Do Changes in Steroid Sensitivity and Melatonin Underpin Seasonal Infertility in Pigs

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

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Executive Summary

Seasonal infertility represents a considerable problem for Australian pork farmers in that it reduces overall fertility and fecundity and causes unpredictable variation in production. One important seasonal effect is the variation in the age at which gilts reach puberty. There has been considerable research effort into the phenomenon and photoperiod and environmental temperature are considered the most likely primary influences, although housing conditions, pheromones and drinking water temperature may also play a role.

In other species with a similar gestation length to that of pigs and which in their wild state are normally weaned onto spring pastures, (eg. sheep), it is well established that daylength (photoperiod) is the prime environmental variable that controls fertility and fecundity. The domestic pig has of course been intensively bred for high fertility and fecundity and the industry expectation is that this potential can be sustained throughout the year.

The aim of this study was to determine if gilts are similar to these other species and show delayed puberty during summer as a result of daylength mediated inhibition of pituitary gland function. If this were the case, then treatment with small pellets of the hormone melatonin could be expected to release the animals from this inhibition and allow first ovulations to occur at or before the age of puberty in the winter months.

Experiments were conducted at a single site (Roseworthy, South Australia) to (1) determine the size of estradiol implants that would allow us to monitor the gradual release from the negative feedback actions of the steroid in ovariectomised gilts, (2) determine the effect of commercially available continuous release melatonin implants on the timing of the release from negative estradiol negative feedback and (3) determine the effect of melatonin implants on the time of the first ovulation, as determined by weekly progesterone measurement. In experiment 2, the studies were conducted in 4 cohorts of animals born in early July, August, September and October, while in experiment 3 they were conducted in 5 cohorts of animals born in early July, August, September, October and November.

We have shown that gilts are extremely sensitive to the negative feedback effects of estradiol prior to puberty, but failed to demonstrate a seasonal change in this feedback. In addition treatment with the melatonin implants failed to alter the timing of the release from the negative feedback effects of estradiol in estrogen treated ovariectomised gilts. Finally, while we showed evidence of delayed puberty onset in gilts achieving the appropriate body weight in mid-summer compared to autumn in 2007/2008, this was not replicated the following season (2008/2009) and was unaffected by melatonin treatment.

The overall conclusion is clearly that treatment of gilts with melatonin from 18 weeks of age does not affect the timing of puberty in the absence of boar stimulation.

There are several limitations/risks involved in making a global judgment that melatonin is not an appropriate treatment for seasonal infertility in pigs.

(1) This study failed to demonstrate a consistent seasonal delay in the age of puberty onset across the 2 seasons of study

(2) The study deliberately investigated the timing of spontaneous ovulations. It is possible that melatonin treatment could overcome the seasonal variation in boar stimulated first ovulations (See Paterson *et al* 1991)

(3) The study used only one starting age for the melatonin treatment, 18 weeks of age. Treatment at 21 or 24 weeks of age may prove to be more effective.

(4) The study was conducted at only one site

(5) The study did not address the seasonal variation in the time to post partum ovulation/estrus of sows

Table of Contents

1. Introduction

Seasonal infertility represents a considerable problem for Australian pork farmers in that it reduces overall fertility and fecundity and causes unpredictable variation in production. One important seasonal effect is the variation in the age at which gilts reach puberty (Paterson et al., 1989; Paterson & Pearce, 1990; Paterson et al., 1991). There has been considerable research effort into the phenomenon and photoperiod and environmental temperature are considered the most likely primary influences, although housing conditions, pheromones and drinking water temperature may also play a role.

In other species with a similar gestation length to that of pigs and which in their wild state are normally weaned onto spring pastures, (eg. sheep, goats and deer), it is well established that daylength (photoperiod) is the prime environmental variable that controls fertility and fecundity (Foster, 1981; Legan & Karsch, 1980). The domestic pig has of course been intensively bred for high fertility and fecundity and the industry expectation is that this potential can be sustained throughout the year. Nevertheless reproductive seasonality does occur in domestic sows kept outdoors (Bassett et al., 2001)

In the past 20 years, the role of photoperiod in the control of seasonality has been studied in considerable depth in sheep and an excellent model has been established that can both determine the extent of the seasonality and provide a template for studying possible interventions. Release from the negative feedback actions of estradiol is a pre-requisite for a pre-ovulatory LH surge and ovulation to occur (Legan & Karsch, 1980). The seasonal change in feedback can be evaluated effectively in ovariectomised, estradiol implanted animals. Studies in sheep have shown that the very low circulating levels of estradiol continuously released into the circulation by the implant suppress the secretion of luteinising hormone (LH) during the non breeding season. During this time levels of LH remain very low, in contrast to the very high levels of LH and FSH in non estrogen implanted castrated ewe. During the transition into the breeding season or puberty, under the influence of changing photoperiod the duration of melatonin secretion lengthens, the hypothalamus is released from the negative feedback and LH secretion increases. Importantly, the timing of these changes coincides faithfully with the onset of ovarian cyclicity in intact animals. This model has not been carefully and systematically evaluated in pigs. The primary flaws in previous studies have been the use of extremely high levels of estradiol (e.g. Compudose) which have compromised the researchers' ability to detect changes in sensitivity to the steroid (Almond & Dial, 1990; Smith et al., 1991). We are not aware of studies addressing the seasonal changes in estradiol negative feedback in pigs in the transition to puberty. There is, however strong evidence that exposure to long photoperiod results in delayed onset of puberty in gilts not exposed to boars (Paterson & Pearce, 1990)

Melatonin has been shown unequivocally to be the key link between the photoperiod and the reproductive system of a wide range of animals from small rodents through to sheep and deer. The role of photoperiod and melatonin in seasonal infertility in pigs has been studied previously but many early studies concluded that the pig is unlike other animals and does not show circadian secretion of the hormone melatonin. Even in those studies that reported a

day/night difference in melatonin secretion in pigs, the authors implied that pigs do not respond to changing daylength with altered melatonin secretion. These conclusions have been shown to be quite false. Recent careful studies (Tast et al., 2001a; Tast et al., 2001c) using a new sensitive and specific melatonin assay have shown that pigs (1) have very low daytime circulating levels of melatonin (<0.5 – 2 pg/ml range) (2) a high amplitude (> 20 fold) rhythm of secretion at night, (3) respond to short and long daylength by appropriately producing long and short duration peaks of circulating plasma melatonin, respectively and (4) maintain melatonin rhythmicity even in a low illuminance light/dark cycle (Tast et al., 2001b).

Extensive studies in sheep have shown the efficacy of melatonin treatment in altering seasonal infertility (Kennaway et al., 1987). The treatments have been in the form of oral administration (Kennaway et al., 1982a) and via implants (Kennaway, 1988; Kennaway et al., 1982b; Williams et al., 1992). There have been few controlled studies addressing the use of melatonin to alter the timing of puberty in pigs. In a study of melatonin in gilts, huge doses of melatonin (4 X 12 mg) were administered to gilts already maintained in continuous short daylength (10L:14D) at an unknown time of the year with no effect on attainment of puberty (Diekman et al., 1997). Paterson et al. (1992) investigated the effects of treatment with melatonin implants on puberty at a single time of year, starting at 18 weeks of age (early December). Gilts were initially treated with two 18 mg implants and 2 further implants 35 and 70 days later (Paterson et al., 1992). Puberty was determined by observation of morphologically normal corpora lutea at slaughter at a set time, 223 days of age. 2 of 24 control gilts and 1 of 24 implanted gilts had attained puberty by this age. The authors concluded that melatonin implants could not overcome the seasonal inhibition of the attainment of puberty in domestic gilts.

In an attempt to address the lack of systematic studies in the area, a series of experiments were conducted at Roseworthy, South Australia. In this study we determined (1) the size of estradiol implants that would allow gradual release of the hypothalamic/pituitary axis from the negative feedback actions of estradiol in ovariectomised gilts, (2) the effect of a single melatonin implant on the timing of the release from negative estradiol negative feedback and (3) the effect of a single melatonin implant on the time of the first ovulation, determined by weekly progesterone measurement. The studies addressing the second aim were conducted in 4 cohorts of animals born in early July, August, September and October 2007 and 2008. An additional cohort, born in November 2008 was used in experiment 3. We hypothesized that melatonin treatment would result in the early release of the hypothalamus/pituitary gland from estradiol negative feedback. When administered to intact gilts we hypothesized that the melatonin would advance the time of the first spontaneous ovulation.

2. Methodology

Experiment 1

The study was conducted between February and May 2007 at the University of Adelaide Pig Research Facility at Roseworthy, South Australia. Gilts (aged 18 weeks; n = 22) were bilaterally ovariectomised under isoflurane anaesthesia and immediately implanted sub-cutaneously with 1 (n =6), 2 (n = 5) or 4 (n = 5) Silastic capsules containing crystalline estradiol or an empty capsule (n = 6). After surgery the gilts were maintained in pens of 6 in a shed that exposed them to natural changes in ambient light and temperature. Blood was collected twice weekly by venepuncture into heparinised vacutainers and plasma removed and stored at -20C.

Two weeks before the termination of the experiment, a subset of the animals was implanted with 1 or 2 Regulin® melatonin implants subcutaneously behind the ear (n=6 for each melatonin treatment) and blood samples collected during daytime from the contra lateral jugular vein 3 and 7 days later.

Plasma samples were assayed for LH, FSH, estradiol and melatonin by radioimmunoassay.

The LH assay used an LH antibody, reference standard and iodination grade LH obtained from Dr AF Parlow.

The FSH assay used an FSH antibody, reference standard and iodination grade FSH obtained from Dr AF Parlow.

Estradiol was assayed with an Ultrasensitive Estradiol assay (DSL) according to the manufacturer's instructions.

Melatonin was assayed using a direct radioimmunoassay obtained from Buhlmann Laboratories, Allschwil, Switzerland. The sensitivity was 1 pg/ml.

Experiment 2

The study was conducted at the Roseworthy piggery on gilts born in the first week of July, August, September and October 2007 from early November 2007 until May 2008, starting when the gilts were 18 weeks old (Figure 1).

Gilts in each of the four cohorts were ovariectomised and assigned to the following groups:

(1) immediately implanted with 1 Regulin implant behind the left ear and 1 subcutaneous estradiol capsule (n=6 in each cohort)

(2) 1 estradiol capsule, but no melatonin (n = 6)

(3) an empty capsule (n = 4)

An additional group of gilts with intact ovaries (n = 6 in each cohort) was used to monitor the normal timing of first ovulation. The animals were maintained in a shed that exposed them to natural changes in sunlight and temperature. Single blood samples were obtained by venepuncture from the right jugular vein, weekly until the gilts were 30 weeks of age for subsequent LH, FSH, melatonin assay and where appropriate, estradiol and progesterone analysis by radioimmunoassay. Gilts

were weighed every 4 weeks and at the end of the experiment, the estradiol implants were retrieved at slaughter.

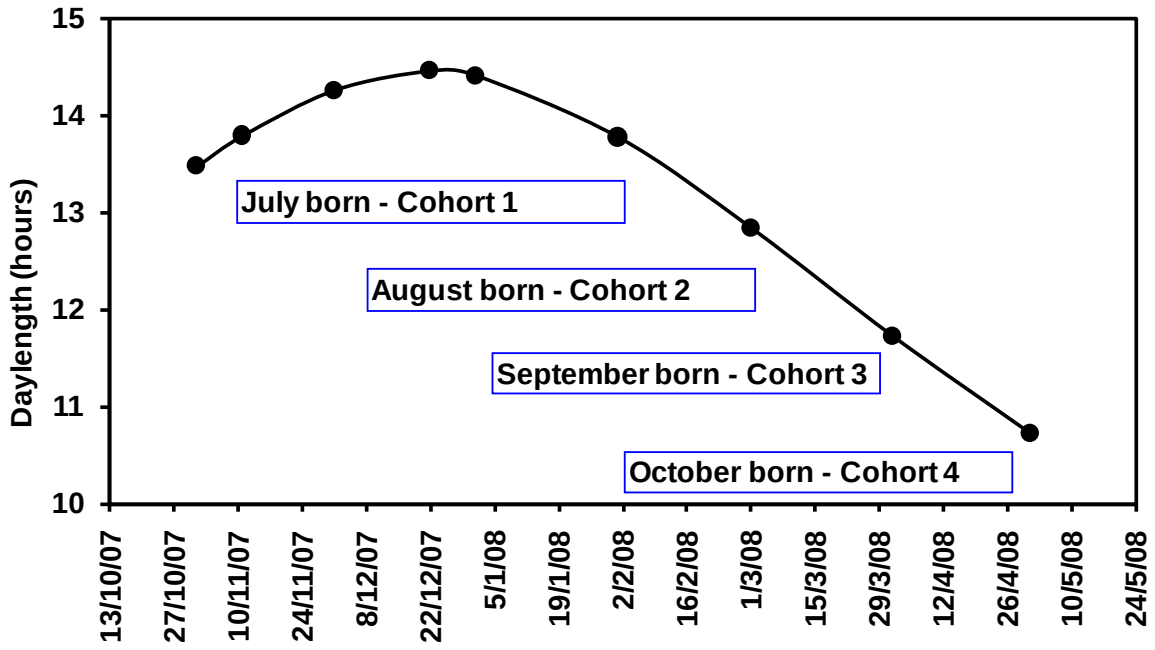


Figure 1. Experimental design for Experiment 2. Four cohorts of gilts were ovariectomised and treated with estradiol, with or without melatonin pellets. The bars indicate the period of study for each cohort from 20 to 30 weeks of age. Also shown is the duration of daylight to highlight the fact that the gilts would be expected to reach puberty at different times across the summer/autumn period.

Experiment 3

The study was conducted at the Roseworthy piggery on gilts born in the first week of July, August, September and October and November 2008 from early November 2008 until May 2009 starting when the gilts were 18 weeks old. A total of 96 gilts were implanted with either 1 Regulin implant or left untreated. The animals were maintained in a shed that exposed them to natural changes in sunlight and temperature. Single blood samples were collected weekly by jugular venepuncture (10 ml vacutainer, Becton Dickson) until the gilts were 30 weeks of age for subsequent progesterone analysis by radioimmunoassay as above.

3. Outcomes

Experiment 1

Plasma LH

When the ovaries were surgically removed at 18 weeks of age, plasma LH increased from approximately 0.5 ng/ml to 1.3 ng/ml within one week and remained within the range of 1 - 2 ng/ml throughout the sampling period (Table 1, Figure 2). Ovariectomised gilts treated with 4 estradiol implants had sustained low LH (at or below the sensitivity of the RIA, 0.4 ng/ml) throughout the study. Ovariectomised gilts receiving 1 or 2 estradiol implants initially had suppressed LH levels but over the course of the study the plasma LH gradually increased into the castration range.

Table 1. LH levels 3 days and 12 weeks after bilateral ovariectomy and the implantation of estradiol capsules into 18 week old gilts.

Age	No capsule	1 implant	2 implants	4 implants
18.5 weeks	1.3 ± 0.2 ng/ml	0.53 ± 0.1 ng/ml	0.53 ± 0.1 ng/ml	0.43 ± 0.0 ng/ml
30 weeks	2.43 ± 0.6 ng/ml	1.43 ± 0.5 ng/ml	1.23 ± 0.5 ng/ml	0.53 ± 0.1 ng/ml
Number of gilts	6	6	5	5

Plasma FSH

When the ovaries were surgically removed at 18 weeks of age, plasma FSH increased from approximately 1.2 ng/ml to 4.7 ng/ml within one week and remained within the range of 6 – 8 ng/ml throughout the sampling period (Table 2, Figure 2). Ovariectomised gilts treated with 4 estradiol implants had sustained low FSH (approximately 1 ng/ml) throughout the study. Ovariectomised gilts receiving 1 or 2 estradiol implants initially had suppressed FSH levels but over the course of the study the plasma FSH gradually increased to be approximately 3 ng/ml and still lower than those animals given no estradiol. The plasma FSH levels were similar in animals treated with either 1 or 2 estradiol implants.

Table 2. FSH levels 3 days and 12 weeks after bilateral ovariectomy and the implantation of estradiol capsules into 18 week old gilts.

Age	No capsule	1 implant	2 implants	4 implants
18.5 weeks	4.7 ± 0.7 ng/ml	2.7 ± 0.2 ng/ml	2.1 ± 0.3 ng/ml	2.3 ± 0.3 ng/ml
30 weeks	5.2 ± 1.3 ng/ml	2.6 ± 0.6 ng/ml	2.7 ± 0.9 ng/ml	1.6 ± 0.5 ng/ml
Number of gilts	6	6	5	5

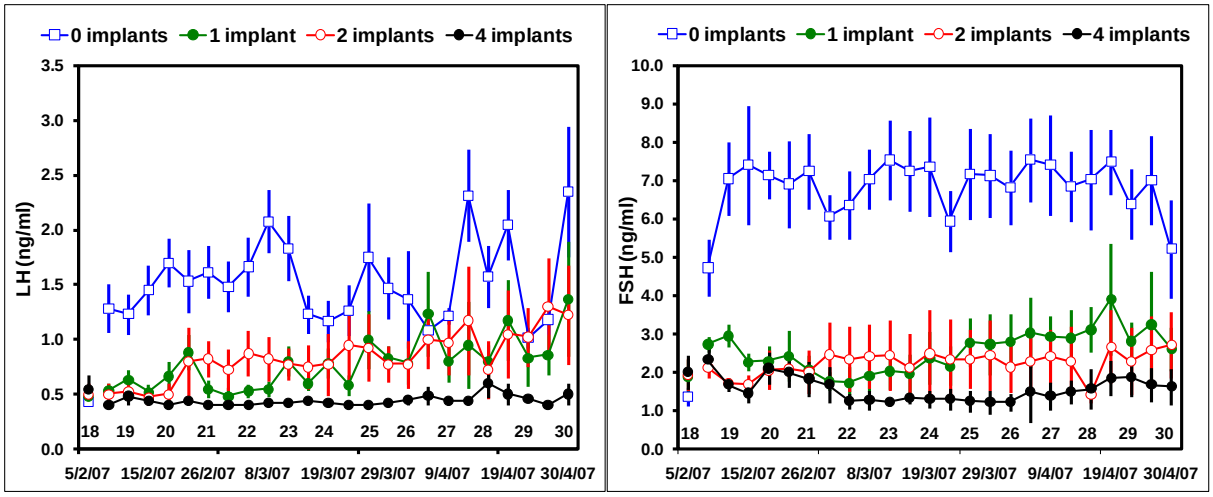


Figure 2. Plasma LH and FSH levels in ovariectomised gilts treated with estradiol capsules. Data are the mean ± SEM (n=5-6).

Plasma estradiol

Plasma estradiol levels were approximately 2 -3 pg/ml in ovariectomised gilts that received no estradiol treatment (Figure 3a). Gilts that received 1 implant had plasma estradiol levels higher than those that received no implant throughout the study. In those gilts treated with either 2 or 4 implants, estradiol levels were extremely high for the first 3 weeks after ovariectomy and then decreased to a steady level over the rest of the experiment. Figure 3b shows that when the estradiol levels were averaged for each animal over the last 5 weeks of the experiment and then group means calculated, there was a clear dose response relationship between the plasma estradiol levels and the number of implants.

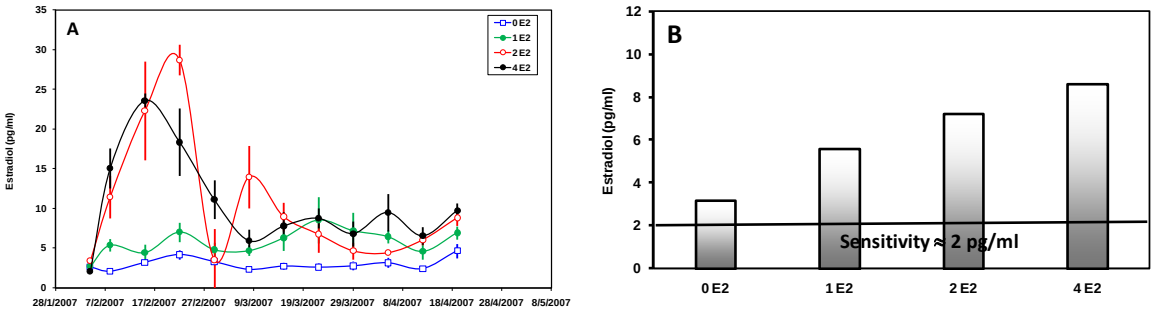


Figure 3. Plasma estradiol levels in ovariectomised gilts treated with 0 (n = 6), 1 (n = 6) 2 (n = 5) or 4 (n = 5) estradiol capsules.
(A) the data are the mean ± SEM (pg/ml) (n =5-6).
(B) the data are the group mean estradiol levels averaged over the last five weeks of the experiment.

Plasma melatonin

Plasma melatonin levels were at or below the sensitivity of the assay (1 pg/ml) in gilts that received no melatonin implants. By contrast, treatment with 1 or 2 Regulin® implants raised the melatonin levels to 49 pg/ml and to more than 60 pg/ml, respectively, within 3 days of treatment.

Table 3 - Melatonin levels in gilts treated with Regulin melatonin implants

	No implant	1 Regulin implants	2 Regulin implants
0	3.7 ±1.1 pg/ml	3.6 ±0.5 pg/ml	3.6 ±0.8 pg/ml
3 days	3.5 ±0.7 pg/ml	49.4 ±7.3 pg/ml	65.2 ±12.2 pg/ml
7 days	5.1 ±3.2 pg/ml	42.8 ±9.6 pg/ml	66.5 ±10.3 pg/ml
Number of gilts	6	7	4

Discussion:

Gilts are extremely sensitive to the negative feedback actions of estradiol on LH secretion, even at the levels secreted by the immature ovary. This is evidenced by the rapid increase in both LH and FSH following ovariectomy. As the gilts reach an age when (if intact) they would normally commence ovarian cyclicity, the sensitivity to the negative feedback action of estradiol diminishes and both LH and FSH increased towards the castration range.

These results provided the basic information required to further test the over-riding hypothesis that photoperiod alters the sensitivity of the estradiol feedback system in pigs as it does in sheep (Karsch et al., 1984).

We predicted that gilts approaching the usual age of puberty in summer would be held back from actually reaching puberty by the photoperiodically driven high estradiol negative feedback.

Based upon these preliminary results we concluded that treatment with one estradiol implant would be sufficient to test the hypothesis that the sensitivity to estradiol changes across the summer. Furthermore, treatment with one Regulin® (melatonin) implant would be sufficient to test the hypothesis that melatonin treatment can decrease the negative feedback sensitivity to estradiol across the summer.

Experiment 2

For each cohort 14-15 gilts were successfully ovariectomised and 12 had a single estradiol capsule inserted subcutaneously immediately following surgery. Of these gilts, 6 also were injected with a single Regulin melatonin pellet. Depending upon the availability of gilts, 2-3 animals were ovariectomised but not treated with either estradiol or melatonin as castrated controls. At slaughter estradiol capsules could not be located in some gilts (Table 4), while in one case there were obvious signs of a leak in the implant which would be expected to result in extremely high plasma estradiol levels (this was confirmed by subsequent analysis). In all these cases, data on LH and FSH was excluded from further analysis.

Table 4. The proportion of gilts that had estradiol capsules retrieved at slaughter.

	Cohort 1	Cohort 2	Cohort 3	Cohort 4
Control	3/6	6/6	4/6	6/6
Melatonin treated	4/6	5/6	6/6	4/6

Evaluation of plasma estradiol throughout the study revealed estradiol-like immunoreactivity in the castrated/non estradiol treated gilts in the range of 4 – 5pg/ml. Implanting the gilts with the estradiol capsules did not result in any consistent increase in the measured estradiol levels (figure 4, 6) . This is consistent with the very low release rate of the capsules. Melatonin increased

from 1 – 2 pg/ml in non-implanted gilts to approximately 30 pg/ml within 1 week and the levels remained constant for the duration of the study (figure 4, 6).

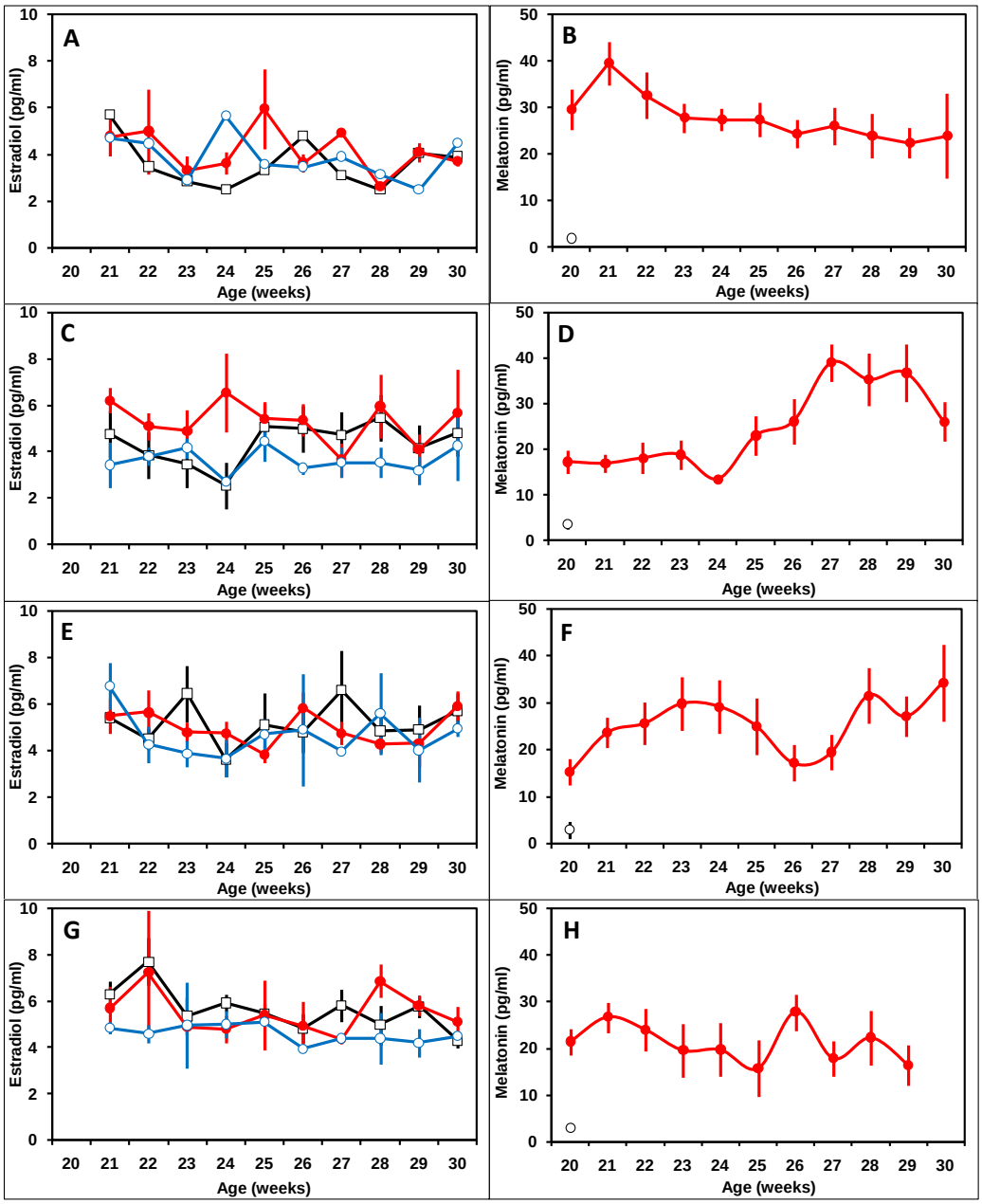


Figure 4 Left panels show the plasma estradiol levels obtained at weekly intervals in gilts ovariectomised and either treated with estradiol alone (□), estradiol plus melatonin (●) or left untreated (○). The right panels show the melatonin levels throughout the study in gilts implanted with melatonin pellets. The normal melatonin levels in non-melatonin treated gilts are indicated by the open symbol in the graphs. The data are the mean ± SEM for 2-6 gilts aged between 20 and 30 weeks. (A, B) gilts that were born in July 2007 and studied from November 2007, (C, D) gilts that were born in August 2007 and studied from December 2007, (E, F) gilts that were born in September 2007 and studied from January 2008, (G, H) gilts that were born in October 2007 and studied from February 2008.

In all 4 cohorts of gilts, ovariectomy without estradiol replacement resulted in an increase in plasma LH and FSH which plateaued after approximately 2 – 3 weeks.

Gilts receiving a single estradiol capsule on the day of surgery had very low LH and FSH within 1 week of surgery, irrespective of whether they had also been treated with melatonin. LH and FSH increased significantly with age (Figure 5; $P < 0.001$) in each cohort, but there was no effect of melatonin treatment ($P > 0.05$) when the entire data set was analysed (Figure 6). Similarly when the analysis was restricted to the hormone levels measured during the last 4 weeks of the study, there was no significant effect of melatonin treatment on LH or any effect of the time of year of the study (Appendix 3). For FSH there was similarly no significant effect of melatonin but there was a significant effect of the time of year of the study on FSH levels ($P < 0.05$) such that FSH was highest in cohort 3 (Appendix 2).

There were no significant effects of the time of year the gilts were born, castration with or without estradiol or melatonin treatment on growth rates (Appendix 3).

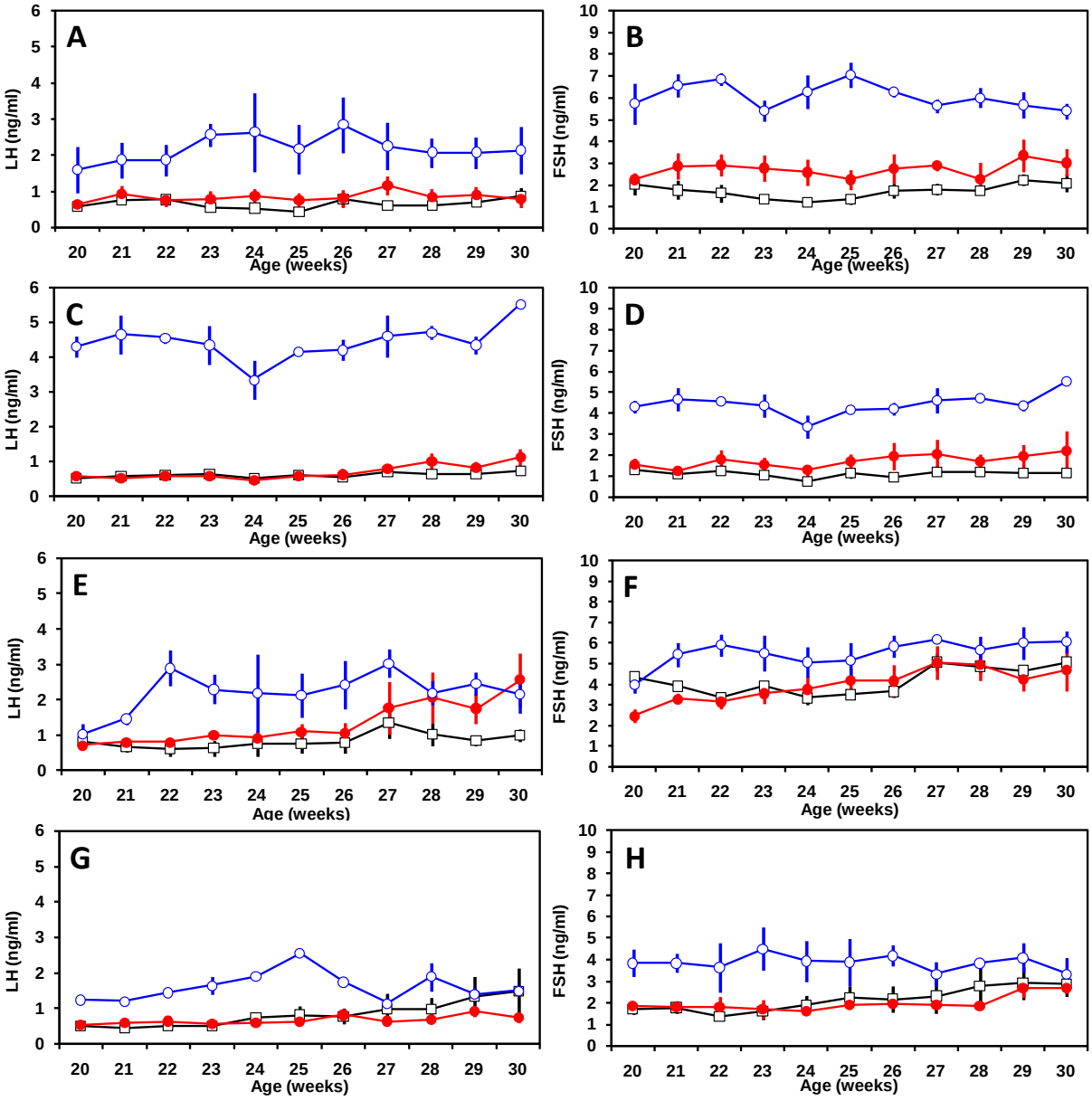


Figure 5. Plasma LH and FSH levels obtained at weekly intervals in gilts ovariectomised and either treated with estradiol alone (□), estradiol plus melatonin (●) or left untreated (○). The data are the mean ± SEM for 2-6 gilts aged between 20 and 30 weeks. (A, B) gilts that were born in July 2007 and studied from November 2007, (C, D) gilts that were born in August 2007 and studied from

December 2007, (E, F) gilts that were born in September 2007 and studied from January 2008, (G, H) gilts that were born in October 2007 and studied from February 2008.

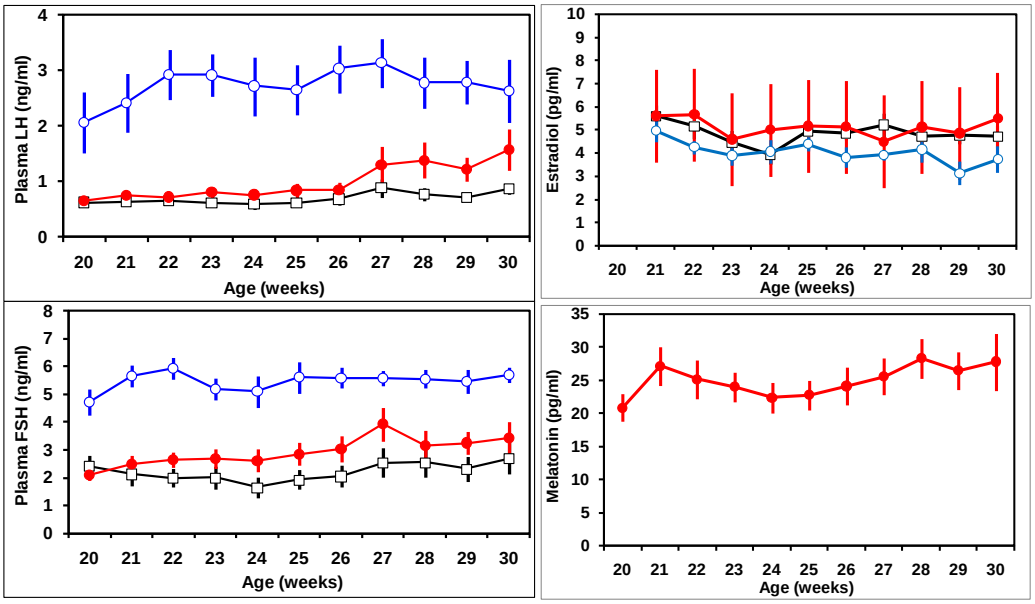


Figure 6. Left panels show the plasma LH and FSH levels obtained at weekly intervals from all the ovariectomised gilts that were either treated with estradiol alone0 (□) (n=19), estradiol plus melatonin (●) (n=19) or left untreated (○) (n=10). The right panels show the estradiol and melatonin levels throughout the study in all the gilts. The data are the mean ± SEM for gilts aged between 20 and 30 weeks.

Normal Gilts

As part of the main study, untreated, ovary-intact gilts were included in each cohort and were blood sampled at weekly intervals. Figure 7 shows the plasma progesterone levels for these animals. In the July born cohort, 3/6 gilts had commenced ovulating (as determined by the increase in plasma progesterone) from the age of 24 weeks. For the August born cohort, only 1/6 gilts was ovulating by the end of the study. For the September born cohort, 5/6 commenced ovulating, while for the October born gilts, 6/6 were ovulating by 30 weeks of age. The median age of the first increase in progesterone was 30 weeks, >31 weeks, 28.5 weeks and 21.5 weeks for cohorts 1 – 4 respectively.

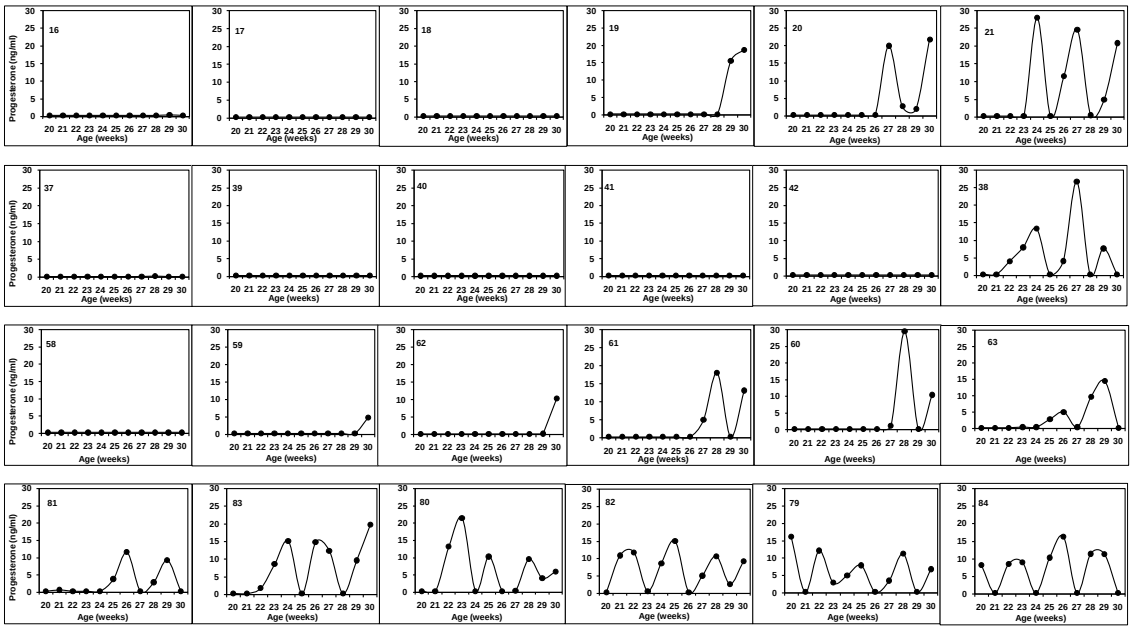


Figure 7. Individual plasma progesterone profiles for the untreated gilts in Experiment 2. Each row represents the successive cohorts of animals in the study, starting with July born gilts.

Figure 8 shows the cumulative sum of gilts that were cycling for the 4 cohorts.

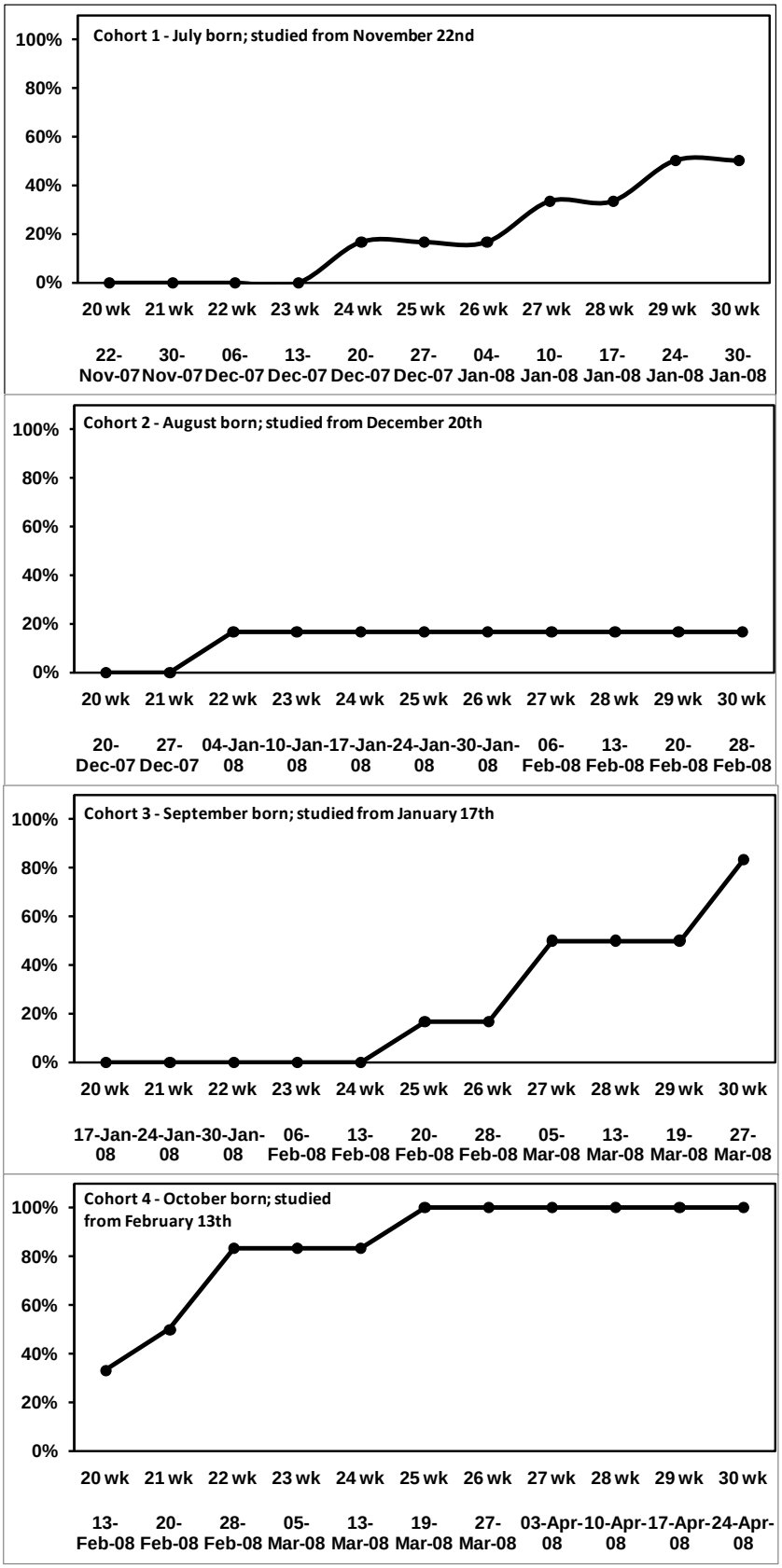


Figure 8. The cumulative sum of gilts that were showing evidence of ovulations as determined by plasma progesterone levels at weekly intervals from figure 6.

Discussion

Successively more gilts showed evidence of ovulation and at an earlier age in the animals born in July, August, September and October as daylength decreased (refer to Figure 1). Thus we have confirmed that puberty is affected by the time of the year. In the main part of the experiment the melatonin implants increased melatonin plasma levels from less than 2.5 pg/ml to over 30 pg/ml, which is approximately two fold higher than normal night-time levels. The single estradiol implants resulted in an increment in plasma estradiol levels of less than 1 pg/ml, smaller than for Experiment 1 but consistent with the low level of release from this type of implant. Despite the extremely low levels of estradiol released from the implants, plasma LH and FSH were maintained at very low levels and only slowly increased up to 30 weeks of age, approaching the levels measured in the non-estradiol treated ovariectomised gilts. The melatonin implants failed to overcome this negative feedback in any of the 4 cohorts of gilts studied.

We can conclude that gilts are extremely sensitive to the negative feedback actions of estradiol. We were unable to confirm a seasonal release of the hypothalamic pituitary axis from the negative feedback. Nevertheless, we have clear evidence that the time of the year that gilts are born affects the timing of their first ovulation.

Experiment 3

In this study of ovary-intact melatonin treated and control gilts, by the end of the study 78% of the melatonin treated gilts of cohort 1 were ovulating regularly compared to 44% of the controls (Figure 8, Appendix 4). The difference between the groups emerged late (after 27 weeks).

In cohort 2, (which was underpowered due to unavailable gilts), all the melatonin treated and control gilts were ovulating by the end of sampling (Figure 8, Appendix Figure 2). There was no evidence that melatonin accelerated the onset of the first ovulation.

In the 3rd, 4th and 5th cohorts less than 50% of the gilts had shown signs of ovulation before the sampling sessions were terminated at 29 weeks of age and there was no effect of melatonin treatment (Figure 8, Appendix 4).

Melatonin treatment did not increase the number of gilts ovulating overall (Figure 9). There was no indication that the peak levels of progesterone were affected by the season or melatonin treatment (Appendix 4).

Table 5 shows the median and average ages that the first progesterone rise was detected across the 5 cohorts. There was no obvious effect of the melatonin treatment on the time of puberty.

Table 5. Median and average ages when progesterone was first detected from weekly blood sampling.

		Cohort 1	**Cohort 2**	**Cohort 3**	**Cohort 4**	**Cohort 5**
Control	Median	30 weeks	27.5 weeks	30 weeks	30 weeks	30 weeks
Melatonin	Median	27 weeks	27 weeks	30 weeks	30 weeks	30 weeks
Control	Average	28.3 weeks	27.2 weeks	28.1 weeks	28.5 weeks	27.9 weeks

Melatonin	Average	27.9 weeks	26.4 weeks	28.8 weeks	29 weeks	28.8 weeks

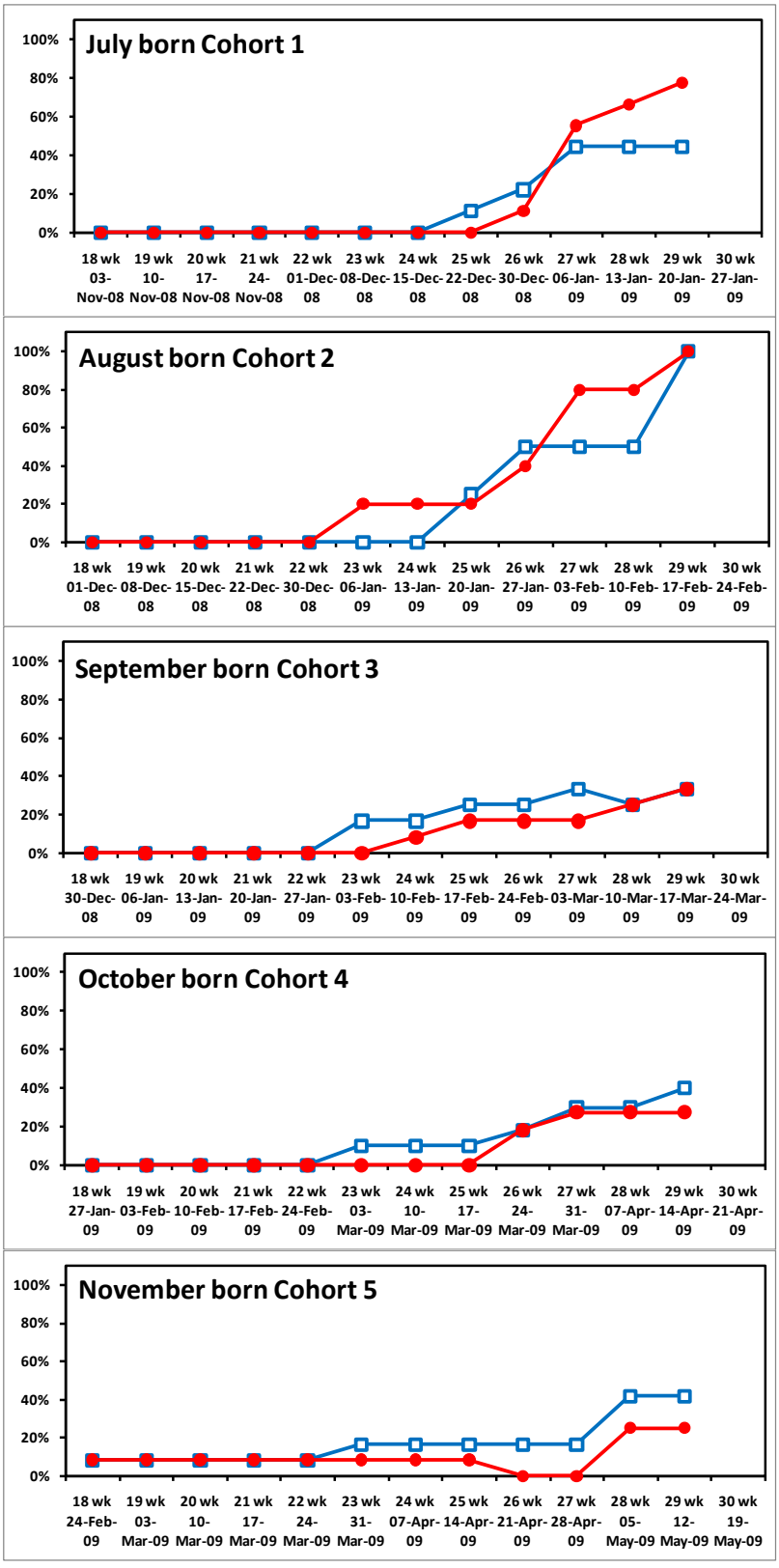


Figure 9 Cumulative percentage of gilts showing evidence of ovulation (determined by plasma progesterone levels) in the five cohorts of Experiment 3. (●) Melatonin treated gilts, (□) untreated

gilts. Note that the ages and dates of the measurements are indicated for each cohort.

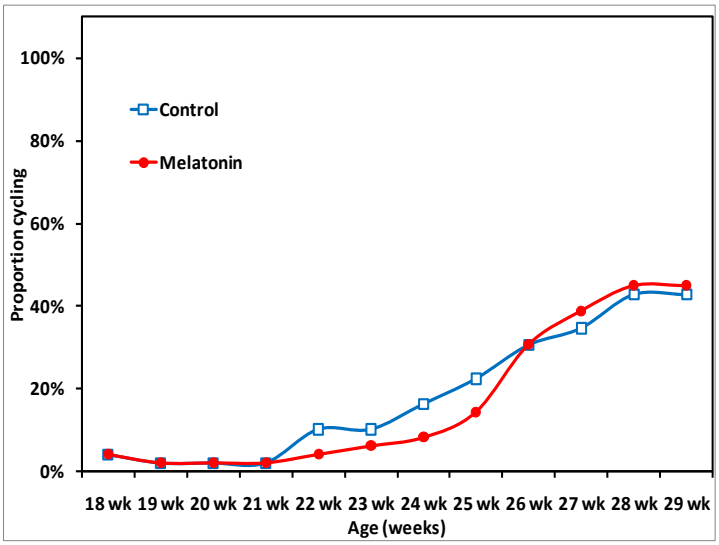


Figure 10. Summary of the cumulative percentage of gilts ovulating for the entire experiment. (●) Melatonin treated gilts, (□) untreated gilts.

Discussion

In Experiment 3 there was no effect of season of birth on the timing of the first progesterone rise in gilts. Indeed even in the gilts that had been born in November (cohort 5), less than50% had had their first ovulation before the experiment ended at 29 weeks of age (203 days of age). Melatonin treatment failed to alter the timing of the onset of the first spontaneous ovulation.

These results are in contrast to those obtained in Experiment 2 where there was a clear advance in the age of first ovulation from the July born to October born gilts. Indeed in 2007/8, all of the October born gilts (Cohort 4) were ovulating by the end of the experiment, with a median age of onset of 21.5 weeks of age, compared to 30 weeks of age in the same birth cohort in 2008/9. We can offer no explanation for the apparent differences between years in the patterns of puberty onset in experiments 2 and 3.

Melatonin treatment had no effect on body weight (Appendix 3).

4. Application of Research

The results of this study have not provided any new opportunities for the application of melatonin treatments to overcome a seasonal delay in puberty in gilts.

5. Conclusion

The aims of this study were to show that the timing of puberty in gilts was affected by season of birth, that it was due to a photoperiod driven, high sensitivity to estradiol negative feedback, and that treatment with constant release melatonin implants would overcome the impact of photoperiod on puberty.

We have shown that gilts are extremely sensitive to the negative feedback effects of estradiol prior to puberty, but failed to demonstrate a seasonal change in this feedback. In addition treatment with the melatonin implants failed to alter the timing of the release from the negative feedback effects of estradiol in estrogen treated ovariectomised gilts. Finally, while we showed evidence of delayed puberty onset in gilts achieving the appropriate body weight in mid-summer, compared to autumn in season 2007/2008, this was not replicated the following year and in fact there was evidence of an opposite trend in that delayed puberty appeared to be in the later born gilts in Experiment 3 whereas in Experiment 2, the earlier born gilts experienced much more delayed puberty attainment. Furthermore, attainment of puberty was unaffected by melatonin treatment in Experiment 3.

6. Limitations/Risks

The overall conclusion is clearly that treatment of gilts with melatonin from 18 weeks of age does not affect the timing of puberty in the absence of boar stimulation.

There are several limitations/risks involved in making a global judgment that melatonin is not an appropriate treatment for seasonal infertility in pigs.

(1) This study failed to demonstrate a consistent seasonal delay in the age of puberty onset across the 2 seasons of study
(2) The study deliberately investigated the timing of spontaneous ovulations. It is possible that melatonin treatment could overcome the seasonal variation in boar stimulated first ovulations (See Paterson et al 1991)
(3) The study used only one starting age for the melatonin treatment, 18 weeks of age. Treatment at 21 or 24 weeks of age may prove to be more effective
(4) The study was conducted at only one site
(5) The study did not address the seasonal variation in the time to post partum ovulation/estrus of sows

7. Recommendations

There is extensive literature indicating that the time of year (season) influences pig fertility in a number of different ways, including age of puberty, post partum return to estrus, maintenance of pregnancy, litter size, etc.

On the basis of these studies being conducted over a couple of years at a single site, it may be unwise to abandon attempts to use melatonin to overcome some of these fertility issues. If found to be effective for any of the seasonal fertility problems the relatively low cost of a Regulin (melatonin) implant of $6 each, would be expected to be a cost effective approach.

8. References

Almond, G W & Dial, G D 1990 Estradiol feedback inhibition of luteinizing hormone concentrations in the anestrous sow. *J Anim Sci* **68** 1077-86.

Bassett, J M, Bray, C J & Sharpe, C E 2001 Reproductive seasonality in domestic sows kept outdoors without boars. *Reproduction* **121** 613-29.

Diekman, M A, Arthington, J A, Clapper, J A & Green, M L 1997 Failure of melatonin implants to alter onset of puberty in gilts. *Animal Reproduction Science* **46** 283-88.

Foster, D L 1981 Mechanism for Delay of First Ovulation in Lambs Born in the Wrong Season (Fall). *Biology of Reproduction* **25** 85-92.

Karsch, F J, Bittman, E L, Foster, D L, Goodman, R L, Legan, S J & Robinson, J E 1984 Neuroendocrine basis of seasonal reproduction. *Recent Progress in Hormone Research* **40** 185-232.

Kennaway, D J 1988 Short- and long-term effects of manipulation of the pineal/melatonin axis in ewes. *Reproduction Nutrition and Development* **28** 399-408.

Kennaway, D J, Dunstan, E A & Staples, L D 1987 Photoperiodic control of the onset of breeding activity and fecundity in ewes. *J Reprod.Fertil.Suppl.* **34** 187-99.

Kennaway, D J, Gilmore, T A & Seamark, R F 1982a Effect of melatonin feeding on serum prolactin and gonadotropin levels and the onset of seasonal estrous cyclicity in sheep. *Endocrinology* **110** 1766-72.

Kennaway, D J, Gilmore, T A & Seamark, R F 1982b Effects of melatonin implants on the circadian rhythm of plasma melatonin and prolactin in sheep. *Endocrinology* **110** 2186-88.

Legan, S J & Karsch, F J 1980 Photoperiodic Control of Seasonal Breeding in Ewes: Modulation of the Negative Feedback Action of Estradiol. *Biology of Reproduction* **23** 1061-68.

Paterson, A M, Hughes, P E & Pearce, G P 1989 The effect of season, frequency and duration of contact with boars on the attainment of puberty in gilts. *Animal Reproduction Science* **21** 115-24.

Paterson, A M, Maxwell, C A & Foldes, A 1992 Seasonal inhibition of puberty in domestic gilts is overcome by melatonin administered orally, but not by implant. *Journal of Reproduction and Fertility* **94** 97-105.

Paterson, A M & Pearce, G P 1990 Attainment of puberty in domestic gilts reared under long-day or short-day artificial light regimens. *Animal Reproduction Science* **23** 135-44.

Paterson, A M, Pearce, G P & D'Antuono, M F 1991 Seasonal variation in attainment of puberty in isolated and boar-exposed domestic gilts. *Animal Reproduction Science* **24** 323-33.

Smith, C A, Almond, G W & Dial, G D 1991 Effects of season and estradiol-17 beta on luteinizing hormone release in ovariectomized sows. *J Anim Sci* **69** 4907-13.

Tast, A, Halli, O, Ahlstrom, S, Andersson, H, Love, R J & Peltoniemi, O A 2001a Seasonal alterations in circadian melatonin rhythms of the European wild boar and domestic gilt. *Journal of Pineal Research* **30** 43-49.

Tast, A, Love, R J, Evans, G, Andersson, H, Peltoniemi, O A & Kennaway, D J 2001b The photophase light intensity does not affect the scotophase melatonin response in the domestic pig. *Animal Reproduction Science* **65** 283-90.

Tast, A, Love, R J, Evans, G, Telsfer, S, Giles, R, Nicholls, P, Voultsios, A & Kennaway, D J 2001c The pattern of melatonin secretion is rhythmic in the domestic pig and responds rapidly to changes in daylength. *Journal of Pineal Research* **31** 294-300.

Williams, A H, McPhee, S, Staples, L D & Reeve, J L 1992 Optimum use of subcutaneous melatonin implants to enhance the reproductive performance of seasonal and non-seasonal sheep joined in spring and early summer. *Animal Reproduction Science* **30** 225-58.

9. Appendices

Appendix 1:

Table 1

Body weights during Experiment 2

		20 weeks	24 weeks	28 weeks	30 weeks
Control	Cohort 1	75.3 ± 1.4	99.7 ± 2.4	119.5 ± 3.6	130.4 ± 4.3
Control	Cohort 2	74.7 ± 3.8	94.3 ± 5.2	116.3 ± 5.3	120.0 ± 5.6
Control	Cohort 3	76.4 ± 2.3	98.0 ± 2.4	117.2 ± 2.8	123.0 ± 2.5
Control	Cohort 4	79.7 ± 3.9	97.3 ± 4.3	123.3 ± 4.4	126.2 ± 4.7
Melatonin	Cohort 1	85.4 ± 2.7	108.0 ± 1.7	128.9 ± 1.8	138.1 ± 2.1
Melatonin	Cohort 2	74.7 ± 3.0	96.9 ± 3.2	121.3 ± 3.3	128.5 ± 3.8
Melatonin	Cohort 3	75.8 ± 3.2	98.2 ± 3.3	119.0 ± 3.3	124.7 ± 3.9
Melatonin	Cohort 4	75.8 ± 1.2	91.5 ± 2.8	121.1 ± 2.0	123.3 ± 2.2
Castrate	Cohort 1	78.2 ± 2.5	100.0 ± 2.8	118.7 ± 5.3	130.0 ± 4.9
Castrate	Cohort 2	76.8 ± 1.0	100.2 ± 1.1	122.3 ± 2.4	127.5 ± 1.0
Castrate	Cohort 3	74.7 ± 2.7	98.0 ± 2.8	114.7 ± 3.2	120.7 ± 3.3
Castrate	Cohort 4	82.5 ± 3.3	101.2 ± 1.9	126.7 ± 1.2	130.0 ± 1.5
Intact	Cohort 1	80.8 ± 4.0	101.7 ± 5.6	121.3 ± 6.5	131.6 ± 5.5
Intact	Cohort 2	76.7 ± 3.7	99.4 ± 4.7	118.4 ± 5.5	128.7 ± 5.4
Intact	Cohort 3	ND	108.8 ± 2.2	124.1 ± 3.5	132.2 ± 3.9
Intact	Cohort 4	91.1 ± 4.9	107.5 ± 3.9	131.9 ± 4.2	135.9 ± 5.0

Appendix 2

Table 2

2-way ANOVA for LH and FSH measurements in the last four weeks of sampling for each cohort in Experiment 2.

Treatment	LH	FSH
	F = 0.84; P = 0.367	F = 0.46; P = 0.456
Control	0.91 ± 0.19 ng/ml	2.69 ± 0.30 ng/ml
Melatonin	1.15 ± 0.18 ng/ml	3.01 ± 0.29 ng/ml

Cohort	LH	FSH
	F = 1.86; P = 0.158	F = 11.4; P = 0.001
Cohort 1	0.78 ± 0.30 ng/ml	2.37 ± 0.48 ng/ml
Cohort 2	0.81 ± 0.24 ng/ml	1.72 ± 0.38 ng/ml
Cohort 3	1.55 ± 0.26 ng/ml	4.83 ± 0.41 ng/ml *
Cohort 4	0.98 ± 0.26 ng/ml	2.49 ± 0.41 ng/ml

Treat X Cohort	LH		FSH	
	F = 1.28	P = 0.300	F = 0.84	P = 0.480
	Control	Melatonin	Control	Melatonin
Cohort 1	0.69 ± 0.46 ng/ml	0.87 ± 0.40 ng/ml	2.00 ± 0.73 ng/ml	2.77 ± 0.63 ng/ml
Cohort 2	0.68 ± 0.32 ng/ml	0.94 ± 0.36 ng/ml	1.18 ± 0.51 ng/ml	2.26 ± 0.56 ng/ml
Cohort 3	1.05 ± 0.40 ng/ml	2.04 ± 0.32 ng/ml	4.90 ± 0.63 ng/ml	4.74 ± 0.51 ng/ml
Cohort 4	1.20 ± 0.32 ng/ml	0.76 ± 0.40 ng/ml	2.72 ± 0.51 ng/ml	2.27 ± 0.63 ng/ml

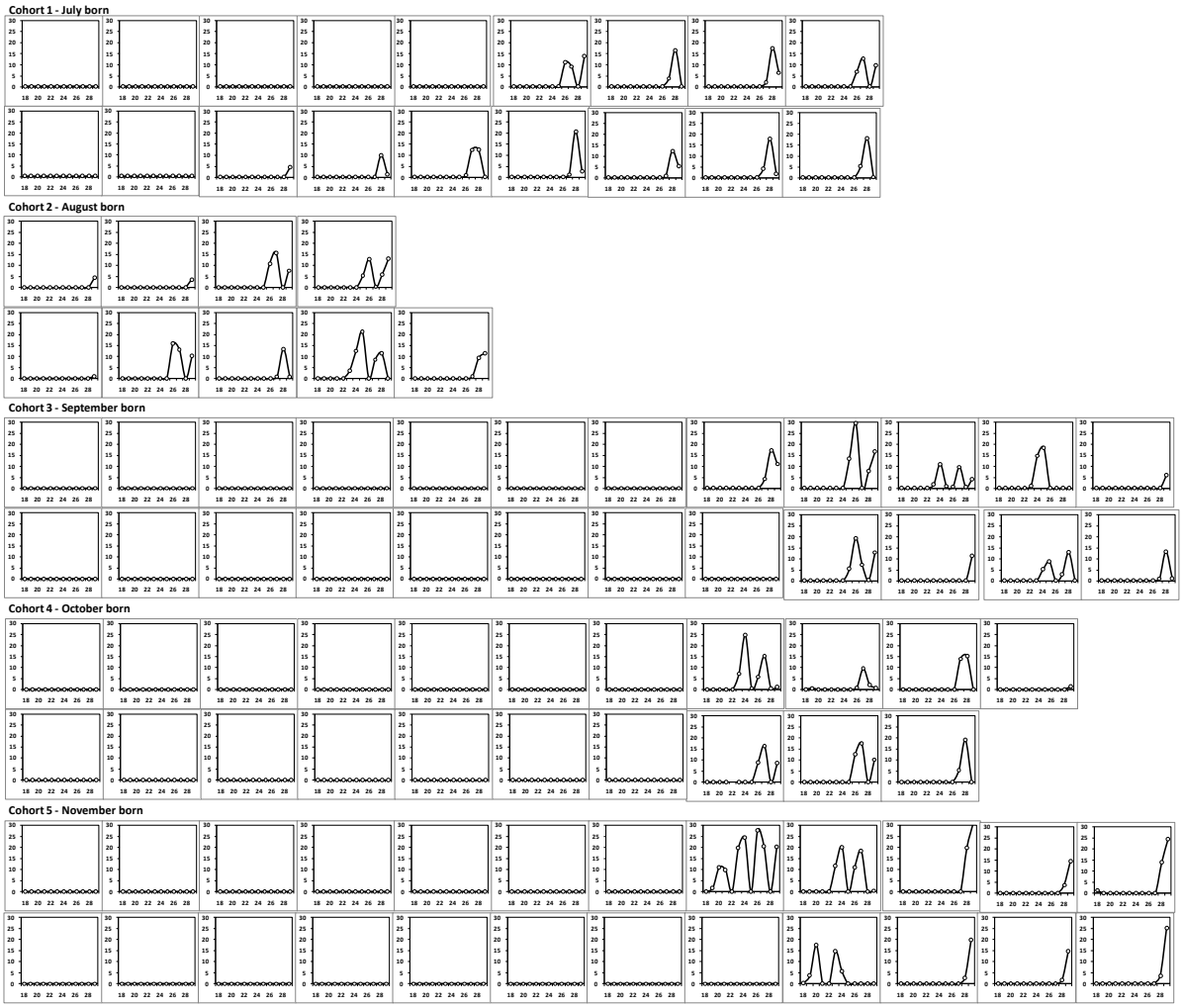
Appendix 3

Table 3
Body weights during Experiment 3

		18 weeks	22 weeks	26 weeks	30 weeks
Control	Cohort 1	80.9 ± 3.5	96.4 ± 3.4	120.2 ± 3.3	135.7 ± 3.8
Melatonin	Cohort 1	76.3 ± 2.5	90.8 ± 2.6	118.2 ± 3.4	130.6 ± 4.0
Control	Cohort 2	70.1 ± 5.0	90.9 ± 3.7	109.1 ± 4.6	117.5 ± 3.7
Melatonin	Cohort 2	66.3 ± 4.2	88.7 ± 4.3	109.4 ± 5.1	120.0 ± 5.2
Control	Cohort 3	75.7 ± 2.6	96.6 ± 2.7	114.9 ± 2.6	127.3 ± 3.2
Melatonin	Cohort 3	78.7 ± 1.8	97.9 ± 1.9	118.8 ± 1.9	132.8 ± 2.5
Control	Cohort 4	78.8 ± 2.4	96.1 ± 3.3	117.3 ± 3.9	142.9 ± 4.9
Melatonin	Cohort 4	76.9 ± 2.2	94.4 ± 2.5	117.7 ± 3.0	140.1 ± 3.7
Control	Cohort 5	74.3 ± 1.9	96.6 ± 2.6	116.4 ± 2.9	123.3 ± 2.5
Melatonin	Cohort 5	76.0 ± 1.4	97.9 ± 2.0	113.3 ± 2.1	122.2 ± 2.7

Appendix 4

Figure S1



The progesterone levels from weekly blood sampling from all the gilts in Experiment 3. Note that the scale is the same for every graph (0 – 30 ng/ml).