaert:** critically revising the manuscript.
**X.L. Li:** design of the experiment, data analysis, and critically revising the manuscript.
All authors read and approved the final manuscript.

## Declaration of interest

The authors declare no conflicts of interest.

## Acknowledgements

We thank Z. X. Wei, L. Cai and Y. P. Li for their assistant in sample collection.

## Financial support statement

This study was financially supported by the National Key R&D Program of China (2018YFD0501000), and the Elite Youth Program of Chinese Academy of Agricultural Sciences (to Xilong Li).

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