In cattle and other mammalian species, an ideal uterine environment is essential to prepare the
uterus for embryonic implantation and growth.¹ Many factors such as hormonal milieu, nutritional status,
clinical or subclinical uterine infections, and potentially other factors may alter uterine environment
resulting in decreased fertility. Methods to accurately evaluate uterine status prior to fertilization could be
useful for predicting fertility, validly determining optimal treatments for individual cows, and further
understanding factors that regulate fertility in cattle or other species.

Some techniques such as uterine cytology, biopsy, reproductive tract scoring (RTS),² and
ultrasound examinations have been used to assess fertility in many species including humans.³ In
bovines, ultrasound evaluation of the reproductive tract has some advantages over the other techniques
because it provides a rapid, noninvasive, and accurate diagnosis of uterine status. Although, RTS has
been shown to be an important tool that can be used to predict fertility performance of heifers,² it tends to
be a more subjective technique as compared to ultrasound measurement of uterine and ovarian structures.
Interestingly, in women, several studies have reported the use of ultrasound as a tool for predicting
fertility. For example, ultrasonographic evaluation of the endometrial thickness (ET) has been used for
over 20 years to evaluate endometrial receptivity during assisted reproduction programs for humans.³
Nevertheless, there is surprisingly little information in the scientific literature relating ultrasound
measurement of ET to fertility in cattle. Recently we described a technique to evaluate endometrial
thickness as a predictor of fertility in lactating dairy cows.⁴ This manuscript is a further analysis of this
ultrasound technique to evaluate endometrial thickness.

Johnson et al⁵ studied the influence of steroid hormones in ewes. They observed that uterine
weights were greater near estrus compared to mid-luteal phase, and that this increase in weight was due to
estrogen-induced endometrial tissue hypertrophy rather than hyperplasia. This is consistent with data
from other species showing hormonal regulation of uterine morphology and function.⁶ Thus,
ultrasonographic measurement of ET near ovulation might be a good indicator of hormonal environment
(low progesterone [P4] and/or high estradiol [E2]) and could be used to estimate whether the uterus has
been exposed to adequate concentrations of hormones compatible with optimal fertility.

Although cows do not lose endometrial tissue due to menses, the thickness of the endometrium⁷
and certain histological aspects⁸,⁹ vary considerably during the estrous cycle. An early study using
Holstein heifers described remarkable ultrasonographic changes in the endometrium of the uterine body
near ovulation. These authors reported an increase in ET at the time of expected luteolysis, with maximal
ET found on the day before ovulation. A schematic representation of their results is shown in Figure 1.
A number of studies have described the decrease in ET and changes in endometrial echotexture after
ovulation, probably due to increasing circulating P4.⁷,¹⁰,¹¹ We observed substantial variation in ET in
lactating dairy cows during the Ovsynch protocol when we were doing routine ultrasonographic
examination of cows in the University of Wisconsin herd.
Figure 1. Schematic representation of the changes in endometrial thickness in the uterine body of heifers throughout the estrous cycle. Data from Pierson and Ginther.

Figure 2. Schematic of the method used for measuring ET. Endometrial thickness was determined by ultrasound, with measurements done using electronic calipers in a 90-degree cross-sectional frozen image acquired at about 2 cm from the internal uterine body bifurcation. Minimum pressure was applied with the ultrasound probe on top of the uterus to avoid deformation of the uterine horns when performing ultrasound measurements of ET. Endometrial thickness was defined as the distance between the edge of the endometrial lumen to the visualized interface between the endometrium and myometrium.

The above results by Pierson and Ginther were obtained by ET measurements on the body of the uterus. We decided to measure ET in the uterine horns in all of our experiments. The uterine horns were an excellent location for validly measuring ET since the internal uterine bifurcation could be used as a guide to provide a consistent location for obtaining measurements from both horns for each animal (see Figure 2). Within an animal, the ET was similar between horns, presumably because cows enrolled in the
study were far enough in lactation to allow for full regression of the previously gravid horn. The average for the two horns was used as a final score for ET. Further, the uterine horns were chosen for measurements in these experiments since a 90° cross-sectional ultrasound image was easier to obtain from uterine structures that are deeper in the abdominal cavity due to the physical space needed for perpendicular placement of the probe on the uterus (see Figure 2 for schematic of measurement method). Finally, the distance between the uterine lumen and myometrium seemed easier to define for measurements using the uterine horns than the uterine body. Endometrial area or endometritis have previously been examined by ultrasound of the uterine horns in cattle.

Our first experiment evaluated the changes in ET associated with the periovulatory period (Figure 3). Eight lactating Holstein cows (four multiparous and four primiparous) were housed in tie-stall barns. Cows were 150.9 ± 1.4 DIM and had milk production of 30.0 ± 0.3 kg/day. All cows received the Ovsynch protocol (gonadotropin releasing hormone (GnRH) - 7d – prostaglandin F2α (PGF2α) - 72h - GnRH). Based on weekly ultrasound examinations at the University herd, all animals were cycling regularly and presented no uterine disorders prior to Ovsynch. In addition, all cows had at least one corpus luteum (CL) and circulating progesterone > 1ng/mL at the beginning of Ovsynch.

All cows had CL regression following PGF2α with circulating P4 decreasing to less than 1 ng/mL within 24 h. As shown in Figure 1, circulating E2 averaged 3.1 pg/mL at the time of PGF2α treatment and was similar at 1 d after PGF2α before increasing to 3.7 pg/mL at 2 d after PGF2α and to 5.1 pg/mL at 3 d after PGF2α, near the time of last GnRH treatment. At 1 d after the final GnRH treatment, circulating E2 decreased from 2.7 pg/mL to 1.2 pg/mL at 2 d after GnRH treatment. The ET increased rapidly after PGF2α treatment from ~ 7 mm to ~ 9.5 mm by 24 h after PGF2α. The ET remained similar at 2 d (9.2 mm) and 3 d (9.1 mm) after PGF2α. However, following the final GnRH treatment there was a decrease to 8.0 mm at 1 d after GnRH and 7.4 mm at 2 d after GnRH (Figure 1). Using data from each individual cow on each day, we found a negative correlation between circulating P4 and ET (r = - 0.28; P = 0.05); whereas, circulating E2 was positively correlated with ET (r = 0.33; P = 0.02).

We found a more acute increase in ET within 24 h after induced luteolysis than described by Pierson and Ginther during the expected time of luteolysis in natural estrous cycles. The increase in ET from 6.9 mm to 9.4 mm corresponds to a 2.5-fold increase in endometrial tissue volume, if calculated as a spherical measurement. This is an astounding increase in volume of endometrial tissue and probably primarily accounted for by increases in endometrial blood flow. The decrease in P4 is the primary hormonal value associated with this dramatic increase in ET. An increase in circulating E2 was not observed during the initial rise in ET; however, it seems likely that elevated circulating E2 needs to be present to facilitate the increase in ET. The reduction in ET near the time of ovulation corresponds to a decrease in E2 with no detected change in P4, providing further evidence that elevated E2 is important for elevated ET.
Our major experiment was performed with lactating Holstein cows, in a total of 942 breedings (581 multiparous and 361 primiparous). There were no significant differences between primiparous vs. multiparous cows for distribution of ET (Figure 4).

Figure 4. Distribution of endometrial thickness in primiparous vs. multiparous dairy cows.

Probably the most interesting findings in our study are illustrated in Figure 5. Even though changes in ET during the estrous cycle in cattle were first described more than two decades ago,7 we found only
two studies\textsuperscript{12,13} that attempted to associate ultrasound measurements of the bovine endometrium with fertility. Both of these studies evaluated the endometrium of Holstein cows in an attempt to diagnose subclinical endometritis in early lactation (40 to 60 days). Uterine cytology was used to define endometritis with results compared with ultrasound findings. Overall, maximal ET was associated with presence of endometritis. However, cows in these previous studies were not at the same stage of the estrous cycle and therefore the wide variation in ET throughout the estrous cycle that was described previously\textsuperscript{7} and that we observed in our study could not be taken into account. In the current study, we did not enroll cows with detectable uterine disorders in the Ovsynch program making the fundamental questions addressed in our study different from the questions addressed in previous studies.

Our results showed a linear increase in pregnancies per artificial insemination (AI) from less than 20\% to more than 40\% when ET increased from 6 to 10 mm (Figure 5B). These differences in fertility were associated with differences in circulating E2 and P4 concentrations. The P4 concentrations were greater in cows with ET of \leq 7 or 8 mm. This is due to a lack of luteolysis in a greater percentage of cows that were found to have lower ET (see Table). This is further illustrated by evaluating the circulating P4 after removal of cows that did not undergo luteolysis (cows with P4 > 0.5 ng/mL).

Although there is a difference between cows with thinner vs. thicker ET prior to removing these cows (see Table), there was no difference in P4 for cows with thinner ET (0.127 ± 0.008 ng/mL; n = 147) vs. thicker ET (0.134 ± 0.004 ng/mL; n = 482; P > 0.10) after removal of cows without luteolysis. This demonstrates that the reason for higher P4 in cows with thinner ET in the original comparison (prior to removal of cows with P4 > 0.5 ng/mL) was due to differences in percentage of cows without complete luteal regression and not due to higher P4 in all cows in this group.

We also found that lower fertility in Ovsynch-treated cows with lower ET seemed to be related to size of the ovulatory follicle. Cows ovulating smaller follicles after Ovsynch might have lower fertility due to lower ovulation rates, due to lower circulating E2 near ovulation, and due to impaired CL function after ovulation. For instance, Vasconcelos et al\textsuperscript{14} observed that cows that were intentionally induced to ovulate smaller follicles had lower circulating E2 prior to AI, had smaller CL after AI, lower circulating P4 after AI, an increase in short luteal phases, and poorer fertility. Any of these same problems could be present in cows with lower ET since they ovulated smaller follicles, had lower circulating E2 prior to AI, and had lower fertility. Cows with lower ET also had about 12\% fewer cows that ovulated to the second GnRH of Ovsynch. This would clearly lower fertility. The luteolysis rate was also lower in the cows with lower ET resulting in an elevated circulating P4. Thus, the lower ET is indicative of cows that were not properly synchronized by the Ovsynch protocol and do not have the proper environment for optimal fertility.
Figure 5. A) Relationship between endometrial thickness (ET) and circulating concentrations of E2 and P4 at 48 h after the PGF₂α treatment of the Ovsynch protocol. Lower ET (≤ 8 mm) was associated with lower E2 and greater P4 concentrations. B) Relationship between ET and pregnancies per AI (P/AI). Lower ET (≤ 8 mm) was associated with lower P/AI.
Table. Differences in various measures in lactating dairy cows with thinner (≤ 8 mm) or thicker (>8 mm) endometrium.

<table>
<thead>
<tr>
<th>Endometrial thickness (mm)</th>
<th>≤ 8</th>
<th>&gt; 8</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCS (scale 1-5)</td>
<td>2.79±0.03</td>
<td>2.84±0.02</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>(174)</td>
<td>(703)</td>
<td></td>
</tr>
<tr>
<td>Anovular before 1st AI (%)</td>
<td>24.7</td>
<td>15.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(21/85)</td>
<td>(67/438)</td>
<td></td>
</tr>
<tr>
<td>Ovulation to 2nd GnRH (%)</td>
<td>85.1</td>
<td>97.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(166/195)</td>
<td>(729/747)</td>
<td></td>
</tr>
<tr>
<td>P/AI (%)*</td>
<td>24.</td>
<td>44.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(48/195)</td>
<td>(329/747)</td>
<td></td>
</tr>
<tr>
<td>Luteolysis rate (%)**</td>
<td>89.2</td>
<td>96.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(174/195)</td>
<td>(718/747)</td>
<td></td>
</tr>
<tr>
<td>Circulating P4 (ng/mL)</td>
<td>0.32±0.05</td>
<td>0.18±0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(177)</td>
<td>(678)</td>
<td></td>
</tr>
<tr>
<td>Circulating E2 (pg/mL)</td>
<td>1.6±0.2</td>
<td>2.5±0.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(151)</td>
<td>(606)</td>
<td></td>
</tr>
</tbody>
</table>

* Pregnant per AI
** Circulating P4 < 0.5ng/mL at TAI

Our results in cattle are surprisingly similar to reported results in humans. Most of the studies done in women indicated that ET of less than 7 mm on the day of ovulation is not likely to result in a pregnancy. For example, some authors\textsuperscript{15-19} have reported a minimal threshold of 5 to 8 mm, below which no pregnancies are likely to occur in women. Another study\textsuperscript{20} found that women with greater ET have greater fertility. Interestingly, Baerwald et al\textsuperscript{17} described a positive association between follicular waves and endometrial growth, assessed by ultrasound, in women. In contrast, some studies have found that ultrasound evaluations of the endometrium are not specific enough to accurately predict future fertility in women.\textsuperscript{20,21} These conflicting results might be related to the day of the ovulation cycle that the endometrium was measured, accuracy and repeatability of ET measurements with ultrasound within and between different technicians, or even to the measurement techniques used. Nevertheless, the strong relationship with fertility that we found in cattle, as well as the previous relationship found between ET and fertility in humans supports the need for future research in this area. From a practical standpoint studies need to be performed that evaluate the variation among technicians, farms, and types of ultrasound machines and probes used for evaluations. In addition, it is not clear whether ET measurements will be of utility for predicting fertility in cows bred to a natural estrus due to potentially greater synchrony of hormonal concentrations during natural estrus.

In conclusion, assessment of ET near ovulation in a timed AI program was a surprisingly good predictor of ovulation failures and pregnancy success. This measurement can be performed routinely in order to detect cows with lower fertility. This finding may open the way for a broader use of ultrasound for measuring ET in practical cattle reproductive management. In addition, fertility research could be enhanced by using this technique to develop more optimized timed AI programs and for understanding
the molecular and physiological mechanisms that underlie optimal uterine fertility. After concluding these studies, it remains to be determined what hormonal, cellular, and molecular mechanisms underlie the reduction in fertility in cows with lower ET.

References