Determining the effects of season on ovarian development and early pregnancy returns

Report prepared for the Co-operative Research Centre for an Internationally Competitive Pork Industry

By

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Executive Summary

Domestic sows commonly experience a depression in fertility during late summer and early autumn. Commonly referred to as seasonal infertility, summer depression of fertility is manifested as a reduced proportion of gilts reaching puberty, extended weaning-oestrus intervals in weaned sows and high anoestrus rates in gilts and sows, as well as increased rates of regular and irregular returns (particularly days 25 – 32 post insemination). While the seasonal effects of photoperiod are relatively well known, growing evidence suggests that exposure to high ambient temperatures and the resultant heat stress negatively affect ovarian function and early embryo development. Betaine, a widely available dietary supplement, acts as a potent organic osmolyte and a major dietary source of the methyl groups required for methionine formation and subsequent DNA methylation. DNA methylation is especially important during embryogenesis. Betaine also acts as an osmoprotectant, increasing the water retention capacity of gut and muscle tissue in pigs, and has the potential to reduce susceptibility to dehydration in response to high ambient temperature, and increase thermotolerance. In total, three studies have been conducted as part of this project.

In summary, the current data demonstrate that betaine supplementation prior to mating can improve reproductive performance of gilts. In particular betaine supplementation reduces the interval from boar exposure to puberty attainment, and appears to alleviate the negative effects of high temperatures on ovulation rate. The failure of betaine supplementation to increase embryo number may reflect the fact that gilts were mated at their pubertal oestrus, resulting in uterine restrictions on embryo survival. Importantly, it is possible that maintaining betaine supplementation after mating would have resulted in an improvement in embryo survival during the period of high temperature. Further research should, therefore, focus on the effects of betaine supplementation on ovulation rate, oocyte quality and embryo survival under conditions of heat stress, preferably under conditions where environmental temperature can be controlled.

The aim of the second study was to determine whether supplementing summer gestation diets with betaine would reduce incidences of irregular returns in summer as well as increasing litter size. A total of 450 sows (parities 1 to 7) were used in this study. Sows were mated between 11th January and 11th February 2008, and were fed either a standard gestation diet (n = 221) or betaine supplemented diet (n = 229) throughout gestation. Daily betaine intake was between 6.5 and 9 g, depending on gestation feeding level. Overall, the current data demonstrate that total litter size and born alive are increased by 0.6 and 0.5 piglets, respectively, when gestation diets are supplementation with betaine during gestation. Importantly, the positive effects of betaine supplementation on litter size are most pronounced in older sows (parity 3 - 7), with total litter size and born alive increased by 1.6 and 1.2 piglets, respectively, compared to control sows of similar parities. The current data demonstrate that adding betaine to gestation diets can significantly improve litter size, and at a marginal price (~ $2.80 per gestation). However, future research is required to maximise the potential benefits of this technology. In particular it is important that the mechanism(s) mediating the beneficial effects of betaine supplementation on litter size are identified.

The primary aims of the third study were: one, to determine whether early or late disruption of pregnancy is related to alterations in progesterone during the peri-ovulatory and implantation periods; and two, to determine if there are seasonal differences in luteal progesterone levels in early pregnancy. To this end, blood samples were collected from 240 sows (120 in summer and 120 in winter) every 4 days between day 3 and 31 of pregnancy. The key findings were that progesterone levels were significantly higher on days 3 and 7 post insemination in summer compared to winter mated sows, and that progesterone levels were significantly lower on days 15 to 23 post-insemination in summer compared to winter mated sows. Together, these findings indicate that progesterone secretion by the luteal cells is altered during summer. Further, the elevated progesterone levels during the first 7 days post-insemination indicate that ovulation may be occurring earlier relative to first detection of behavioural oestrus during summer, which may require artificial insemination protocols to be altered to accommodate this.

Based on these data, a number of research opportunities have been identified:

1. To determine whether betaine increases litter size when added to the gestation diets of sows mated in winter
2. To determine the optimal dose of betaine required to maximise the increase in litter size
3. To determine the mechanisms whereby betaine is improving litter size, and thus the optimal timing of its inclusion in gestation diets
4. Further identification of the interaction between dietary levels of betaine and folate, and their effects on embryo and foetal survival may make further improvement in litter size possible
5. To determine whether seasonal differences exist in the timing of ovulation relative to the onset and duration of behavioural oestrus, with the view to generating recommendations for insemination protocols during summer
6. To determine if the development and function of luteal cells are different in summer compared to winter, and to investigate the existence of an interaction between luteal cell function and peri-implantation embryo development
7. To conduct a further bleeding trial on a different facility, to confirm the seasonal differences in progesterone levels observed in this study

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1. Introduction

Domestic sows commonly experience a depression in fertility during late summer and early autumn. Referred to as seasonal infertility, summer depression of fertility is manifested as a reduced proportion of gilts reaching puberty, extended weaning-oestrus intervals in weaned sows and high anoestrus rates in gilts and sows, as well as increased rates of regular and irregular returns (particularly days 25 – 32 post insemination) (Tast et al., 2001; Peltoniemi et al., 2005).

Changes in photoperiod drive seasonal breeding cycles, and the domestic pig resembles the sheep by being a short day breeder. Current understanding of the mechanisms underlying seasonal infertility in the domestic pig is poor. However, Peltoniemi and Virolainen (2006) suggested it was unlikely the central mechanisms mediating seasonal effects on reproductive activity would differ between pigs and other short-day breeders (e.g. sheep). Nocturnal secretion of melatonin by the pineal gland is the underlying mechanism whereby changes in photoperiod synchronise the start of the breeding season (Lincoln, 1992; Peltoniemi and Virolainen, 2006). Melatonin has a stimulatory effect on episodic release of gonadotrophin releasing hormone (GnRH) and luteinising hormone (LH), most likely through the suppression of the negative feedback effects of oestrogen on the GnRH pulse generator. Melatonin production is reduced during exposure to long photoperiods (i.e. during the summer months) essentially suppressing episodic LH release and thus restricting reproductive activity. Studies involving sheep demonstrate a significant reduction in LH pulsing during the summer (anoestrous) period, with additional studies indicating that the depth of this anoestrus (i.e. the frequency of episodic LH release) has a major influence on the ewe's ability to exhibit ovarian follicle growth and ovulation in response to oestrus stimulation such as ram contact (Martin et al., 1980; Martin et al., 1986).

While the seasonal effects of photoperiod are relatively well known, there is growing evidence from studies in cattle that high ambient temperatures and the resultant heat stress negatively affect ovarian function and early embryo development (Wolfenson et al., 2001). There is now considerable evidence that the growth of the oocyte prior to ovulation, in particular the environment experienced during growth and maturation, has a profound influence on its ability to undergo the early stages of embryo development and survive implantation (Prunier and Quesnel, 2000; Ferguson et al., 2003; Hunter et al., 2004). More specifically, the developmental competence of the oocyte determines its ability to successfully complete the first six to nine days of embryonic development, and the ovulation of meiotically immature oocytes is responsible for a large portion of the embryo losses that occurs prior to day 10 of pregnancy (Geisert and Schmitt, 2002).

Despite a lack of data in the pig, seasonal reductions in the fertility of dairy cattle have been attributed to a decrease in the size and composition of the antral follicle pool, suppression of ovarian steroidogenesis and the final stages of follicle growth, as well as a reduction in oocyte quality, impaired embryo quality and altered progesterone secretion post-ovulation (Zeron et a., 2001; Rensis and Scaramuzzi, 2003; Silva et al., 2006). Although season has little or no effect on conception, gilts and sows affected by seasonal infertility appear to lose embryos during the implantation period, causing the loss of the whole litter (Love et al., 1993). Additionally, studies involving cattle indicate that high ambient temperatures during the pre-implantation period impair embryonic secretion of signals required for maternal recognition of pregnancy and CL maintenance (Wolfenson et al., 2000), and that exposure to high ambient temperatures during the implantation period itself can compromise pregnancy outcomes and increase the risk of foetal losses (Garcia-Ispierto et al., 2006). It is likely that failure of both the first and second embryonic signals for maternal recognition of pregnancy may reflect impaired development within the cohort of developing embryos, and that absolute failure of the signal required for the continuation of pregnancy may occur if a high proportion of the developing embryos are of a poor quality. Importantly, studies in cattle demonstrate that heat stress during the summer months actually manifests as alterations in ovarian function, oocyte quality and early embryo development during the cooler autumn months (Wolfenson et al., 2001).

Betaine and folic acid are both major dietary sources of methyl groups required for methionine formation and subsequent DNA methylation (Lindemann, 1993; Niculescu and Zeisel, 2002). DNA methylation is especially important during embryogenesis (Niculescu and Zeisel, 2002; Valee et al., 2002), and large quantities of choline, the precursor of betaine, are transported to the developing embryo, with low betaine levels associated with abnormal pregnancies in humans (Velzing-aarts et al., 2005). As well as increasing feed utilisation and increasing uptake of nutrients from the diets, betaine acts as an osmoprotectant, increasing the water retention capacity of gut and muscle tissue in pigs (Eklund et al., 2005). Interestingly, the addition of betaine to sow lactation diets has been demonstrated to increase subsequent litter size; however, it is not known whether this reflects an increase in the efficiency of feed utilisation or a direct effect on reproductive function. Therefore, in addition to a potential direct beneficial effect on litter size, dietary betaine supplementation of pre-mating and gestating diets has the potential to reduce susceptibility to dehydration in response to high ambient temperature, thus increasing thermotolerance and 'protecting' the pig from the negative effects of heat stress.

In total three studies were conducted as part of this project. Study one determined whether dietary betaine supplementation can improve puberty attainment and embryo development in replacement gilts and whether there is a season x betaine interaction. The aim of study two was to determine whether supplementing summer gestation diets with betaine will reduce incidences of irregular returns in summer as well as increasing embryo survival and litter size. The third study had two primary aims; one, to determine whether early and/or late disruption of pregnancy is related to alterations in progesterone profiles during the peri-ovulatory and implantation periods; and two, to determine if there are seasonal differences in luteal progesterone levels in early pregnancy.

2. Methodology

Study 1: Betaine supplementation of pre- and peri-pubertal diets will alleviate the negative effects of high summer temperatures on reproductive performance of replacement gilts

This experiment was conducted at the University of Adelaide's piggery, Roseworthy, South Australia, with approval from the animal ethics committee of The University of Adelaide. The experimental design was a 2 x 2 factorial, incorporating two seasons at mating (summer versus winter) and two feeding treatments (standard versus betaine supplementation). The experiment used 168 purebred maternal (Large White) / terminal (Duroc) line gilts, and was conducted in two replicates per season (n = 21 gilts / treatment / replicate). Replicates one and two were run in June to August 2007 and July to September 2007, respectively (winter / spring), replicates three and four were run in December 2007 – February 2008 and January – March 2008, respectively (Summer / autumn).

Animals, housing and feeding

At 18 weeks of age, gilts were weighed, stratified according to weight and allocated to receive either a standard diet or a betaine supplemented diet (6 g betaine / day). Between 18 and 21 weeks of age, gilts received 3.0 kg per day of a standard finisher diet (14 MJ/kg DE, 15% Protein, 0.065 av. Lys/ MJ DE). At 21 weeks of age, gilts commenced their dietary treatments, and received 3.0 kg of food per day. The daily ration was split into two feeds, a morning and an afternoon feed, with the daily ration spread on the floor of the pen.

Gilts were housed in finisher pens from selection through to their first artificial insemination (AI), and between 21 and 25 weeks of age were penned in groups of seven. The finisher sheds contained no male pigs, and were fitted with adjustable side blinds. Following their first AI gilts were moved to individual stalls, where they remained until slaughter. From their second AI through to slaughter gilts received 2.3 kg/day of a dry sow diet (13.0 MJ DE, 14% protein, 0.5 g available lysine/MJ).

Boar contact and oestrus detection

From selection at 18 weeks of age until the commencement of boar exposure gilts had no contact with male pigs. From 22 weeks of age through to 25 weeks of age, gilts were checked daily, in their pens and without boar contact, for vulval swelling and reddening as well as signs of behavioural oestrus. All gilts commenced boar exposure at 25 weeks of age. Each group of gilts, as penned, were taken daily to a detection-mating area (DMA), where they received twenty minutes of supervised full contact with a vasectomized boar. Boar exposures began at 08:00 hours. Three vasectomized boars, greater than 10 months of age, were used in rotation. The DMA was situated in a separate building approximately 100 m from the grower rooms in which gilts were housed, and consisted of four pens measuring 3 m × 3.5 m and lined on two sides by inward facing boar pens. The attainment of puberty was defined as the first signs of a standing reflex, either in response to the backpressure test or mounting by the boar, and the timing of the pubertal oestrus was recorded for all gilts. Days-to-puberty refers to the number of days from the start of boar

exposure to the start of the pubertal oestrus. Gilts not detected in oestrus by 35 days after commencement of boar exposure were slaughtered to confirm pre-pubertal status.

Artificial inseminations

All gilts received two AIs, once at detection of oestrus and again 24 hours later. All AIs took place in the DMA, with fence-line contact with a boar during the procedure. Inseminations were performed as per standard industry practice using disposable spirette catheters, with each insemination consisting of an 80 ml dose of fresh, extended semen (3 $\times 10^9$ spermatozoa per inseminate≤4 -day-old). Semen used for this experiment was purchased from a commercial artificial insemination collection centre (SABOR Pty. Ltd., Clare, South Australia).

Animal measurements

Gilt liveweight and body composition measurements

Gilts were weighed prior to being fed and P2 backfat depth and maximum eye muscle depth (MMD) were measured at 18, 21 and 25 weeks of age, and also at the start of the pubertal oestrus and at slaughter. P2 backfat and MMD were measured over the last rib 65 mm down from the vertebrae by an experienced operator using a 3.5 MHz linear probe (Piemedical).

Reproductive parameters: ovulation rate and embryo measurements

All gilts were slaughtered at a commercial abattoir. In the winter treatment, 11 gilts per dietary treatment were slaughtered on day 5 post-mating (where day 0 refers to the first 24 hours after oestrus was initially detected), and the reproductive tract of each gilt recovered. Embryos were retrieved by treating each uterine horn to two consecutive flushes of 20 ml of saline (O'Leary et al., 2004). Embryos were transported to the lab, where their developmental stage was recorded and cell number was counted following staining with Geimsa. However, due to inaccuracies relating to timing of embryo collection relative to time of ovulation, it was decided not to collect day 5 embryos from gilts in the summer treatment block.

In the winter and summer treatment groups, 30 and 42 gilts / treatment, respectively, were slaughtered 30.6 ± 0.14 days after their first mating (mean ± S.E.M.) and the reproductive tract of each gilt recovered. The ovaries were weighed and where necessary dissected to determine the number of corpora lutea. The number of corpora lutea was taken to represent the number of oocytes ovulated at the oestrus of AI. The uterus was trimmed of mesentery and dissected, the number of viable and non-viable embryos recorded, and embryo crown-to-rump length measured. Embryos were described as viable or non-viable based on their gross morphology and crown-to-rump length—embryos were classified as non-viable if their crown-to-rump length was more than two standard deviations less than the mean for that gilt. Each embryo was weighed, and the placenta for each embryo was dried and weighed. The embryo:placental weight ratio was calculated as a measure of placental efficiency. The total number of viable embryos observed in both uterine horns was expressed as total embryo number. Embryo survival

was calculated based on the total number of viable embryos, and expressed as a percentage of the number of CLs observed in both ovaries. The uterine horns were weighed after removal of all conceptus tissue to provide an empty uterine weight.

Statistical Analysis

Values in the text are expressed as mean ± standard error. A general analysis of variance model, with block built in, housing pen and day of gestation at slaughter included as co-variates, was used to study the effects of dietary treatment and season on days-to-puberty, ovulation rate, embryo number and embryo survival, as well as embryo crown-rump length, embryo weight and placental efficiency. Between treatment differences were examined using least significant difference. All analyses were performed using Genstat, eighth edition (Committee of the Statistics Department, Rothamsted Experimental Station, Harpenden).

Study 2: Supplementing gestation diets with betaine will protect the animal from heat stress, therefore alleviating summer suppression of fertility and increasing litter size

Animals, housing and feeding

A total of 450 primiparous and multiparous sows were used in this study. Sows were selected from 10 mating days: 11th, 14th, 18th, 21st, 25th and 28th January and 1st, 4th, 8th and 11th February 2008. All sows had a weaning-to-first mating interval of 4 days. Sows were weighed within 2 days of their first artificial insemination (AI), stratified according to weight and parity and allocated to receive either a standard gestation diet (Control: n = 221) or a betaine supplemented gestation diet (Betaine: n = 229). All sows were fed at the same level during gestation (**Table 1**). The Betaine diet was first fed from day 3 ± 1 of gestation, with betaine inclusion rate altered during gestation to ensure a daily intake of between (7.6 and 9.0 g / sow; **Table 1**). The specification of the diet used is detailed in **Table 2**

Table 1 Daily feed intake and betaine intake during gestation for Control and Betaine sows.

Period of gestation	Daily feed intake (kg / sow)	Betaine inclusion rate (g / kg feed)	Daily betaine intake (g / sow / day)
Day 3 – 42	1.9	4	7.6
Day 43 – 84	2.5	3	7.5
Day 85 - Farrowing	3.0	3	9.0

Table 2 Specifications of diet fed during gestation

	Inclusion rate
Digestible energy (MJ/kg)	13.02
Crude Protein (%)	15.07
Fat (%)	4.41
Crude Fibre (%)	4.80
Lysine (%)	0.674
Methionine (%)	0.253
Choline (mg / kg)	1335.23

Animal measurements

Sows were weighed, and P2 backfat and maximum eye muscle depth (MMD) measured on day 2 after mating, on day 1 of lactation (where day 0 = day of farrowing) and on the day after weaning. P2 backfat and MMD were measured over the last rib 65 mm down from the vertebrae by an experienced operator using a 3.5 MHz linear probe (Piemedical). For all sows, total litter birthweight was recorded within 24 hours of farrowing. For a subset of sows (n = 85 litters / treatment), live born piglets were weighed individually within 24 hours of farrowing. For all sows, total litter weaning weight was recorded on day 24 of lactation.

Reproductive parameters

Sows returning to oestrus approximately 21 days after first artificial insemination were identified and recorded as regular returns. Transcutaneous ultrasound was performed on day 35 of gestation to determine pregnancy status, the number of pregnant and non-pregnant sows was recorded. At farrowing, the total number of piglets born was recorded. The number of piglets born alive, stillborn and mummified were also recorded.

Statistical analysis

Values in the text are expressed as Mean ± S.E.M. A general analysis of variance model, with block built in, was used to study the effects of dietary treatment and sow parity on all variates. Between treatment differences were examined using least significant difference. All analyses were performed using Genstat, eighth edition (Committee of the Statistics Department, Rothamsted Experimental Station, Harpenden).

Study Three: Determining the existence of a correlation between peri-ovulatory and implantation circulating progesterone concentrations and incidences of summer induced early disruption of pregnancy

Animals, housing and sample collection

A total of 224 primiparous and multiparous sows were used in this study, which was conducted in summer and winter/spring. In summer, 111 sows were randomly selected from 3 mating days: 5th, 8th and 12th January 2008. In winter / spring, 113 sows were randomly selected from four mating days: 8th and 15th August 2008 and 26th and 29th September 2008. Plasma samples were collected by jugular venipuncture on day 3, 7, 11, 15, 19, 23, 27 and 31 post-mating, where day 0 = the first 24 hours after first detection of oestrus and artificial insemination. All sows were artificially inseminated at their first oestrus after weaning, were housed individually in stalls until approximately day 42 of pregnancy and then moved to concrete floored, group housing facilities. During the course of pregnancy, animals were classified according to pregnancy outcomes into the following four groups;

- Regular returns, those expressing oestrus approximately 21 days after first AI;
- Irregular returns, those showing no signs of oestrus on day 21 but determined to be not-pregnant by ultrasound on day 35 of pregnancy;
- Late-pregnancy failures, those sows determined to be pregnant on day 35 of pregnancy but losing their litter prior to farrowing;
- Farrowings, those sows successfully completing their pregnancy

Plasma samples from all sows classified as regular returns, irregular returns and late pregnancy failures, as well as 64 farrowing sows (32 from the summer block and 32 from the winter/spring block) were selected for analysis of progesterone on all sampling days. In addition, samples collected from the remainder of farrowing sows on days 3 post-AI were analysed for progesterone. Following analysis of progesterone profiles, sows were re-classified into six categories:

- Regular returns or conception failures: sows displaying a progesterone profile typical of the luteal phase of the porcine oestrous cycle
- Irregular returns or early disruption of pregnancy: sows with an extended luteal phase and elevated progesterone on day 19 post-AI
- Late pregnancy failures: sows with elevated progesterone on day 31 post-AI but losing their pregnancy prior to farrowing shed entry
- Sows inseminated at the inappropriate time: as evidenced by excessively high progesterone profiles, or declining progesterone levels during the 7 days post-AI – Data not shown in this report
- Anoestrus sows: those with no progesterone present in samples during the first 11 days post-AI.
- Farrowed: those sows with elevated progesterone profiles on day 31 post-AI and farrowing a litter of piglets

Classification of elevated progesterone profiles, and evidence of a luteal activity, was based on a progesterone level in excess of 5 – 9 ng/ml. Classification of regular returns /

conception failures and irregular returns / early disruption of pregnancy conducted based on profiles reported by Peltoniemi et al. (2002).

In addition, a subset of samples from regular returns, irregular returns, late pregnancy failures, and farrowed sows were assayed for the following metabolites: IGF-I, samples collected on days 3 and 15 post-AI; homocysteine, samples collected on days 3, 15 and 31 post-AI; and oestrone sulphate, samples collected on days 23 and 27 post-AI.

Reproductive measures

For all sows from which samples were collected the following measures were recorded: litter size (total born, born alive) at the previous farrowing; lactation length and number of piglets weaned; the interval from weaning to oestrus and successful artificial insemination; subsequent litter size (total born, born alive)

Metabolite hormone assays

Plasma Progesterone was assayed in 25ul of sample by radioimmunoassay using reagents obtained from Beckman Coulter/DSL, according to the manufacturer's instructions. Plasma Oestrone sulphate was assayed in 100ul of sample by radioimmunoassay using reagents obtained from Beckman Coulter/DSL, according to the manufacturer's instructions. Plasma Homocysteine was assayed in 25ul of sample by enzyme immunoassay using reagents obtained from BioRad, according to the manufacturer's instructions. Briefly in this assay, protein-bound Homocysteine is reduced to free Homocysteine, and is enzymatically converted to S-adenosyl-L-homocysteine (SAH) in a separate procedure prior to the immunoassay.

IGFs were extracted from plasma by size exclusion high performance liquid chromatography at pH 2·5, using a modification of the original procedure (Scott & Baxter 1986), as described previously (Owens, et al. 1990). Four fractions of eluate (fraction 1, containing IGFBP; fraction 2, inter-peak; fraction 3, containing IGF; and fraction 4, post-peak) were routinely collected for each acidified plasma sample, using collection times based on elution times of ^{125}I-labelled IGF-I and IGF immunoreactivity. Maternal plasma samples were extracted in three HPLC runs with an average recovery of ^{125}I-IGF-I of 81.8 ± 0.0% (mean ± SEM). Samples were assayed in triplicate in each assay. Plasma IGF-I concentrations were measured by analysis of neutralised HPLC fraction 3, in an RIA specific for IGF-I (Francis et al., 1989), using a rabbit polyclonal antibody to human IGF-I (GroPep, Australia). Total IGFBP concentrations were measured by analysis of neutralised fraction 1 in the same assay. Because IGFBP bind to and sequester ^{125}I-IGF-I in this assay, they can be measured due to their effect of reducing the amount of ^{125}I-IGF-I in the immunoprecipitated pellet, giving an apparent IGF concentration that reflects the total amount and binding affinity of IGFBP present in plasma. Covariance for IGF-I extraction and assay of a maternal porcine plasma QC containing 131 ng/ml IGF-I and included in each HPLC run of maternal samples was 18.9 %, with an intra-assay CV of 7.2% (n = 3 HPLC runs, 5 assays). Covariance for total IGFBP determined in neutralised fraction 1 from the same QC samples was 7.9%, with an intra-assay CV of 6.5% (n = 3 HPLC runs, 5 assays).

Statistical analysis

Values in the text are expressed as Mean ± S.E.M. Plasma progesterone and metabolite samples were analysed using a linear mixed model (REML) with repeated measures. Pig was considered as the random effect and day of sample, pregnancy status (conception

failure versus early disruption of pregnancy versus pregnant) and season (summer versus winter) as the fixed effects to test the effects of pregnancy status and season on progesterone and metabolite concentration. A two-way analysis of variance (ANOVA) was used to test the effects of season and pregnancy status on previous litter size, previous lactation length, number of piglets suckled at the previous lactation and weaning-to-oestrus interval. A one-way analysis of variance was used to test the effect of season on subsequent litter size of pregnant sows. A linear regression model was used to compare progesterone levels on days 3 and 11 post-AI with subsequent litter size. All analyses were performed using Genstat, tenth edition (Committee of the Statistics Department, Rothamsted Experimental Station, Harpenden).

3. Outcomes

Study 1: Betaine supplementation of pre- and peri-pubertal diets will alleviate the negative effects of high summer temperatures on reproductive performance of replacement gilts

General results

Of the 168 gilts allocated to this experiment, one gilt was removed from the trial as a result of locomotive disorders. The body composition data, puberty attainment data relates to 84 and 83 gilts from the Control and Betaine treatments, respectively. The ovulation rate, embryo number, survival and embryo-placenta data relates to 72 and 71 gilts from the Control and Betaine treatments, respectively.

Growth characteristics

There was no effect of dietary treatment on gilt liveweight, P2 backfat, MMD or average daily liveweight gain (ADG) during the experimental period (**Table 3**); however, betaine gilts gained significantly more ($P < 0.05$) P2 backfat between weeks 21 and 25: 2.0 ± 0.16 versus 1.4 ± 0.23 mm). There was a significant ($P < 0.05$) effect of season on gilt body composition, with gilt liveweight, P2 backfat and MMD at 21 and 25 weeks of age higher in the summer compared to the winter blocks (Table 3). There was no effect of season on ADG or gain of P2 backfat; however, MMD gain was significantly higher ($P < 0.05$) in the winter compared to summer blocks (7.1 ± 0.50 versus 5.1 ± 0.68 mm; Table 3). Gilt liveweight at puberty attainment was similar for Control and Betaine gilts (114.5 ± 0.99 and 114.7 ± 0.94 kg), but tended ($P = 0.06$) to be higher in summer compared to winter (116 ± 1.06 versus 113.0 ± 0.83 kg).

Table 3 Effect of dietary treatment (Control versus Betaine) and season (winter versus summer) on gilt liveweight, P2 backfat and MMD

	Dietary treatment		Season		*Pooled SEM*
	Control (n = 84)	Betaine (n = 83)	Winter (n = 84)	Summer (n = 83)	
Body composition: 21 weeks of age					
LW (kg)	85.4	85.9	83.4[a]	87.9[b]	*0.60*
P2 backfat (mm)	10.7	10.3	10.0[a]	10.9[b]	*0.26*
MMD (mm)	49.0	48.1	47.5[a]	49.6[b]	*0.77*
Body composition change					
ADG (kg / day)	0.84	0.85	0.84	0.84	*0.03*
P2 backfat (mm)	1.4[a]	2.0[b]	1.9	1.6	*0.21*
MMD (mm)	5.5	6.7	7.1[b]	5.1[a]	*0.68*
Body composition: 25 weeks of age					
LW (kg)	108.4	109.1	106.9[a]	110.5[b]	*0.75*
P2 backfat (mm)	11.9	12.3	11.7[a]	12.5[b]	*0.29*
MMD (mm)	54.6	54.8	54.6	54.8	*0.47*

[ab] within row, and main effect, indicates differences between means: $P < 0.05$

Puberty attainment

Days-to-puberty were significantly shorter (P < 0.05) for Betaine compared to Control gilts (7.5 ± 0.74 versus 9.6 ± 0.82 days), but this was unaffected by season (**Table 4**). Treatment effects on the timing and synchrony of puberty attainment are described in Figure 1. The pattern of puberty attainment was similar for Control and Betaine gilts, and was unaffected by season (Figure 1).

Table 4 Effects of dietary treatment (Control versus Betaine) and season (winter versus summer) on days-to-puberty and gilt liveweight (LW) at puberty

	Dietary treatment		Season		Pooled SEM
	Control (n = 84)	Betaine (n = 83)	Winter (n = 84)	Summer (n = 83)	
Days-to-puberty	9.6[b]	7.5[a]	8.5	8.6	0.73
LW at puberty (kg)	114.5	114.6	113.2*	115.9*	1.01

[ab] within row, and main effect, indicates differences between means: P < 0.05. *P = 0.06

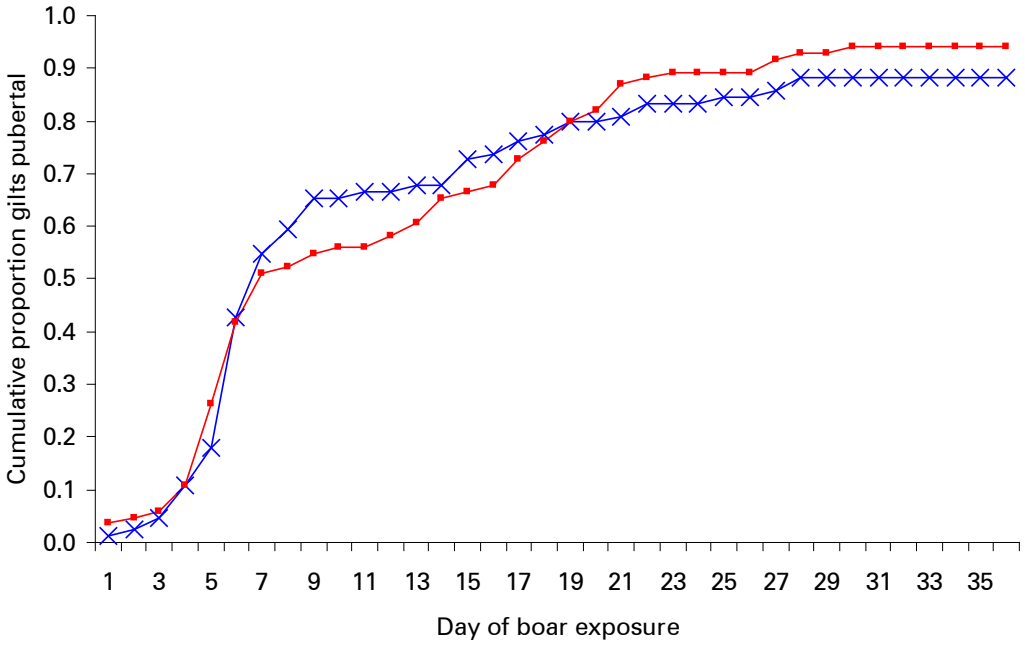

Figure 1 Cumulative proportion of gilts attaining puberty in response to daily, physical contact with a vasectomised boar. (■) Control diet; (x) Betaine supplemented diet.

Potential litter size

The effects of dietary treatment and season on ovulation rate, embryo number and embryo survival are described in **Table 5**. Ovulation rate tended to be higher (P < 0.1) for Betaine compared to Control gilts (14.1 ± 0.26 versus 13.6 ± 0.24). Ovulation rate tended to be higher in summer compared to winter (P = 0.08) (Table 5). There was an interaction between treatment and season for ovulation rate (P = 0.08), with ovulation rate higher for Betaine fed gilts in summer compared to Betaine fed gilts in winter and control fed gilts in summer and winter: 14.6 ± 0.36 versus 13.5 ± 0.35, 13.5 ± 0.34 and 13.6 ± 0.35. The number of embryos present on day 30 of gestation was unaffected by dietary treatment or season (Table 5). The proportion of embryos surviving to day 30 tended to be lower (P =

0.06) for Betaine compared to Control gilts (0.76 ± 0.02 versus 0.82 ± 0.02; Table 5), and was significantly lower (P < 0.05) in summer compared to winter: 0.82 ± 0.03 versus 0.76 ± 0.02 (Table 5).

Table 5 Effects of dietary treatment (Control versus Betaine) and season (winter versus summer) on ovulation rate, embryo number, embryo survival and ovarian weight of gilts mated at the pubertal oestrus

	Dietary treatment		Season		
	Control (n = 72)	Betaine (n = 71)	Winter (n = 80)	Summer (n = 81)	*Pooled SEM*
Ovulation rate	13.6*	14.1*	13.6	14.1	*0.26*
Embryo Number	11.1	10.7	11.1	10.6	*0.35*
Embryo survival (%)	0.82**	0.76**	0.82[b]	0.76[a]	*0.02*

[ab] within row, and main effect, indicates differences between means: P < 0.05. *P = 0.09. ** P = 0.07

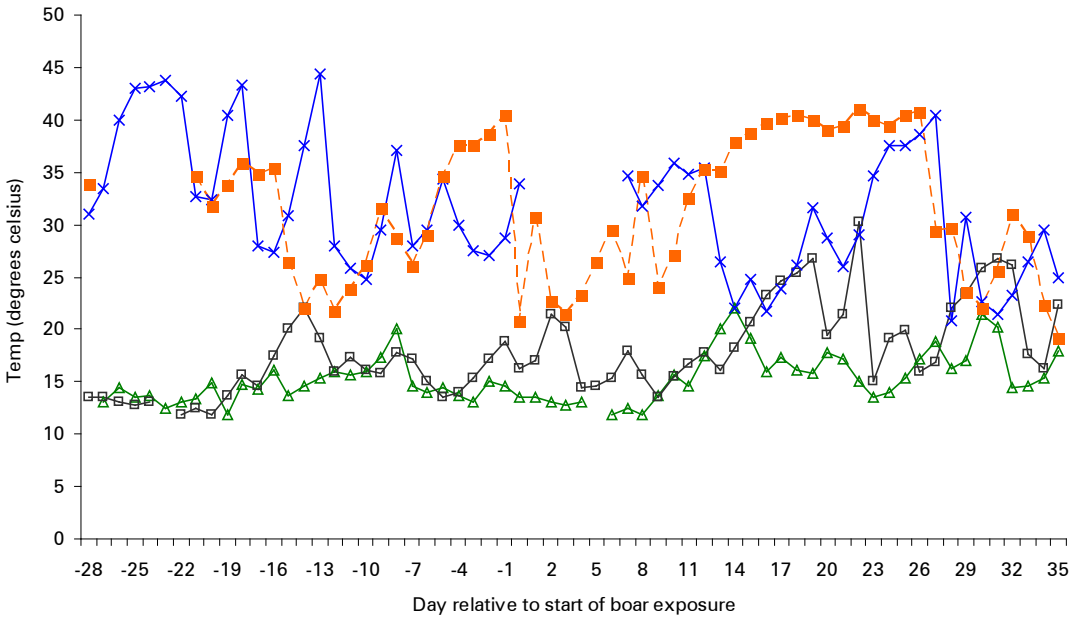

Figure 2 Mean daily room temperature from the start of dietary treatment through to the end of boar exposure. (Δ) Replicate 1 – June/August 2007; (□) Replicate 2 – July / Sept 2007; (x) Replicate 3 – Dec 2007 / February 2008; (▮) Replicate four – Jan / March 2008.

The effect of dietary betaine on ovulation rate was most evident in block 4 which coincided with a prolonged period of high ambient temperature (Figure 2). In block four, ovulation rate was significantly higher for Betaine compared to Control gilts (14.4 ± 0.50 versus 12.3 ± 0.52). A significant effect of experimental replicate on both embryo number and embryo survival was also evident. Embryo number and embryo survival were significantly lower (P < 0.05) in block four compared to blocks 1, 2 and 3: 9.1 ± 0.49 versus 11.4 ± 0.57, 10.9 ± 0.53 and 11.8 ± 0.44 embryos, and 0.69 ± 0.03 versus 0.82 ± 0.04, 0.83 ± 0.04 and 0.82 ± 0.03 %, respectively.

Study 2: Supplementing gestation diets with betaine will protect the animal from heat stress, therefore alleviating summer suppression of fertility and increasing litter size

Sow body composition

Sow liveweight gain during gestation and liveweight on day 1 of lactation were similar for Control and Betaine sows: 58.3 ± 1.75 and 55.7 ± 1.67 kg, and 269.7 ± 2.35 and 269.5 ± 2.29 kg (**Table 6**). There was no effect of gestation feeding treatment on MMD on day 1 of lactation or MMD gain during gestation (**Table 6**). However, compared to Betaine sows there was a tendency (P = 0.08) for Control sows to gain more P2 backfat during gestation and have a higher P2 backfat on day 1 of lactation: 5.3 ± 0.56 versus 4.0 ± 0.53 mm; 22.8 ± 0.43 versus 21.9 ± 0.42 mm (**Table 6**).

Table 6 Body composition on day of gestation and day 1 of lactation as well as body composition changes during gestation of Control and Betaine sows

	Control sows	Betaine sows
LW day 2 gestation	213.5 ± 2.69	215.5 ± 2.70
P2 day 2 gestation	18.2 ± 0.40	17.7 ± 0.40
MMD day 2 gestation	52.5 ± 0.44	52.2 ± 0.43
Gestation LW gain (kg)	58.3 ± 1.75	55.7 ± 1.67
Gestation P2 backfat gain (mm)	5.3 ± 0.56*	4.0 ± 0.53*
Gestation MMD gain (mm)	5.8 ± 1.25	3.8 ± 1.20
LW day 1 of lactation (kg)	269.7 ± 2.35	269.5 ± 2.29
P2 backfat day 1 of lactation (mm)	22.8 ± 0.43*	21.9 ± 0.42*
MMD day 1 of lactation (mm)	60.4 ± 2.90	56.5 ± 2.84

* P = 0.08

Reproductive parameters

The proportion of sows that were not pregnant on day 35 of gestation was similar for the Control and Betaine treatment (0.13 ± 0.02 and 0.16 ± 0.03; Figure 3). However, there was a tendency (P = 0.065) for a lower proportion of Betaine sows to lose their pregnancy between day 36 of gestation and 113 of gestation (0.04 ± 0.01 versus 0.06 ± 0.01; **Figure 3**).

The total number of piglets born tended to be higher (P = 0.06) for Betaine compared to Control sows (12.7 ± 0.24 versus 12.1 ± 0.25), and there was a tendency (P = 0.08) for the number of piglets born alive to be higher for Betaine compared to Control sows; 11.6 ± 0.21 versus 11.1 ± 0.22 (**Table 7**). Gestation diet had no effect on either the mean number of stillborns or mummified foetuses (**Table 7**). When sows were blocked according to two parity groups within treatment, the total number of piglets born was significantly higher (P < 0.01) for Betaine-fed parity 3 – 7 sows compared to all other sows (**Table 8**). The number of piglets born alive tended (P = 0.06) to be higher for Betaine-fed parity 3 -7 sows compared to all other sows (**Table 8**). Total litter birth weight and individual birth weights of live born piglets were similar for Betaine and Control sows: 17.6 ± 0.24 and 18.1 ± 0.25 kg, and 1.51 ± 0.02 and 1.52 ± 0.02 kg, respectively.

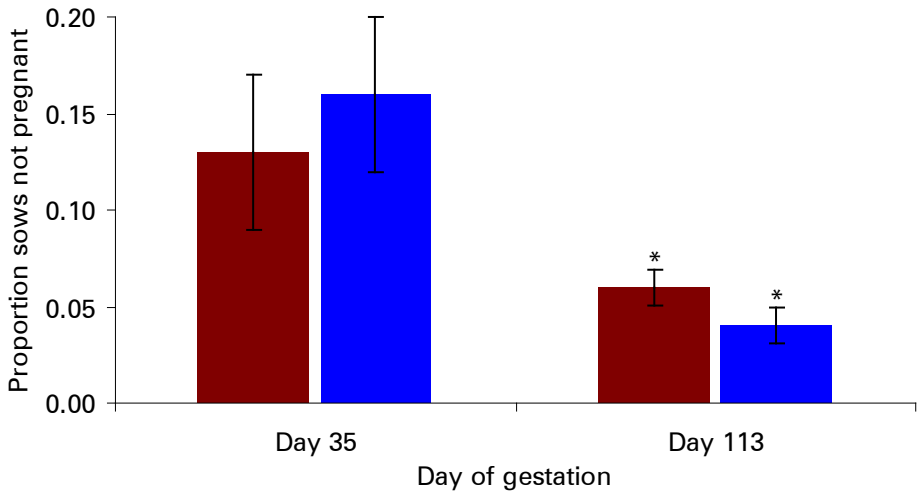

Figure 3 Proportion of Control and Betaine sows not pregnant on day 35 and day 113 of gestation; (■) represents Control sows; (■) represents Betaine sows. * P = 0.07

Table 7 Total number of piglets born, number of piglets born alive, number of stillborns and mummified foetuses for Control and Betaine sows

	Control sows (n = 174)	Betaine sows (n = 181)
Total litter size	12.1 ± 0.25*	12.7 ± 0.24*
Piglets born alive	11.1 ± 0.22**	11.6 ± 0.21**
Stillborns	1.0 ± 0.12	1.1 ± 0.12
Mummified foetuses	0.11 ± 0.04	0.16 ± 0.04

*P = 0.064; ** P = 0.08

Table 8 Total litter size and number of piglets born alive for Control and Betaine Parity 1 and 2 and parity 3 to 7 sows

	Control diet		Betaine diet	
	Parity 1 and 2 (n = 79)	Parity 3 to 7 (n = 95)	Parity 1 and 2 (n = 93)	Parity 3 to 7 (n = 88)
Total litter size	12.3 ± 0.38[a]	12.0 ± 0.30[a]	11.9 ± 0.34[a]	13.6 ± 0.35[b]
Piglets born alive	11.4 ± 0.33[c]	10.8 ± 0.30[c]	11.2 ± 0.30[c]	12.0 ± 0.30[d]

[ab] within row indicate significant difference; P < 0.05. [cd] P = 0.06

Study Three: Determining the existence of a correlation between peri-ovulatory and implantation circulating progesterone concentrations and incidences of summer induced early disruption of pregnancy

General Results: pregnancy outcomes and reproductive performance

There was no interaction between season of mating and pregnancy outcome for lactation length, weaning-to-oestrus interval or the number of piglets weaned at the previous litter, As a consequence, only the main effects of season and pregnancy outcome are presented in Table 9 and Table 10, respectively. The data presented in Table 9 demonstrate that the mean parity of the sows bled, the total number of piglets born at the previous farrowing, the number of piglets weaned, the interval from weaning to oestrus (WOI) and subsequent litter size were similar for the sows bled in summer and winter. However, previous lactation length was significantly ($P < 0.05$) shorter in summer compared to winter: 24.0 ± 0.40 versus 25.3 ±0.39 (Table 9). The number of piglets weaned at the previous lactation and weaning-to-oestrus interval were similar for all sows, regardless of pregnancy outcome (Table 9). However, the length of the previous lactation was 3.8 days shorter ($P < 0.05$) for sows subsequently experiencing conception failure compared to those maintaining their pregnancy through to farrowing (Table 9).

Table 9 Performance data (Mean ± SEM) for all sows sampled in summer and winter

	Summer	Winter
Total number of sows	111	113
Parity at Artificial insemination	3.3 ± 0.19	3.9 ± 0.20
Previous total born	11.6 ± 0.33	11.63 ± 0.32
Number piglets weaned	9.1 ± 0.29	8.7 ± 0.29
Lactation length (days)	24.0 ± 0.40[a]	25.3 ± 0.39[b]
Weaning-to-oestrus interval (days)	10.9 ± 1.36	8.1 ± 1.33
Subsequent total born	11.3 ± 0.36	11.8 ± 0.35

[ab] within row indicate significant difference; $P < 0.05$.

Table 10 Performance data (Mean ± SEM) for all sows experiencing conception failure, early disruption of pregnancy or farrowing in summer and winter

	Conception failure	Early disruption of pregnancy	Pregnant
Total number of sows	9	6	184
Lactation length	21.8 ± 1.26[a]	23.8 ± 1.48[ab]	25.6 ± 0.46[b]
Number piglets weaned	8.1 ± 1.02	8.7 ± 1.20	9.6 ± 0.37
Weaning-to-oestrus interval (days)	9.6 ± 3.9	4.8 ± 4.59	8.1 ± 1.42

[ab] within row indicate significant difference; $P < 0.05$.

The data presented in Table 11 describe the pregnancy outcomes based on physical examination (checks for oestrus post-AI) and transcutaneous ultrasound on day 35 post-AI approximately for the sows from which blood samples were collected. The data presented in Table 12 describes the pregnancy outcomes based on analysis and characterization of the progesterone profiles of the sows from which blood samples were collected.

Table 11 Timing of pregnancy losses for summer and winter mated sows based on farm records

	Summer N (%)	Winter N (%)
Total number of sows	111	113
Total number of pregnancy failures	23 (20.7%)	18 (15.9%)
Returning to oestrus ~day 21 post AI	6 (5.4%)	1 (0.9%)
Not Pregnant at ultrasound check on ~day 35	9 (8.1%)	11 (9.7%)
Pregnancy lost between ~ day 35 and farrowing	8 (7.2%)	6 (5.3%)

Table 12 Timing of pregnancy losses for summer and winter mated sows based on progesterone profiles

	Summer			Winter		
	No.	% bled	% of NIPs	No.	% bled	% of NIPs
conception failure (regular return to oestrus post-AI)	6	5.4%	60%	3	2.7%	50%
Early disruption of pregnancy (irregular return post-AI)	3	2.7%	30%	3	2.7%	50%
Late pregnancy failure	1	0.9%	10%	0	0%	0%

Early pregnancy hormone levels: progesterone and oestrone sulphate

As shown in Table 13 and Figure 4, progesterone levels were similar for conception failures, early pregnancy failures and pregnant sows on days 3, 7, 11 and 15 post-AI. However, on day 19 post-AI progesterone levels were significantly lower for conception failures compared to early pregnancy failures and pregnant sows (Table 13). On day 23 post-AI, progesterone levels were similar for conception failures and early pregnancy failure, but significantly ($P< 0.05$) higher for pregnant sows (Table 13).

The data described in Table 13 and Figure 5 demonstrate that progesterone levels on days 3 and 7 post-AI were significantly higher ($P < 0.05$) in summer compared to winter: 4.4 ± 0.46 versus 2.7 ± 0.48 ng / ml and 13.1 ± 1.02 versus 9.3 ± 1.06 ng/ml respectively. Conversely, compared to sows mated in winter, summer-mated sows possessed significantly lower ($P < 0.05$) progesterone levels on day 15 (16.7 ± 0.96 versus 19.1 ± 1.13 ng/ml), day 19 (11.5 ± 0.60 versus 13.4 ± 0.65 ng/ml) and day 23 (9.7 ± 0.48 versus 10.9 ± 0.49 ng/ml) (Table 13). There was no interaction between season and pregnancy outcomes in terms of progesterone levels on any of the days on which blood samples were collected. Comparison of plasma samples collected from all pregnant sows on day 3 post-AI, demonstrate that progesterone is significantly higher ($P < 0.01$) in summer compared to winter (4.6 ± 0.34 versus 3.3 ± 0.31 ng/ml). There was no correlation between progesterone on day 3 post-AI and the total number of piglets born or the number of piglets born alive at the subsequent farrowing.

Table 13 Progesterone levels (Mean ± SEM; ng/ml) for sows experiencing conception failure, early disruption of pregnancy and farrowing in summer and winter

Day after first AI	Progesterone (ng/ml)				
	Pregnancy outcome			Season of AI	
	Conception failure (n=9)	Early Disruption of pregnancy (n = 6)	Pregnant (n=64)	Summer (n = 42)	Winter (n=38)
D3	2.3 ± 1.03	3.4 ± 1.16	4.0 ± 0.37	4.4 ± 0.46^{b}	2.7 ± 0.48^{a}
D7	8.5 ± 2.33	10.4 ± 2.63	11.7 ± 0.81	13.1 ± 1.02^{b}	9.3 ± 1.06^{a}
D11	15.4 ± 2.41	17.5 ± 2.99	16.6 ± 1.00	16.4 ± 1.36	16.6 ± 1.41
D15	15.2 ± 3.04	19.3 ± 2.50	17.9 ± 0.78	16.7 ± 0.96^{a}	19.1 ± 1.13^{b}
D19	0.5 ± 1.32^{c}	11.4 ± 1.72^{d}	14.2 ± 0.49^{d}	11.5 ± 0.60^{a}	13.4 ± 0.65^{b}
D23	0.8 ± 1.21^{c}	2.0 ± 1.34^{c}	11.9 ± 0.37^{d}	9.7 ± 0.48^{a}	10.9 ± 0.49^{b}
D27	8.4 ± 1.40^{c}	5.4 ± 1.41^{c}	11.7 ± 0.39^{d}	10.5 ± 0.51	11.7 ± 0.51
D31	11.7 ± 1.73	10.4 ± 1.74	11.8 ± 0.38	11.3 ± 0.51	12.2 ± 0.52

Different superscripts within row, and main effect, indicate significant difference: ^{cd} between pregnancy outcomes; ^{ab} between seasons

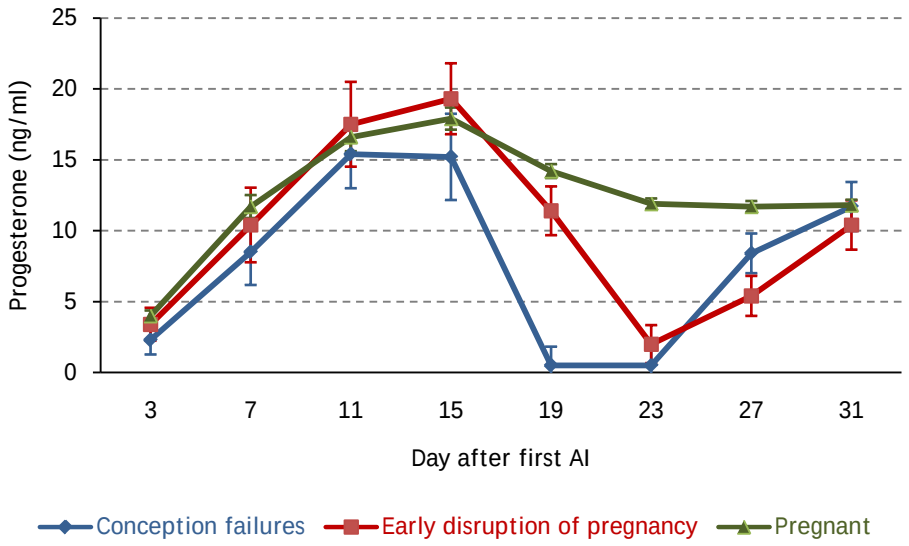

Figure 4 Progesterone levels (ng/ml) on days 3 to 31 post-AI for conception failures, early disruption of pregnancy and pregnant sows

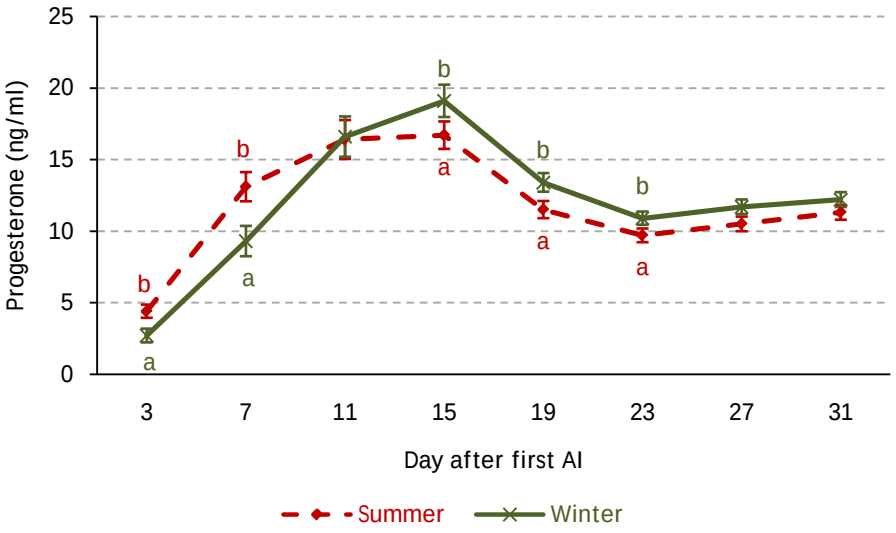

Figure 5 Progesterone levels (ng/ml) on days 3 to 31 post AI for pregnant sows mated in summer and winter ([ab] within day indicates significant difference; P < 0.05)

Plasma concentrations of oestrone sulphate were similar in summer and winter, regardless of day of collection (Table 14). However, plasma collected from pregnant sows on days 23 and 27 post-AI contained significantly higher (P < 0.05) levels of oestrone sulphate compared to samples collected from either conception failures or early pregnancy failures (Table 14).

Table 14 Oestrone Sulphate concentration (ng/ml) in plasma collected from sows in summer and winter

Day post AI	Pregnancy outcome		Season of AI		
	Conception failure	Early pregnancy failure	Pregnant	Summer	Winter
Day 19	0.1 ± 0.18	0.1 ± 0.24	0.37 ± 0.07	0.3 ± 0.09	0.3 ± 0.08
Day 23	0.1 ± 0.46[a]	0.3 ± 0.46[a]	1.6 ± 0.14[b]	1.4 ± 0.19	1.4 ± 0.19
Day 27	0.0 ± 0.73[a]	0.5 ± 0.82[a]	3.8 ± 0.23[b]	3.2 ± 0.30	3.4 ± 0.30

[ab] within row, and main effect, indicate significant difference; P < 0.05.

Early pregnancy hormone levels: metabolites

Plasma concentrations of homocysteine were similar in summer and winter, regardless of day of collection (Table 15). However, on day 3 post-AI, plasma collected from early pregnancy failures contained 6 ng /ml and 4.6 ng / ml more (P < 0.05) homocysteine compared to samples collected from conception failures and pregnant sows (Table 15). Similarly, plasma samples collected from early pregnancy failures on day 31 post-AI contained 8 ng / ml more homocysteine (P < 0.05) compared to those of conception failures (Table 15).

Table 15 Homocysteine samples (µM) in plasma collected from sows in summer and winter

Day post AI	Pregnancy outcome			Season of AI	
	Conception failure	Early pregnancy failure	Pregnant	Summer	Winter
Day 3	9.0 ± 1.61[a]	15.0 ± 1.27[b]	10.4 ± 0.47[a]	11.4 ± 0.63	10.5 ± 0.63
Day 15	9.1 ± 1.69	13.1 ± 1.33	10.9 ± 0.49	11.5 ± 0.65	10.6 ± 0.60
Day 31	5.9 ± 2.85[c]	13.9 ± 1.64[d]	11.1 ± 0.58[cd]	11.4 ± 0.83	11.0 ± 0.83

Different superscripts within row, and main effect, indicate differences: [ab] $P < 0.05$; [cd] $P = 0.053$

As shown in Table 16, plasma concentrations of IGF-I on days 3 and 15 post-AI were 14% and 18% higher, respectively, in summer compared to winter ($P = 0.097$). Although, plasma IGF-I levels were unaffected by pregnancy outcome on day 15 post-AI, IGF-I levels in samples collected on day 3 post-AI from early pregnancy failures were significantly higher ($P < 0.05$) than those collected from conception failures and numerically higher compared to those collected from pregnant sows (Table 16). On day 3 post-AI, concentrations of IGF-BP were significantly higher ($P < 0.01$) in plasma samples collected in winter compared to summer (996.3 ± 60.12 versus 875.0 ± 54.43 ng/ml), and samples collected from pregnant compared to conception failures (1008.2 ± 48.69 versus 703.4 ± 99.13 ng / ml). On day 3 post-AI, the ratio of IGF-I to BP (IGF-I: BP), which is a measure of IGF-I bioavailability was significantly higher ($P < 0.05$) in summer compared to winter, and for early pregnancy failures compare to pregnant sows (Table 16). However, there was no effect of season or pregnancy outcome on IGF-I, IGFBP or IGF-I: BP levels on day 15 post-AI (Table 16). Plasma concentrations of insulin were similar for all pregnancy outcomes, regardless of day of collection (Table 16).

Table 16 IGF-I (ng/ml), IGF-BP (ng/ml), Total IGF (IGF-I + BP; ng/ml), IGF-I: IGF-BP (%), and insulin (mU/L) in plasma collected from sows in summer and winter

Day post-AI	Pregnancy outcome			Season of AI	
	Conception failure	Early pregnancy failure	Pregnant	Summer	Winter
IGF-I (ng/ml)					
Day 3	103.8 ± 12.73[a]	148.9 ± 13.22[b]	118.1 ± 6.25[ab]	127.2 ± 6.99	111.3 ± 7.72
Day 15	87.4 ± 12.50	112.8 ± 12.98	115.3 ± 6.14	117.9 ± 6.86	99.8 ± 7.58
IGF-BP (ng/ml)					
Day 3	703.4 ± 99.13[a]	888.4 ± 103.0[ab]	1008.2 ± 48.69[b]	875.0 ± 54.43[a]	996.3 ± 60.12[b]
Day 15	755.8 ±107.62	1059.7 ± 107.34	886.6 ± 50.73	910.3 ± 61.79	866.9 ± 61.79
IGF-I:BP (%)					
Day 3	0.16 ± 0.02[ab]	0.21 ± 0.02[b]	0.12 ± 0.01[a]	0.16 ± 0.01[b]	0.12 ± 0.01[a]
Day 15	0.12 ± 0.03	0.11 ± 0.03	0.15 ± 0.01	0.14 ± 0.02	0.13 ± 0.02

[ab] Different superscripts within row, and main effect, indicate difference; $P < 0.05$

4. Application of Research

Betaine supplementation of breeding sow diets during summer

The results described in this report clearly demonstrate the potential benefits of adding betaine to the diets of breeding gilts and sows. More specifically, it is evident that betaine supplementation during gestation can improve the reproductive performance of sows mated during the summer, particularly older parity sows. Overall, the current findings demonstrate that both total litter size and the number of piglets born alive are increased by 0.6 and 0.5, respectively, when gestation diets are supplemented with 6.5 to 9.0 g of betaine each day. In particular, the present findings suggest the beneficial effects of supplementary betaine on litter size are most evident in older parity sows (parity 3 – 7), with total litter size increased by 1.6 piglets compared to non-supplemented sows of the same parity. Further, it also appears that betaine supplementation prior to mating can reduce the interval to puberty, and importantly, has the potential to prevent heat-induced reductions in ovulation rate, and by inference ovarian follicle growth.

The present experiment is the first to evaluate the effect of betaine supplementation during gestation on litter size, and to demonstrate that supplementary betaine reduces incidence of pre-natal mortality. However, there is a growing body of evidence demonstrating that supplementing gestation diets with other metabolites of the methionine cycle (i.e. Folate, glycine, Vitamin B12) can be beneficial for embryo and placental development and can also increase litter size in multiparous sows (Lindemann et al., 1993; Guay et al., 2002; Simard et al., 2007). The potential of maternal betaine supplementation to affect the uterine environment and embryo development has yet to be investigated. Betaine is an obligatory intermediate in the catabolism of choline, and acts as a source of methyl groups for the conversion of homocysteine to methionine (Finkelstein, 1990), and there is growing evidence to support an inverse relationship between dietary betaine intake and plasma homocysteine levels (Ueland et al., 2005; Holm et al., 2008). High doses of betaine (> 6 g per day) reduce circulating homocysteine in humans (Holm et al., 2004). Although not measured in this study, the available literature suggests that betaine supplementation of gestating sow diets would reduce homocysteine levels. A strong, positive association between plasma and uterine homocysteine levels has previously been demonstrated (Di Simone et al., 2004; Guay et al., 2002). Homocysteine is a recognised teratogen, with elevated homocysteine levels resulting in abnormal embryo development, increased pre-natal mortality and miscarriages in a number of species (Guay et al., 2002; Di Simone et al., 2004).

The findings of the described studies are particularly pertinent to the Australian pig industry and its breeding animals which are exposed to prolonged periods of high temperature during the summer period. It is, therefore, suggested that betaine represents a nutritional strategy to alleviate the heat stress component of summer infertility. Betaine represents a relatively cheap remedial strategy for seasonal infertility and lower litter sizes in older parity sows, with the cost of its inclusion in gestation diets estimated to be approximately $3.00. Furthermore, it is extremely easy for producers, nutritionists and feedmills to adopt, as it is added to diets. Importantly, an AusPig simulation has been conducted to evaluate the cost benefit of adding betaine to gestation diets. Based on the results of these studies, it is expected that betaine supplementation of gestation diets will

increase litter size by 0.5 to 1.0 piglets/litter/sow. An increase of 0.5 piglets per sow per litter increases profitability by $4.42 per pig sold (or 5 c/kg), and an increase of 1.0 piglets per sow per litter increases profitability by $7.89 per pig sold (or 10 c/kg). Based on the current findings as well as the low cost and ease of adoption, it is clear that the research conducted as part of this project will have a significant impact on the commercial industry.

Seasonal differences in early pregnancy steroid and metabolite profiles

Overall, the current data indicate that early disruption of pregnancy occurs in both summer and winter, and that significant differences exist between summer and winter in peripheral progesterone concentrations at key time points during the first 31 days of pregnancy. Of particular importance is the elevated progesterone observed on days 3 and 7 post-AI in summer mated sows, which is indicative of either earlier timing of ovulation relative to first AI or altered progesterone production and secretion by the luteal cells. Equally significant is the reduced progesterone levels observed on days 15, 19 and 23 post-AI in summer-mated sows, most likely reflecting reduced progesterone secretion by the luteal cells.

To our knowledge, the current study is the first conducted in Australia characterising progesterone profiles during the first 31 days post-mating of sows inseminated during summer and winter. Analysis of the progesterone profiles of animals which failed to maintain their pregnancy or returned to oestrus within 35 days of mating, demonstrate that conception failure and early disruption of pregnancy are important causes of pregnancy loss on this farm. It is worth noting that true pregnancy failures, that is conception failures and early disruptions of pregnancy, were only marginally higher in summer compared to winter, accounting for 9.0% and 5.4% of artificial inseminations, respectively. In the current study, the plasma progesterone profiles during the first 31 days post-AI of sows experiencing conception failure or early disruption of pregnancy were similar to those reported by Tast et al. (2002) for a commercial breeding unit in Southern Finland. The current findings also demonstrate that late pregnancy losses, namely those animals with high progesterone profiles on days 7 through to 31 post-mating but which subsequently lost their pregnancy, equate to less than 1% of animals inseminated.

Previous studies have concluded that summer infertility is characterized by an increase in the number of sows exhibiting either an extended, irregular return to oestrus (> 26 days post-mating) (Paterson et al., 1978; Peltoniemi et al., 1999) or early disruption of pregnancy (Tast et al., 2002). Specifically, studies conducted in Finland demonstrate a 2% increase in incidences of irregular returns to oestrus post-mating (Peltoniemi et al., 1999) and a 6.4 fold increase in early disruption of pregnancies (Tast et al., 2002) in summer compared to winter. Further, Paterson et al. (1978) reported increased incidences of irregular returns to oestrus when temperatures were in excess of 32^{o}C for the 7 days prior to insemination. In contrast, the current data suggest that incidences of early disruption of pregnancy were actually higher in winter compared to summer, accounting for 50% and 30% of early pregnancy losses respectively. In contrast, incidences of conception failure were higher in summer compared to winter, accounting for 60% compared to 50% of early pregnancy losses. The current data appear to be consistent with findings of Williamson et al. (1980) which were based on the collection of a single blood sample on day 18 post-mating from sows inseminated in May and June on a large, Australian, commercial piggery.

The data of Williamson et al. (1980) suggest that incidences of early disruption of pregnancy are also prevalent in winter, with 46% of sows which exhibited a delayed, or irregular, return to oestrus following insemination in May and June possessing high progesterone levels on day 18 post-mating. An elevation in progesterone on day 18 is consistent with the profiles reported by Tast et al. (2002) for sows experiencing early disruption of pregnancy. However, Williamson et al. (1980) only collected a single sample on day 18 after mating, and it is possible the elevation in progesterone may have reflected a delay in ovulation relative to insemination, rather than an extension of the luteal phase due to the existence of the first signal for maternal recognition of pregnancy.

The cause of the discrepancies between the current study and those of Paterson et al. (1978) and Tast et al. (2002) as to the manifestation of seasonal infertility is not clear. Certainly, the temperature patterns experienced during summer and winter in the current study would have been similar to those occurring during the study of Paterson et al. (1978). However, it is worth noting that the findings of Paterson et al. (1978) were based on farm records with no samples taken for progesterone analysis, consequently the exact causes of the delay in return to oestrus cannot be elucidated. Equally, the seasonal infertility reported by Tast et al. (2002) occurred in the absence of any changes in temperature, with temperature averaging 16 to 17^{o}C in both summer and winter, suggesting that changes in photoperiod were likely responsible for the increased incidences of early disruption of pregnancy.

The current finding that only 1% of pregnancy failures occur after day 31 contrasts with the recent report of Bertoldo et al. (2009), in which incidences of late pregnancy losses amongst sows mated during summer ranged from 7% to 33% on three large, Australian commercial herds. However, the records of the farm on which the current study was conducted also indicated that incidences of late pregnancy loss were 7.2% and 5.4% in summer and winter, respectively, figures which are similar to those reported by Bertoldo et al. (2009). In the current study, the observed discrepancy between records of mating outcomes based on transcutaneous ultrasound compared to those based on progesterone profiles suggest inaccuracies in ultrasonic testing for pregnancy outcome. Specifically, it seems likely the additional 6.2% and 5.4% of late pregnancy losses recorded on farm during summer and winter, respectively, were due to falsely identifying pregnancy in a number of sows. Progesterone concentrations were not obtained in the study of Bertoldo et al. (2009), with analysis of pregnancy status reliant solely on transcutaneous ultrasound conducted between days 26 and 35 post-mating depending on farm. Consequently, it is reasonable to suggest the high incidences of late pregnancy loss reported by Bertoldo et al. (2009) reflected inaccuracies in pregnancy detection, specifically falsely identification of not-pregnant sows as pregnant.

The physiological mechanisms responsible for increased incidences of sows failing to maintain their pregnancy in summer have yet to be fully identified. However, photoperiod induced changes in the pattern of luteinising hormone, mediated via melatonin, combined with extreme and prolonged exposure to elevated ambient temperature appear largely responsible for reproductive failure during summer (Peltoniemi et al., 2000; Tast et al., 2002; Peltoniemi and Virolainen, 2006; Halli et al., 2008). Equally, stress and a reduction in voluntary feed intake due to elevated temperatures have also been implicated as possible contributing factors to increased reproductive failure during summer (Wan et al., 1994; Love et al., 1995). It is generally accepted that early disruption of pregnancy during

summer is caused by failure of the developing embryos to produce the second signal for maternal recognition of pregnancy, resulting in regression of the corpora lutea and, thus, pregnancy failure. Maintenance of corpora lutea function beyond day 12 requires LH support (Tast et al., 2000), and an alteration in the pattern of LH release during summer has been reported on day 14 of gestation (Peltoniemi et al., 1997). However, by immunising pregnant sows against gonadotrophin releasing hormone (GnRH), Tast et al. (2000) demonstrated that a prolonged and complete blockade of LH release is necessary for regression of corpora lutea to occur. Such an extreme impairment of LH release is not evident during summer, suggesting the reduced amplitude of LH pulsing observed during summer may simply impair luteal progesterone production (Wrathel et al., 1986; Tast et al., 2000). Variations in progesterone production prior to and during implantation elicit alterations in the pattern of uterine secretions (van Wettere and Hughes, 2007), suggesting that sub-optimal progesterone release during summer would create an unfavourable intra-uterine environment for embryo development, resulting in failure to produce the second oestrogenic signal for maternal recognition of pregnancy (Tast et al., 2000; Tast et al., 2002; reviewed by Peltoniemi and Virolainen, 2006).

The current findings indicate no seasonal differences in the progesterone concentrations of sows experiencing conception failure or early disruption of pregnancy. However, sows inseminated during summer and maintaining their pregnancy through to farrowing did possess significantly higher progesterone levels on days 3 and 7 post-mating and significantly lower progesterone concentrations on days 15, 19 and 23 post mating compared to sows inseminated during winter. Variation in either the rate or timing of the rise in progesterone is responsible for differences in progesterone concentrations during the first few days of pregnancy. However, it is not clear from the current data whether the elevation in early pregnancy progesterone concentrations during summer reflect an alteration in the timing of luteinisation relative to ovulation or an earlier ovulation relative to onset of behavioural oestrus. The available literature demonstrate the sensitivity of the corpora lutea to alterations in environmental and nutritional conditions both prior to, and after, ovulation. Photoperiod or temperature induced alterations in LH release and follicle function prior to ovulation have recently been shown to affect the developmental competence of oocytes collected from weaned sows (Bertoldo et al., 2009b), and it is logical to suggest that corpora lutea function would also be affected. In support of this, exposure to hyperthermic conditions (heat stress) in vitro has been shown to stimulate premature luteinisation of bovine follicular cells in vitro (Bridges et al., 2005). It is, therefore, suggested that elevations in temperature prior to ovulation may stimulate earlier luteinisation in the pig, thus explaining the increased progesterone concentration observed during summer in the current study. Alternatively, Langendijk et al. (2008) provided tentative data to support a positive correlation between peripheral IGF-I and progesterone during the first 12 days post-mating. The positive relationship exists between nutritional status and IGF-I (van Wettere et al., 2006). A positive effect of IGF-I on progesterone production by the luteal cells has been demonstrated in vitro (Ptak et al., 2003; Ptak et al., 2004), and the current data demonstrate a numerical increase in IGF-I and a significant increase in the bioavailability of IGF-I on day 3 post-mating in summer compared to winter. The reduced energy requirements for maintenance of temperature during summer compared to winter as well as the slightly longer interval from weaning to oestrus may explain the improved nutritional status of summer mated sows.

Although the porcine corpora lutea can operate independently of LH prior to day 12 post-ovulation (Peltoniemi et al., 2000), inhibiting LH release or immunising against LH during days 3 to 8 post-ovulation can reduce size and progesterone production of the corpora lutea (Ziecik et al., 2006). It is, therefore, plausible that photoperiod induced alterations in the pattern of gonadotrophin release during the first 12 days post-ovulation may affect progesterone production by the corpora lutea during this period. Following the 12th day post-ovulation, maintenance of the corpora lutea and progesterone production depends on luteinising hormone (Peltoniemi et al., 2000). The current data clearly demonstrate that progesterone concentrations, and presumably luteal progesterone production, are significantly lower on days 15 to 23 post-mating for pregnant sows mated in summer compared to winter. A direct link between the pattern of luteinising hormone release during days 12 to 18 post-mating and luteal progesterone production has not been clearly established. It is, therefore, plausible the observed reduction in progesterone was caused by environmental and nutritional influences on growth and function of the follicle cells prior to ovulation. However, regardless of the exact cause, the available data indicate that reductions in progesterone production by the corpora lutea of pregnant sows will alter the uterine environment, thus affecting embryo development. Further, it is plausible that seasonally induced alterations in the uterine environment will increase sensitivity to the negative impact of nutritional perturbations on endometrial secretion of the growth factors required for optimal embryo development. In other words, if progesterone production is already sub-optimal, or bordering on sub-optimal, during summer, then embryo development and pregnancy maintenance will be more susceptible to the negative effects on factors such as under nutrition during this period.

Preliminary support for a metabolic effect on pregnancy outcomes is evident from the observed differences in peripheral concentrations of homocysteine and IGF-I between sows experiencing conception failure, early pregnancy failure or maintaining their pregnancy. Specifically, concentrations of homocysteine were higher on days 3, 15 and 31 post-mating for sows experiencing early disruption of pregnancy compared to sows maintaining their pregnancy or experiencing conception failure. Further, early disruption of pregnancy was associated with increased concentrations of IGF-I and increase IGF-I bioavailability on day 3 post-mating compared to conception failure. The cause of these differences are unclear; however, studies conducted in rodent species and humans suggest a negative impact of elevated homocysteine during early pregnancy on embryo and foetal development (Petrie et al., 2002; House et al., 2002; Rees et al., 2006). Known to be embryotoxic (Greene et al., 2003), homocysteine promotes apoptosis of trophoblast cells, potentially impairing or inhibiting implantation (Di Simone et al., 2003; Di Simone et al., 2004). Alterations in homocysteine concentrations can reflect imbalances in dietary amino-acid intake, with catabolism of excess methionine resulting in increased homocysteine production (Rees et al., 2006). Changes in methionine intake have been associated with imbalances in one-carbon metabolism (Rees et al., 2006). Folate and vitamin B12 are also integrally involved in the remethylation of methionine from homocysteine, with inadequate intakes associated with elevated homocysteine concentrations (Rees et al., 2006). Homocysteine has been confirmed as a sensitive biochemical marker of folate status in rodents (House et al., 2003). Although studies in gestating sows indicate no or marginal effects of folic acid supplementation on plasma homocysteine (Guay et al., 2002a; Guay et al., 2002b), a reductive effect of supplementary vitamin B12 on plasma homocysteine has been demonstrated in gestating sows (Simard et al., 2007). Supplementing with both folic acid and glycine can significantly decrease concentrations of homocysteine within embryos

collected on day 25 of gestation (Guay et al., 2002a), and also appear to alter prostaglandin metabolism by the uterine endometrium (Guay et al., 2004), potentially effecting embryonic implantation and subsequent survival. Although a causative role for elevated plasma homocysteine cannot be established from the current data, based on the available literature it is plausible that the elevated plasma homocysteines levels of sows experiencing early disruption of pregnancy could be symptomatic of imbalances within the methionine and folic acid cycle. These imbalances, combined with elevated homocysteine levels within the utero-embryonic environment could impair embryo implantation, resulting in failure of the second signal for maternal recognition of pregnancy.

5. Conclusion

Betaine supplementation of breeding sow diets during summer

In conclusion, the present data clearly demonstrate that total litter size is increased when betaine is added to the gestation diets of sows mated in summer. However, identification of the physiological mechanisms responsible for the increased prenatal survival in response to betaine supplementation is necessary if the potential of this cheap and easy to implement technology is to be realised. In particular, future work should determine whether dietary betaine supplementation improves pre-implantation or peri-implantation embryonic survival or causes a reduction in foetal losses. Equally important is the need to establish if a relationship exists between elevated homocysteine levels during gestation and foetal survival, and to determine whether supplementing the diets of gestating sows with other metabolites of the methionine cycle (i.e. Folate, glycine, Vitamin B12) will reduce foetal losses and increase litter size. Finally, considering the potential for alterations in DNA methylation, and thus gene activation, in response to elevated homocysteine levels, the potential effects of hyperhomocysteinemia during gestation on foetal and post-natal growth of progeny should also be explored.

The present data also demonstrate that betaine supplementation prior to mating has the potential to alleviate the negative impact of high ambient temperatures on gilt reproductive performance.

Seasonal differences in early pregnancy steroid and metabolite profiles

In conclusion, the current findings increase our understanding of the nature and timing of pregnancy failures during both summer and winter. In particular, it is clear that sequentially measuring progesterone profiles is a more accurate method of determining pregnancy status than transcutaneous ultrasound, with the latter appearing to provide too much opportunity for operator error. The impracticalities associated with sequential measuring of progesterone to determine pregnancy status preclude its use on a commercial scale. However, this method of pregnancy diagnosis has potential for use on facilities with extremely high levels of pregnancy loss. Equally, identifying a less frequent sampling timeline would improve the commercial benefits, as would the development of a faecal progesterone assay.

The existence of seasonal differences in progesterone production has also been identified, differences which likely result in a pregnancy which is more sensitive to external perturbations. Importantly, the present data also demonstrate that early disruption of pregnancy is a common cause of pregnancy failures in winter as well as summer, and provides preliminary evidence that metabolic imbalances may be a causative factor. However, further studies are required to identify the physiological mechanisms responsible for early pregnancy losses. In particular, it is important to identify the cause of the elevated progesterone profiles during the first 7 days post-mating. Specifically, if timing of ovulation relative to oestrus detection is indeed earlier in summer than winter then artificial insemination protocols will require alteration if fertility rates are to be maximised. Equally important is the need to determine if seasonally induced alterations in luteal function are responsible for the observed differences in progesterone concentrations.

6. Limitations/Risks

In order to confirm the benefits of gestation betaine supplementation it will be necessary to conduct large scale studies on a number of commercial facilities. The effect of adding betaine to gestation diets during winter also needs to be established. The other major limitation to these findings is that the physiological mechanism mediating the effect of betaine on conceptus survival and ovarian development has not been identified. However, due to the low cost and ease of adoption of this technology there are very few risks associated.

To confirm the observed differences in progesterone release it will be necessary to conduct at least one other similar study on a different commercial facility.

7. Recommendations

As a result of the outcomes in this study the following recommendations have been made:

- An additional study should be conducted, on a different facility to:
    - Confirm the observed differences in progesterone levels during summer
    - Confirm the timing and causes of pregnancy losses
    - Develop a reliable faecal progesterone assay
- Additional work should be conducted to determine:
    - seasonal differences in progesterone production by the corpora lutea, and the importance of pituitary LH support and temperature as determinants of these differences
    - the existence of seasonal differences in the timing of ovulation relative to oestrus detection / expression
- Betaine should be added to summer gestation diets at a dose of 7.6 – 9.0 g / day
- Betaine should be included throughout the gestational period
- This area of research should be continued, specifically future work should be conducted to:
    - Identify the physiological mechanisms responsible for mediating the effects of betaine supplementation on conceptus survival
    - Determine the sow's requirements for other metabolites of the methionine cycle (i.e. Folic Acid, Vitamin B12), and to determine the interaction between these and betaine
    - Confirm whether adding betaine to gilt diets prior to mating or indeed to sow diets during the weaning-to-oestrus period will improve reproductive performance during summer

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