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Effects of folic acid and vitamin B_{12} supplements on folate and homocysteine metabolism in pigs during early pregnancy

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The present experiment aimed to determine the effects of supplements of folic acid (FA) alone or in combination with vitamin B_{12} on folate and homocysteine metabolism in gestating nulliparous Yorkshire–Landrace (YL) and multiparous Landrace (LD) occidental sows and multiparous Chinese Meishan–Landrace (ML) sows. LD sows were randomly assigned to two treatments: 0 or 15 mg FA/kg diet while YL and ML sows were assigned to three treatments: 0 mg FA/kg diet, 15 mg FA/kg or 15 mg FA + 160 μg vitamin B_{12}/kg diet. Supplements were given from the oestrus preceding insemination up to slaughter on day 15 of gestation. At slaughter, a uterine flush was collected to determine uterine contents of homocysteine, methionine, tetrahydrofolate (THF), 5-methyl-THF, pyridoxal 5-phosphate (P5P) and vitamin B_{12}. Blood samples were taken at first oestrus, at insemination and on days 5, 10 and 15 of gestation to determine plasma concentrations of homocysteine, methionine, THF, 5-methyl-THF, P5P, vitamin B_{12} and relative total folate-binding capacity. In occidental sows (YL and LD), the FA supplement tended to decrease uterine flush content of homocysteine ($P=0{\cdot}06$) and concentrations of plasma homocysteine ($P=0{\cdot}09$). Nulliparous YL sows had lower concentrations of plasma homocysteine, methionine, THF and 5-methyl-THF ($P<0{\cdot}05$) than multiparous LD sows. Multiparous ML and LD sows had similar concentrations of plasma THF, 5-methyl-THF, methionine and vitamin B_{12}, but ML sows had lower concentrations of plasma homocysteine ($P<0{\cdot}05$). The vitamin B_{12} supplement increased concentrations of plasma vitamin B_{12} ($P<0{\cdot}05$) both in multiparous ML and nulliparous YL sows, but had no effect on the composition of either uterine flush or plasma. The present results showed also that sows had a low vitamin B_{12} status (<200 pg/ml) and high circulating homocysteine levels (>15 μM) during the first 15 d of gestation. Furthermore, the vitamin B_{12} content in uterine secretions represented between 180 and 300 % of the total content in plasma. The low plasma concentrations of homocysteine in multiparous ML sows suggest a more efficient remethylation pathway which may not be dependent upon dietary supply of FA or vitamin B_{12}. In nulliparous YL sows, low concentrations of both homocysteine and methionine suggest that the methionine requirement for protein deposition might have reduced the amount of methionine available for the methylation pathway. The results of the present experiment suggest that the reduction of uterine homocysteine may be an important aspect of the role of FA supplement on the uterine environment in occidental sows. The presence of high levels of vitamin B_{12} in uterine secretions merits further investigation in relation to embryonic development.

Folates: Homocysteine: Gestation: Sow

Abbreviations: FA, folic acid; FBC, folate-binding-capacity; LD, Landrace sow; ML, Meishan–Landrace sow; P5P, pyridoxal 5-phosphate; THF, tetrahydrofolate; YL, Yorkshire–Landrace sow.

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The death of initially viable embryos is one of the major limitations to improving litter size in pigs. From several studies it has been accepted that during the first 25–30 d of gestation, embryonic mortality is approximately 30 % and occurs especially during embryonic attachment, day 12–15 of gestation (Pope & First, 1985; Wilmut *et al.* 1985). Recent advances in the understanding of embryonic development and uterine physiology have shown that embryonic mortality is controlled by several factors, such as nutritional status and genotype of sows (Haley & Lee, 1993; Ashworth, 1994).

A dietary supplement of folic acid (FA) during pregnancy increases litter size by 10 % in multiparous sows (Matte *et al.* 1984). This increased litter size is associated with modifications in endometrial secretions and decreased embryonic mortality in early pregnancy (Tremblay *et al.* 1989; Matte *et al.* 1996). However, effects of FA on litter size, embryonic mortality and endometrial secretions are less marked in nulliparous sows (Lindemann & Kornegay, 1989; Matte *et al.* 1993; Duquette *et al.* 1997). Chinese sows of the Meishan genotype are recognized for their higher litter size and lower embryonic mortality rate than occidental sows (Haley & Lee, 1993). In a recent study, it has been shown that there was no effect of FA supplementation on litter size and embryo survival in Meishan crossbred sows during the first 25 d of gestation (Guay *et al.* 2002).

In human subjects, FA supplementation is known as a reliable therapeutic tool to reduce the concentration of plasma homocysteine and the incidence of neural-tube defects (Czeizel & Dudas, 1992; Jacques *et al.* 1999). Maternal accumulation of homocysteine, a metabolite of the methionine cycle, has been linked to neural-tube defects in the child and associated with defective chorionic villous vascularization in women with recurrent early pregnancy loss (Mills *et al.* 1995; Nelen *et al.* 2000). *In ovo*, treatment of avian embryos with various concentrations of exogenous homocysteine during the processes of gastrulation, neurulation and crano–truncal separation showed homocysteine induced oro–facial, coro–truncal and neural-tube defects and reduced embryo survival (Rosenquist *et al.* 1996, 1999), but the physiological basis of effects of homocysteine on embryo–fetal development remains to be determined. However, a recent study has shown that homocysteine could inhibit conversion of retinal to retinoic acid in avian embryos (Limpach *et al.* 2000). Retinoic acid is important in the regulation of gene expression prior to and during rapid trophoblastic elongation in porcine conceptus and is also involved in the development and growth of the porcine placenta (Yelich *et al.* 1997; Johnsson *et al.* 2001). Retinoic acid also controls the formation of diverse embryonic structures, including face, heart and limb as well as neural-tube closure (Smith *et al.* 1998), which takes place at day 18 of pregnancy in the porcine embryo (Perry, 1981). In pigs, the concentration of plasma homocysteine is reduced following FA supplementation in growing animals (Ambrosi *et al.* 1999). However, whether this occurs in pregnant sows, which have characteristically high plasma concentrations of homocysteine (Barkow *et al.* 2001), is not known.

The regulation of homocysteine by the methionine synthase pathway requires another B-complex vitamin, vitamin B_{12}, for the demethylation of 5-methyl-tetrahydrofolate (THF) (Bässler, 1997). A deficiency in vitamin B_{12} induces an accumulation of homocysteine and 5-methyl-THF in plasma. This reduced utilization of folates is called 'methyl trapping' (Scott & Weir, 1981). Furthermore, in human subjects, neural-tube defects was associated with lower concentrations of vitamin B_{12} in amniotic fluid (Steen *et al.* 1998). Although such an association has not been established in the pig, it is known that vitamin B_{12} influences swine reproduction and neonatal survival (Frederick & Brisson, 1961). In a recent study, we reported low plasma concentrations of vitamin B_{12} (<200 pg/ml) in gestating multiparous Meishan–Landrace (ML) and nulliparous Yorkshire–Landrace (YL) as compared with multiparous YL sows. These results suggest a possible suboptimal status of vitamin B_{12} in these gestating sows (Guay *et al.* 2002).

The objectives of the present study were to evaluate, during early pregnancy, the effects of supplements of FA alone, or in combination with vitamin B_{12}, on folate and homocysteine metabolism in nulliparous and multiparous occidental sows (nulliparous effect) and multiparous ML sows (Meishan genotype effect) during the first 15 d of gestation.

Materials and methods

Animals and treatments

Ten multiparous ML (three to four parities, body weight 197·5 (SE 6·7 kg)) and twelve multiparous Landrace (LD, three to four parities, body weight 255·6 (SE 7·5 kg)) sows (Génétiporc Inc., St-Bernard, Qué., Canada), were compared, to evaluate impacts of the Meishan genotype. Eleven nulliparous YL sows (body weight 125·7 (SE 3·5 kg)) (Génétiporc Inc.) were also compared with multiparous LD sows to evaluate effects of 'nulliparous status' on responses of homocysteine and folate metabolism to FA supplementation. After weaning, multiparous sows in ML and LD groups were fed with the gestation diet and transported a few days after weaning to the Research Centre. Before their arrival, nulliparous YL sows were fed with breed–gestation diet and were not cycling upon arrival at Research Centre. Beginning on arrival and for at least 2 weeks, all groups of sows received 2·5 kg experimental diet/d (Table 1) without FA supplement. After these 2 weeks, heat detection was performed twice per d, between 08.00 and 09.00 hours, and 16.00 and 17.00 hours, by introducing a boar into the pen. On the first observed oestrus (day -21) (monitored from at least 2 weeks after their arrival), the sows were allocated (Table 2) to one of the following two treatments: basal diet (0 mg FA/kg) or basal diet with 15 mg FA/kg. As a results of findings in a previous study (Guay *et al.* 2002), a third treatment, basal diet supplemented with 15 mg FA and 160 μg vitamin B_{12}/kg, was also used in multiparous ML (n 5 sows) and nulliparous YL (n 6 sows) to evaluate the impact of FA plus vitamin B_{12} on folate utilization and homocysteine metabolism. At second observed oestrus, the

Table 1. Composition of the basal experimental diet*†

Ingredients	g/kg
Maize	500
Barley	200
Wheat bran	200
Soyabean meal (480 g crude protein (N × 6·25)/kg)	50
Limestone	25
Dicalcium phosphate	14
Salt	5
Mineral premix‡	1
Vitamin premix§	5

* The analysed dietary concentrations of folates in the 0 mg/kg, 15 mg/kg and 15 mg/kg + B_{12} diets were 1·02 (SD 0·12), 16·8 (SD 1·4) and 16·3 (SD 2·3) mg/kg respectively. The added folic acid was pteroylglutamic acid, $C_{19}H_{19}N_7O_6$ (molecular mass 441·4; ICN Canada Ltd, Montreal, Que., Canada).

† The analysed dietary concentrations of vitamin B_{12} in the 0 mg/kg, 15 mg/kg and 15 mg folic acid/kg + B_{12} were 15·7 (SD 2·2), 17·4 (SD 1·9) and 192·3 (SD 17·1) μg/kg respectively. The added vitamin B_{12} was cyanocobalamin, $C_{63}H_{88}N_{14}O_{14}PCO$ (molecular mass 1355·4; ICN Canada Ltd, Montreal, Que., Canada).

‡ Provided (/kg diet): Mn as manganous oxide 30 mg, Zn as zinc oxide 100 mg, Fe as ferrous sulfate 100 mg; Cu as copper sulfate 15 mg; I as calcium iodate 300 μg, Se as selenite 300 μg.

§ Provide (/kg diet): retinol acetate 3·44 mg, cholecalciferol 50 μg, DL tocopherol acetate 35 mg, menadione 2·2 mg, thiamin 2 mg, riboflavin 5 mg, niacin 25 mg, pantothenic acid 16 mg, pyridoxine 2 mg, biotin 250 μg, choline 350 mg.

sows were inseminated twice with commercial semen (pooled semen from three Duroc boars of proven fertility; CIPQ Inc., St-Lambert, Qué., Canada), 12 h and 24 h after initial oestrus detection. The first day of the second oestrus was considered to be day 0. Dietary treatments were given to day 15 of pregnancy.

Blood samples and tissues collected

At first oestrus (day −21, baseline value), second oestrus (insemination, day 0), and days 5, 10 and 15 of gestation, blood samples were collected in morning prior to feeding (after a 20–24 h fast) with whole-blood collection tubes (Becton Dickenson, Franklin Lakes, NJ, USA) containing EDTA as anticoagulant and centrifuged for 10 min (1800 ***g***). Plasma was frozen at −25°C, and later analysed for vitamin B_{12}, pyridoxal 5-phosphate (P5P), total folate-binding capacity (FBC), THF, 5-methyl-THF, homocysteine and methionine. For THF and 5-methyl-THF determinations, 0·5 ml ascorbic acid (100 g/l) was added to 10 ml blood to avoid oxidation. On day 15 of gestation, sows were killed according to the recommended code of practices (Canadian Council on Animal Care, 1993). The reproductive tract was taken and transported to the laboratory. The uterine horns were separated from the mesometrium. The ovaries, oviducts and cervix were also removed. One horn, chosen at random, was flushed with 20 ml PBS according to a method described by Laforest *et al.* (1992). The fluid collected was defined as 'uterine flush' and the total volume was recorded. After assessing the presence of filamentous blastocysts, pregnant uteri were kept for further analysis. The fluid was centrifuged at 86 ***g*** for 5 min to precipitate the blastocysts and uterine flush was collected and stored at −25°C.

Plasma determination

Concentrations of plasma vitamin B_{12} were measured by radioassay (Quantaphase II, B_{12} radioassay; Bio Rad Laboratories (Canada) Ltd, Montreal, Qué., Canada) following the validated procedure described by Bilodeau *et al.* (1989). The inter-assay CV was 6·1 %. Total FBC were determined as described by Guay *et al.* (2002). The inter- and intra-assay CV for total FBC were 10·9 and 5·4 % respectively. Concentrations of plasma homocysteine and methionine were measured following sample preparation as described by Malinow *et al.* (1989) and quantified by HPLC (Melnyk *et al.* 1999); inter- and intra-assay CV for homocysteine were 8·7 and 2·8 % and for methionine 11·0 and 5·0 % respectively. Plasma concentrations of P5P were determined by the fluorometric method of Srivastava

Table 2. Composition of the uterine flush of sows slaughtered on day 15 of pregnancy as affected by nulliparous status, Meishan genotype and folic acid supplement‖¶

	Multiparous ML				Multiparous LD				Nulliparous YL			
Dietary folate (mg/kg)	0		15		0		15		0		15	
	LS Mean	SEM	LS Mean	SEM	LS Mean	SEM	LS Mean	SEM	LS Mean	SEM	LS Mean	SEM
Sows (*n*)	5		5		5		7		6		5	
Flush volume (ml)	18·2	0·8	18·1	0·5	17·7	0·3	18·3	0·5	18·7	0·4	19·0	0·6
Methionine (nmol)	786	130	819	202	544	89	769	317	754	242	710	101
Homocysteine (nmol)	117	31	102	31	115	16	84†	18	138	23	98†	12
5-methyl-THF (ng)	423‡	103	514‡	138	314	47	283	30	222§	76	185§	31
P5P (nmol)	56·5	7·0	45·8	11·3	26·6*	3·2	34·3*	5·3	46·8	5·8	36·3	4·2
Vitamin B_{12} (ng)	1339	254	1286	339	1207	167	1346	141	1077	171	1031	106

ML, Meshian–Landrace sows; LD, Landrace sows; YL, Yorkshire–Landrace sows; THF, tetrahydrofolate; P5P, Pyridoxal 5 Phosphate.
For P5P, values in LD sows were lower than in ML and YL sows whatever the dietary folic acid supplement: *$P<0·05$.
For homocysteine, values in occidental sows (YL and LD) tended to be lower than in those fed 15 *v.* 0 mg FA/kg diet: †$P=0·06$.
Values for ML sows were higher than for LD sows: ‡$P=0·09$.
Values for YL sows were lower than for LD sows: §$P=0·09$.
‖ For details of diets and procedures, see Table 1 and p. 254.
¶ Total content = concentration × volume of uterine flush.

& Beutler (1973) with some modifications as described by Matte *et al.* (1997). Under these conditions, the validation tests showed satisfactory parallelism between 170 and 500 μl sample (CV among dilutions 5·1 %) and recovery tests yielded 102 %. Plasma concentrations of THF and 5-methyl-THF were determined by HPLC using a method developed by Wigertz & Jägerstad (1995) following the extraction procedures of Schieffer *et al.* (1984). Briefly, 0·7 ml plasma was added to 0·7 ml 0·01 M-phosphate buffer pH 2·5 containing ascorbic acid (10 g/l) in an assay tube to allow the separation of folates from binding proteins. Thereafter, the sample was loaded in a disposable strong anion-exchange column (Bondesil, Sax 40 μm; Varian Analytical Instruments, Walnut Creek, CA, USA) pre-treated with 1 ml methanol and 1 ml water followed by 1 ml acetic acid pH 2·3 and washed with 2 ml ultrapure water. Folates were eluted twice from the column with 350 μl sodium chloride solution (100 g/l) pH 2·0 (adjusted with citric acid) containing sodium ascorbate (10 g/l) and β-mercaptoethanol (2 ml/l). Plasma THF and 5-methyl-THF were separated by HPLC coupled to a Beckman solvent delivery system (model 126; Beckman Instrument (Canada) Inc., Mississauga, Ont., Canada). A reverse-phase C_{18} Luna column (5 μm; 250 × 4·60 mm; Phenomenex, Torrance, CA, USA) was used. An isocratic elution was performed at ambient temperature, using acetonitrile (80 ml/l acetic acid) pH 2·3 as the mobile phase and a flow rate of 1·5 ml/min. Plasma samples (100 μl) were directly injected onto the columm using a Beckman autosampler, model 507E (Beckman Instrument (Canada) Inc.). Folates were detected following HPLC separation with a LC 240 Perkin Elmer detector (Perkin-Elmer Limited, Beaconsfield, Bucks., UK) at 297 nm excitation and 353 nm emission. Peak area analyses for THF and 5-methyl-THF were performed by GOLD Nouveau software (Beckman Instrument (Canada) Inc.). Validation tests showed satisfactory parallelism (CV, for 600–800 μl plasma were 10·1 and 9·2 % for THF and 5-methyl-THF respectively) and recovery tests yielded 99·6 and 98·7 % for THF and 5-methyl-THF respectively. Inter- and intra-assay CV were 15·7 and 7·8 % for THF and 16·2 and 8·2 % for 5-methyl-THF respectively.

Uterine flush assays

Concentrations of vitamin B_{12} were determined directly (Quantaphase II, B_{12} radioassay; Bio Rad Laboratories (Canada) Ltd) following appropriate dilution with PBS. Validation tests showed satisfactory parallelism among dilution (between 1:40 and 1:320, CV 4·7 %) and recovery tests yielded 109 %. Inter-assay CV was 4·8 %. Concentrations of uterine P5P were determined as described previously for plasma. Validation tests showed satisfactory parallelism (CV among dilutions 1:2 and 1:8 was 9·9 %) and recovery tests yielded 99·9 %. Intra-assay CV was 7·1 %. Concentrations of homocysteine and methionine were determined as previously described for plasma. Validation tests showed satisfactory parallelism for 100–300 μl sample (CV 10·3 % and 8·1 % for homocysteine and methionine respectively) and recovery tests yielded 101 and 102 % for homocysteine and methionine, respectively. The inter- and intra-assay CV were 4·8 and 4·2 % for homocysteine 4·9 and 3·0 % for methionine, respectively. Concentrations of THF and 5-methyl-THF were measured as previously described for plasma; THF was however, undetectable in uterine flushings. Validation tests showed satisfactory parallelism among dilutions (for 300–500 μl CV was 1·7 %) and recovery tests yielded 98·7 %. The inter- and intra-assay CV were 11·1 and 7·7 %, respectively.

Statistical analysis

The effects of Meishan genotype and nulliparous status were analysed using the Mixed procedure of the SAS (SAS Institute, Inc., Cary, NC, USA), according to a 3 × 2 factorial arrangement with the comparison between mutiparous ML and LD, and nulliparous YL and dietary supplements (0 or 15 mg FA/kg) as the main factors. The following model was used: $Y_{ij} = \mu + B_i + F_j + (B_i \times F_j) + e_{ij}$, where μ is the mean, Y_{ij} is the dependent variable, B_i is the type of sow, F_j is the FA supplement and e_{ij} is the residual error. Preplanned single df contrasts were used to compare (1) Meishan genotype effects between multiparous ML and LD sows; (2) 'nulliparous effects' between multiparous LD and nulliparous YL sows; (3) specific effects of FA supplementation within occidental sows. To evaluate the effect of vitamin B_{12} on folate metabolism, an analysis using the Mixed procedure (SAS Institute Inc.) was also done within multiparous ML and nulliparous YL groups using the dietary supplement of vitamin B_{12} as the main variable; comparisons were performed between 15 mg FA/kg and 15 mg FA + 160 μg B_{12}/kg using the following model: $Y_{ij} = \mu + F_i + e_{ij}$, where μ is the mean, F_i is the vitamin B_{12} supplement and e_{ij} is the residual error. For plasma FBC, THF, 5-methyl-THF, homocysteine, methionine, vitamin B_{12} and P5P, days of pregnancy (days 0, 5, 10, 15) were added to the model as a third factor and were analysed using the repeat option of the Mixed procedure (SAS Institute Inc.) with the autoregressive option and then sow was considered as a random effect and included in statistical analysis. To determine effects of FA alone or FA + vitamin B_{12} on concentrations of plasma total FBC, THF, 5-methyl-THF, homocysteine, methionine and P5P, values measured at treatment allocation were used as covariables. For plasma concentrations of total FBC, THF and 5-methyl-THF, a logarithm transformation was performed to normalize for experimental errors.

Results

Meishan genotype and nulliparous effects

During early gestation, multiparous LD and ML sows had similar concentrations of plasma THF, 5-methyl-THF and total FBC (Figs 1 and 2; $P > 0{\cdot}10$), whereas multiparous LD had higher plasma concentrations of THF, 5-methyl-THF and FBC than nulliparous YL sows ($P < 0{\cdot}05$; Figs 1 and 2).

Multiparous LD sows also had higher concentrations of homocysteine than multiparous ML and nulliparous YL

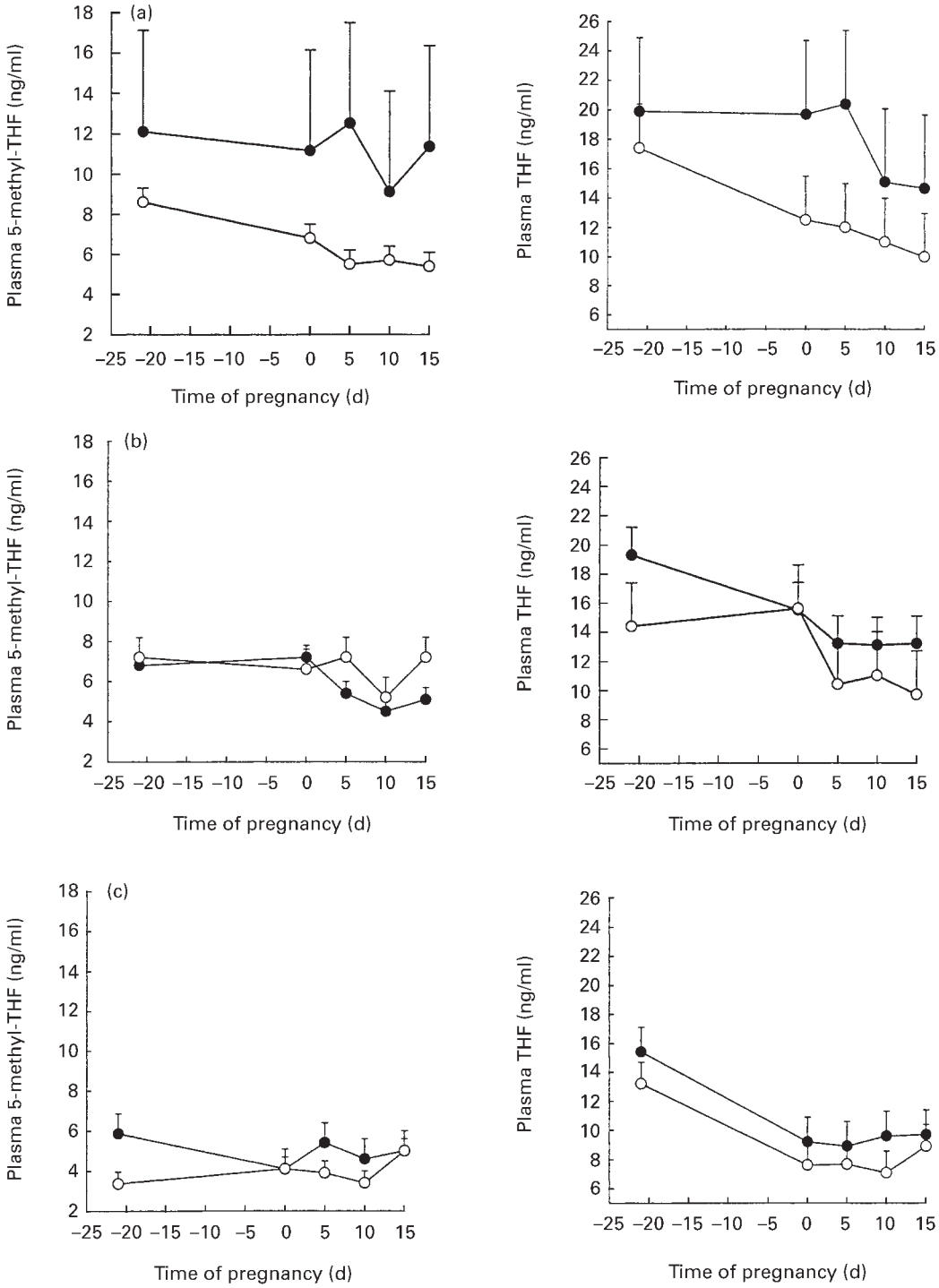
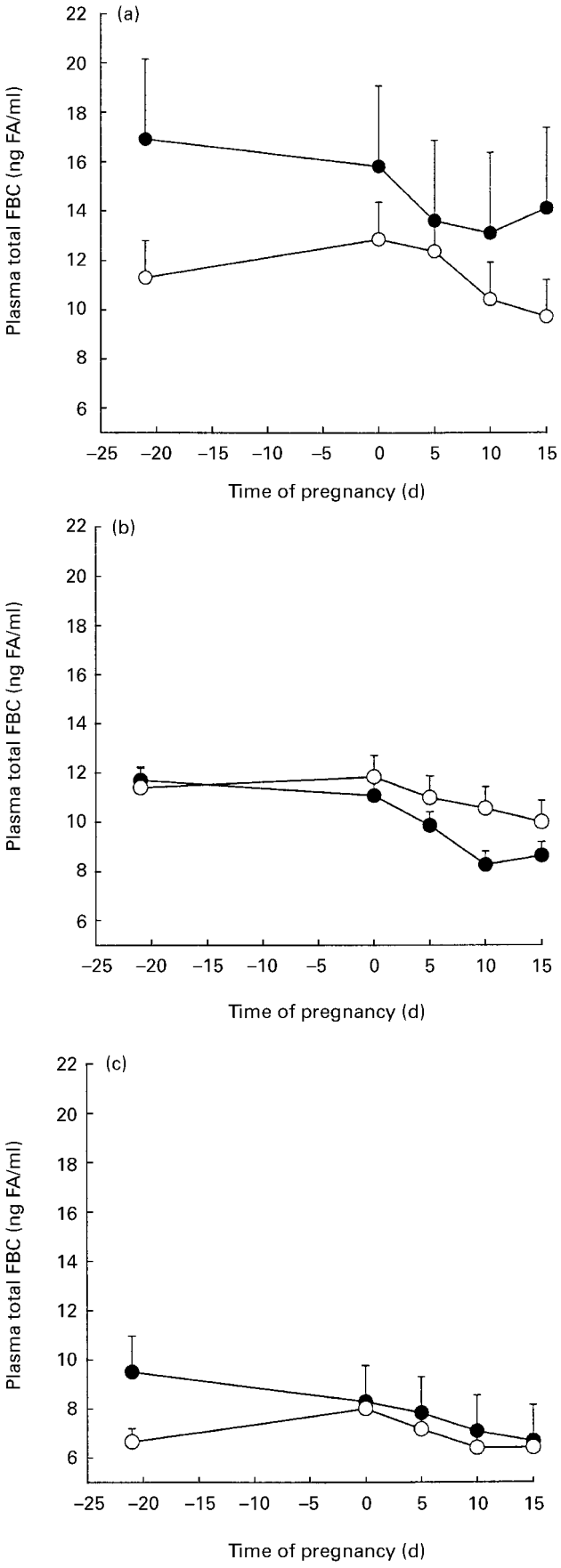


Fig. 1. Effects of nulliparous status, Meishan genotype and supplement with folic acid (FA) on concentrations of plasma tetrahydrofolate (THF) and 5-methyl-THF from the oestrus preceding insemination to day 15 of gestation for (a) multiparous Meishan–Landrace, (b) multiparous Landrace, (c) nulliparous Yorkshire–Landrace sows. (●), 0 mg FA/kg diet; (○), 15 mg FA/kg diet. For details of diets and procedures, see Table 1 and p. 254. Values are least squares means with standard errors shown by vertical bars. Mean values for THF and 5-methyl-THF for Yorkshire–Landrace sows were significantly different from those for landrace sows: $P<0{\cdot}05$. (Statistical analyses were performed on logarithm values.)

sows (Fig. 3; $P<0{\cdot}05$). However, the reduction of homocysteine concentrations during the first 15 d of gestation was more marked in multiparous LD sows than in multiparous ML and nulliparous YL sows (Fig. 3; Genotype × Time and Nulliparous × Time, $P<0{\cdot}05$).

Multiparous LD and ML sows had similar concentrations of plasma P5P (0·27 (SE 0·012) *v.* 0·29 (SE 0·015 μM) respectively) and methionine (39·9 (SE 2·1) *v.* 35·4 (SE 2·2) μM respectively) while multiparous LD sows had higher plasma concentrations of methionine



(39·9 (SE 2·1) *v.* 34·5 (SE 1·1) μM respectively $P<0·05$) than nulliparous YL sows. Concentrations of plasma P5P were higher (0·40 (SE 0·016) *v.* 0·27 (SE 0·012) μM respectively, $P<0·01$) in nulliparous YL than multiparous LD sows. Finally, multiparous ML and LD, and nulliparous YL sows had similar concentrations of vitamin B_{12} (227·1 (SE 51·0), 158·5 (SE 27·6) *v.* 147·5 (SE 5·7) pg/ml respectively, $P>0·10$).

In the uterine flush, nulliparous YL and multiparous LD and ML sows had similar total contents of methionine, homocysteine and vitamin B_{12} (Table 2; $P<0·10$). Multiparous ML sows tended to have higher uterine contents of 5-methyl-THF than multiparous LD sows whereas contents of 5-methyl-THF tended to be lower in nulliparous YL compared with multiparous LD sows (Table 2; $P=0·09$). The content of P5P was higher in multiparous ML and nulliparous YL than in multiparous LD sows (Table 2; $P<0·05$).

Folic acid effects

The FA supplement did not increase plasma concentrations of THF and 5-methyl-THF (Fig. 1; $P>0·10$). However, the FA supplement increased concentrations of plasma total FBC in multiparous ML and LD, and nulliparous YL sows during the first 15 d of gestation (Fig. 2; $P<0·05$).

The concentrations of plasma vitamin B_{12}, P5P, methionine (results not shown) and homocysteine (Fig. 3) were not altered by the FA supplement during the first 15 d of gestation ($P>0·10$). However, if analyses were done within occidental sows only, FA supplement tended to decrease concentrations of plasma homocysteine by 10 % in multiparous LD and nulliparous YL sows (Fig. 3; $P=0·09$).

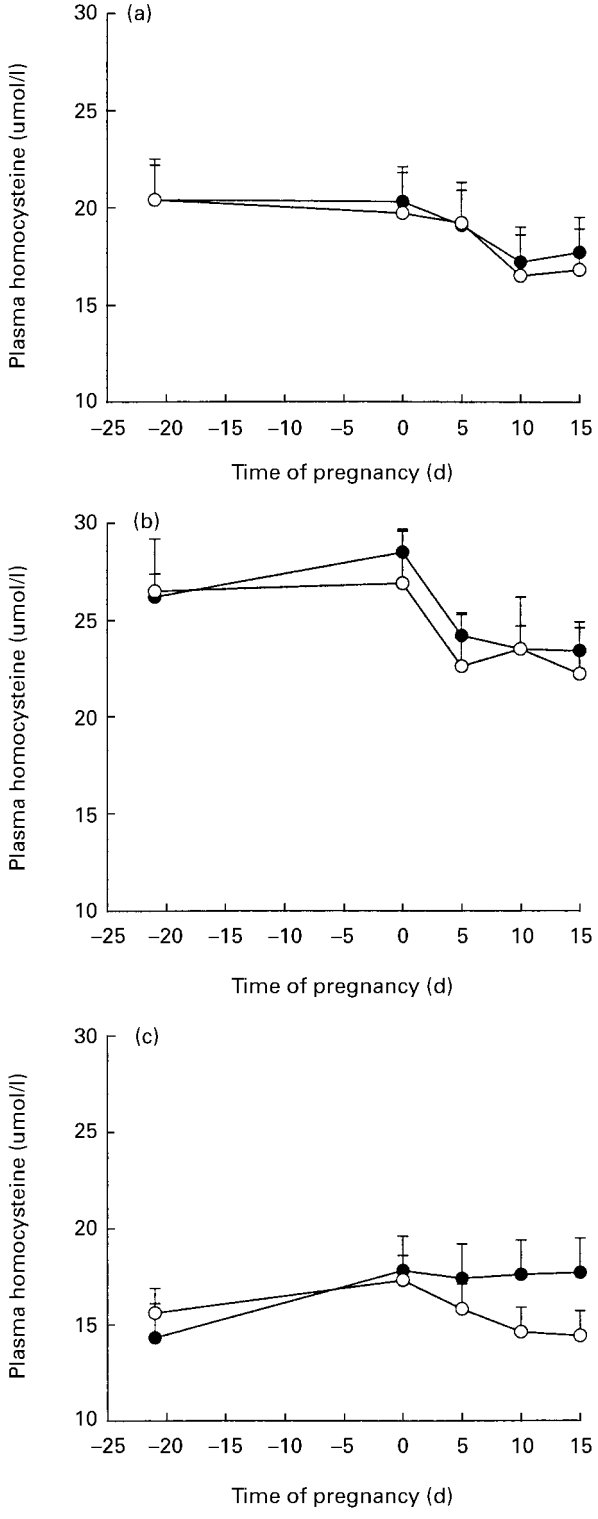
The FA supplement had no significant effect on the composition of the uterine flush in nulliparous YL, and multiparous LD and ML sows (Table 2; $P>0·10$). However, there was a strong tendency for the FA supplement to decrease the total content of homocysteine in occidental sows (Table 2; $P=0·06$).

Vitamin B_{12} effects

In multiparous ML and nulliparous YL sows, vitamin B_{12} had no effect on the concentration of plasma homocysteine, methionine, THF, 5-methyl-THF and P5P (results not shown). Nevertheless, the addition of vitamin B_{12} to the

Fig. 2. Effects of folic acid (FA) supplement, nulliparous status and Meishan genotype on plasma concentrations of total folate-binding capacity (FBC) from the oestrus preceding insemination to day 15 of gestation for (a) multiparous Meishan–Landrace, (b) multiparous Landrace, (c) nulliparous Yorkshire–Landrace sows. (●), 0 mg FA/kg diet; (○), 15 mg FA/kg diet. For details of diets and procedures, see Table 1 and p. 254. Values are least squares means with standard errors shown by vertical bars. Mean values for Yorkshire–Landrace sows were significantly different from those of Landrace sows: $P<0·05$. Mean values for 0 mg FA/kg diet were significantly different from those for 15 mg FA/kg diet: $P<0·05$ (Statistical analyses were performed on logarithm values.)

FA supplement increased the plasma concentrations of vitamin B_{12} compared with FA supplement alone (Fig. 4; $P<0{\cdot}05$). The addition of vitamin B_{12} had no significant effect on the composition of uterine flush either in nulliparous YL or ML sows (results not shown).



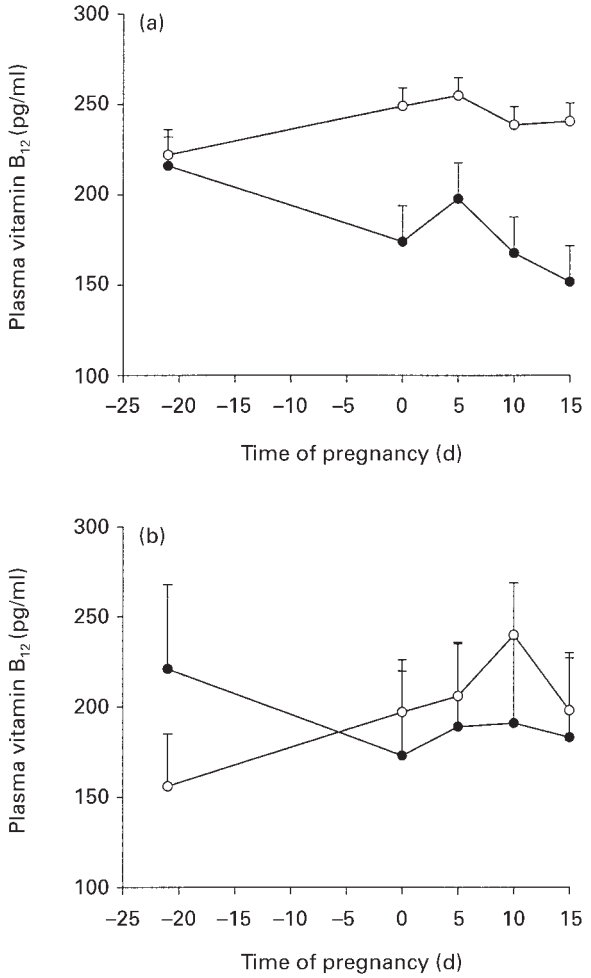


Fig. 4. Effects of addition of vitamin B_{12} to folic acid (FA) supplement on plasma concentrations of vitamin B_{12} from the oestrus preceding insemination to day 15 of gestation for (a) multiparous Meishan–Landrace and (b) nulliparous Yorkshire–Landrace sows. (●), 15 mg FA/kg diet; (○), 15 mg FA and 160 μg vitamin B_{12}/kg diet. For details of diets and procedures, see Table 1 and p. 254. Values are least squares means with standard errors shown by vertical bars. Mean values for Meishan–Landrace and Yorkshire–Landrace sows given FA and vitamin B_{12} were significantly different from those given FA only: $P<0{\cdot}05$. (Statistical analyses were performed on logarithm values.)

Fig. 3. Effects of folic acid (FA) supplement, nulliparous status and Meishan genotype on plasma concentrations of homocysteine from the oestrus preceding insemination to day 15 of gestation for (a) multiparous Meishan–Landrace, (b) multiparous Landrace sows, (c) nulliparous Yorkshire–Landrace sows. (●), 0 mg FA/kg diet (○), 15 mg FA/kg diet. For details of diets and procedures, see Table 1 and p. 254. Values are least squares means with standard errors shown by vertical bars. Mean values for Meishan–Landrace sows were significantly different from those for Landrace sows: $P<0{\cdot}05$. Mean values for Yorkshire–Landrace sows were significantly different from those for Landrace sows: $P<0{\cdot}05$. Statistical significance of (Meishan–Landrace *v.* Landrace) × Time, $P<0{\cdot}05$; (Yorkshire–Landrace *v.* Landrace) × Time, $P<0{\cdot}05$. Mean values for occidental sows (Landrace and Yorkshire–Landrace) fed 15 mg FA/kg diet were significantly different from those fed 0 mg FA/kg diet.

Discussion

To the best of our knowledge, the present experiment was the first to evaluate the effects of FA alone and the effects of FA with vitamin B_{12} on folate and homocysteine metabolism of different types of sows during early pregnancy. In growing pigs and adult human subjects, supplementation with FA reduces the concentration of plasma homocysteine (Ambrosi *et al.* 1999; Jacques *et al.* 1999). In the present experiment, the FA supplement reduced the homocysteine content in uterine flush by approximately 30 % and by 10 % in plasma in occidental sows (multiparous LD and nulliparous YL sows). In the first 15 d of gestation, large amounts of folates are taken up by the uterine tissues and transferred into the uterine lumen (Matte *et al.* 1996; Vallet *et al.* 1999*a*). Those folates can be readily used by the conceptus in order to respond to the intense metabolic activity and cellular division rates at this stage of development (Pusateri *et al.* 1990). This high rate of cellular division is likely to increase FA requirements and induces an increased uterine uptake of FA to allow an efficient methylation of homocysteine within the uterine environment. In culture of post-implantation rat embryos, homocysteine is recognized to be teratogenic for 10-d-old rat embryos (VanAerts *et al.* 1994). The teratogenic effect of homocysteine may be associated with the specific ability of homocysteine to inhibit conversion of retinal to retinoic acid which could regulate gene expression prior to and during rapid trophoblastic elongation in the porcine conceptus and also be involved in the development and growth of the porcine placenta (Yelich *et al.* 1997; Limpach *et al.* 2000; Johansson *et al.* 2001). According to Menezo *et al.* (1989), during early embryonic development of the mouse, homocysteine would act also as an inhibitor of methylation, would decrease the uptake of methionine and the *S*-adenosyl methionine pool, and would partially impair blastocyst formation.

Several aspects of folate and homocysteine metabolism in early pregnancy appear to be influenced by the Meishan genotype. In the present study, multiparous ML sows had lower concentrations of plasma homocysteine although multiparous LD and ML sows had similar concentrations of plasma THF, 5-methyl-THF, vitamin B_{12}, P5P and methionine. Such result suggests that the remethylation pathway is more efficient in multiparous ML than in LD sows (Scott, 1999). In human subjects, polymorphisms of methionine synthase and methylene tetrahydrofolate reductase have been identified. Mutations in human methionine synthase and methylene tetrahydrofolate reductase genes (A2756G and C677T mutations respectively) are a fairly common polymorphism and have been significantly associated with modified homocysteine concentrations (Goyette *et al.* 1994; Jacques *et al.* 1996; Chen *et al.* 1997; Schwartz *et al.* 1997; Harmon *et al.* 1999). Although polymorphisms of methionine synthase and methylene tetrahydrofolate reductase have not yet been identified in pigs, it is possible that different DNA polymorphisms for methionine synthase and methylene tetrahydrofolate reductase exist between Chinese Meishan and occidental sows that could explain the homocysteine metabolism discrepancy observed between multiparous ML and LD sows (Signer *et al.* 2000).

Although the vitamin B_{12} supplement had no effect on folate and homocysteine metabolism, the vitamin B_{12} supplement did increase concentrations of plasma vitamin B_{12}, suggesting a relative efficiency of absorption of the dietary supplement. Moreover, according to the present results, the transfer of vitamin B_{12} towards the uterus is an important process in early pregnancy of pigs. Indeed, the total content of vitamin B_{12} in uterine horns represents 180–300 % of the total content in plasma, assuming that the total volume of plasma represents 4 % of pig body weight (Matte & Girard, 1996). In human subjects, the receptor of transcobalamin II–vitamin B_{12} complex is present in placental tissues (Friedman *et al.* 1977), allowing active transport of vitamin B_{12}. In pigs, the receptor of transcobalamin II has not yet been characterized in endometrial and placental tissues, but transcobalamin I was identified in endometrial tissues (Pearson *et al.* 1998). Although there was no effect of vitamin B_{12} supplement on homocysteine metabolism when sows were fed a dietary supplement of FA, such a large additional uterine transfer of vitamin B_{12} suggests an important requirement for embryo development and for uterine metabolism during first 15 d of gestation.

At the endometrial level, multiparous ML and LD sows had similar uterine flush content of vitamin B_{12}, homocysteine and methionine. For 5-methyl-THF, multiparous ML sows had a higher uterine flush content than multiparous LD sows in spite of similar plasma concentrations. Such results suggest a more efficient uterine transfer of 5-methyl-THF in ML sows. One component of the mechanism controlling folate delivery to tissues are folate-binding proteins (Antony, 1996) that have been isolated in porcine endometrium during first 15 d of gestation (Vallet *et al.* 1999*b*). However, such an explanation is unlikely to apply in the present case since Vallet *et al.* (1999*a*) have shown that the amount of folate-binding protein in uterine secretions are similar between pure-bred Meishan and Large White sows from day 10–15 of pregnancy. Alternatively, it is possible that accumulation of 5-methyl-THF in the uterine flush of multiparous ML sows was due to a reduced utilization of 5-methyl-THF for conceptus cellular division in early pregnancy. It is recognized that Meishan conceptuses are smaller and have fewer cells compared with Yorkshire conceptuses on day 12–15 of gestation (Rivera *et al.* 1996; Wilson & Ford, 1997).

In the present experiment, multiparous LD and nulliparous YL sows differed not only by their nulliparous status, but also by a heterosis effect (multiparous LD *v.* nulliparous YL). Nevertheless, it was assumed that, in such a situation, the nulliparous status was the dominant factor in the difference between multiparous LD and nulliparous YL sows (Johnson, 1981; Yen *et al.* 1987). Moreover, in nulliparous YL, lower concentrations of THF and 5-methyl-THF agree with previous results reported by Guay *et al.* (2002), where concentrations of total folates were lower in nulliparous YL compared with multiparous YL sows. The lower plasma concentration of folates in nulliparous YL as compared with multiparous LD sows may be due to a greater utilization of folates required for both gestation and growth (Matte *et al.* 1993). An age effect was also reported by Natsuhori *et al.* (1996), who

showed that plasma THF and 5-methyl-THF were higher in adult pigs than in 158-d-old pigs. This low concentration of plasma folates during the growing period could also be linked to an increase of folate catabolism, as observed in rats (McNulty *et al.* 1995).

Plasma homocysteine was lower in nulliparous YL than in multiparous LD sows. In nulliparous YL sows, the increased utilization of methionine to sustain protein synthesis and rapid growth (Whittemore *et al.* 1988) is likely to reduce the amount of circulating methionine and methionine available for methyl transfer. Moreover, it is known that the activity of methionine synthase and betaine methyltransferase decreases with ageing, whereas the activity of P5P-dependent cystathionine β synthase (Finkelstein, 1990), an alternative pathway to catabolism of homocysteine to cysteine, increases (Nakagawa & Kimura, 1968). It is possible that in nulliparous YL sows, the reduced level of free methionine combined with intense activity of methionine synthase would bring about a decrease in concentrations of homocysteine, whereas in multiparous LD sows, the lower activity of methionine synthase, along with a lower concentration of P5P, an essential cofactor for the activity of cystathionine β synthase (Smolin *et al.* 1983), increases concentrations of homocysteine.

Conclusion

The reduction of systemic and uterine homocysteine induced by the FA supplement might be a mode of action of this vitamin in the uterine environment and on embryonic development in occidental sows. In multiparous ML sows, low circulating homocysteine suggests an intrinsically more efficient remethylation pathway of homocysteine which did not seem to be dependent on folate or vitamin B_{12} status. In nulliparous sows, the high requirement for both gestation and growth would decrease the amount of methionine available for methyl transfer, decreasing the formation of homocysteine. Finally, the important uterine transfer of vitamin B_{12} during pregnancy merits further investigations with regard to its metabolic role in embryonic development.

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