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Marcinkowski, David Paul

ESTRADIOL AND PROGESTERONE CONCENTRATIONS IN THE BLOOD SERUM AND FOLLICULAR FLUID OF THE SUPEROVULATED PREPUBERTAL CALF

The Ohio State University

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ESTRADIOL AND PROGESTERONE CONCENTRATIONS
IN THE BLOOD SERUM AND FOLLICULAR FLUID
OF THE SUPEROVULATED PREPUBERTAL CALF

Dissertation
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
David Paul Marcinkowski, A.A.S., B.S., M.S.

* * * * *
The Ohio State University
1980

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Very few graduate students are as fortunate as I have been in terms
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Thank you all,
VITA

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| | Artificial Insemination  
| | Embryo Transfer and Culture |
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INTRODUCTION

The successful superovulation of prepubertal calves can offer many possibilities for the genetic improvement of cattle. The calf is born with a large number of germ cells of which only a very small portion are used to produce viable oocytes capable of fertilization. The remainder of these cells are wasted. Superovulation of females offers a method by which animal breeders can utilize more of these germ cells from outstanding females. However superovulation at the present is used on older well proven animals. Researchers report that by the time a calf is 280 days old atresia begins to decrease the number of potential oocytes. By using the prepubertal calf one can greatly increase the number of offspring from a given female in a lifetime, even over present embryo transfer techniques. The superovulation of prepubertal calves increases the length of time in which embryos can be collected. One of the benefits of using young calves is that the generation interval can be reduced so more genetic progress can be made during a given period of time. Selection differentials can be increased so that fewer animals have to be saved as parents of the next generation. The genetic potential of females can be estimated earlier in life with the very best animals being used as the dams of the next generation of AI sires.

Researchers have shown that prepubertal animals can be successfully superovulated using gonadotropin treatments. The treatments used
usually contain either PMSG or FSH. PMSG is a glycoprotein hormone produced by the endometrial cups of the pregnant mare and has a longer half-life than FSH. Because of this longer half-life, PMSG can be administered in a single injection while FSH requires numerous injections. Comparisons between PMSG and FSH show little difference in their effectiveness.

There are still many problems with the present techniques of superovulation of prepubertal calves which have to be solved before the technique will become practical. Some of the problems include variable responses, poor ovulation rates and low fertilization rates. Part of the problem is probably the immaturity of the reproductive tract while other problems are due to our lack of knowledge. The immature tract has been shown to be a hostile environment to the developing embryo. Superovulation coupled with in vitro fertilization may be an effective way of bypassing some of these problems.

Follicular fluid is the fluid which accumulates around the oocyte in the developing follicle. Follicular fluid contains many different compounds, including ions, carbohydrates, lipids, mucopolysaccharides, proteins, enzymes and hormones. Many of these components have been implicated in various reproductive processes including oocyte maturation, ovulation, oviductal transport and fertilization.

The hormones present in the follicular fluid include gonadotropins, glucocorticoids, insulin, androgens, estrogens and progestogens. Estradiol is the major steroid in bovine follicular fluid. The follicular concentrations of estradiol are thousands of times higher than levels found in the blood plasma. Synthesis of estradiol occurs in the
theca and granulosa layers which surround the follicle. The two-cell theory of estrogen synthesis states that the theca and granulosa layers must be in close association to produce sufficient quantities of estrogen. The theca produces androgens but lacks the aromatizing enzymes necessary to convert androgens to estrogens. The androgens diffuse into the follicle and to the granulosa layer where they are converted to estrogens. Disruption of the association between the theca and granulosa causes altered steroid production.

Follicular fluid steroid levels are good indicators of follicular function and represent the steroid production in the follicle. Increasing levels of estradiol in the follicular fluid have been found to be essential in normal follicular development. In cases of abnormal development such as in atretic and cystic follicles, follicular fluid steroid concentrations are significantly changed. In abnormal follicles estrogens levels are lower than the levels of progesterone and androgens. Researchers have used steroid levels in the follicular fluid as indicators of normal and abnormal follicular function.

The concentrations of steroids in follicular fluid of the superovulated prepubertal calf have not been determined. By studying steroid levels in the follicular fluid we may better understand steroidogenesis and follicular development in the immature female. Determination of steroid concentrations may also lead to a better understanding of follicular fluid functions in the reproductive processes of the female.
The objectives of this experiment are:

1. To examine the levels of estradiol and progesterone in the follicular fluid and blood serum of the superovulated prepubertal calf.

2. To determine if any relationships exist between estradiol or progesterone and the follicular response of prepubertal calves to gonadotropin treatments.
The Superovulation of Prepubertal Calves

The female calf is born with a full complement of germ cells designed to last a lifetime. Erickson (39) found tremendous variability among calves in the number of primordial germ cells they possess. Numbers can range from 0 to 700,000 germ cells with a mean of approximately 160,000 cells per calf. Germ cells numbers decrease slowly throughout the lifetime of the animal with numbers approaching zero by 20 years of life.

Age also affects the quality of the germ cells throughout life. At the age of 8 months, the quality of the primordial follicles begins to decrease so that by ten years of age the majority of the follicles in the bovine ovary are in various stages of atresia (39). The numbers of good quality oocytes are largest in immature females, making them the greatest potential source of embryos. The superovulation of prepubertal calves would enable animal breeders to obtain more offspring from outstanding females than is possible with present techniques.

The nonsuperovulated calf has considerable follicular development. The ovaries of calves at birth were found to possess an average of seven vesicular follicles. However, by two months of age follicular populations in the calf are similar to populations in the mature cow (19). Howe et al. (53) found several follicles greater than 5 mm in diameter.
on the ovaries of one month old calves. From birth to five months of age ovarian weight increases nearly four times faster than body weight (28). Desjardins and Hafs (28) attributed the rapid ovarian growth in large part to increased numbers of vesicular follicles. Ovarian growth and follicular development reach a plateau from 5 to 8 months of age with an average of about 20 antral follicles present on both ovaries (28). Morrow (82) found that a majority of heifer calves prior to puberty had one or more palpable follicles, 5 to 20 mm in diameter. Increased follicular activity in heifers was observed 20 to 40 days prior to the first ovulation. The first ovulation in holstein heifers occurred at about 42 weeks of age (82).

In the early 1900's, researchers (106) found that anterior pituitary extracts could increase follicular development and the numbers of follicles which ovulate during a given cycle in laboratory animals. Later it was found that sexually immature animals could also be superovulated. Casida et al. (20) in 1943 used pituitary extracts for the first time to superovulate cows and calves. Calves showed greater follicular development than mature cows, however cows ovulated more frequently and produced more fertile embryos. Superovulation of immature females has been accomplished not only in cattle and lab species but also in sheep (69) and swine (82).

Hammond and Bhattacharva (49) in 1944 were the first to utilize the follicle stimulating activity of Pregnant Mare Serum Gonadotropin (PMSG) for the superovulation of cattle. There are few data that compare the use of Follicle Stimulating Hormone (FSH) with PMSG in the induction of superovulation in cattle and calves. Both are extracts whose purity and
activity vary greatly among batches. The half-life of FSH in cattle is approximately five hours (62), while that of PMSG is 2 to 4 days depending on the sialic acid content of the preparation (2). Superovulation with PMSG can usually be accomplished with a single injection whereas FSH requires numerous injections.

Researchers have found conflicting results when FSH was compared with PMSG in the induction of superovulation. Onuma et al. (86) found five daily injections of FSH superior to a single injection of PMSG in causing follicular development in calves. However, PMSG treated calves had significantly more ovulations. W.D. Foote et al. (45) found that calves treated with PMSG had greater numbers of large follicles (15-20 mm) and greater numbers of mature oocytes than calves treated with FSH. Seidel, Larson and Foote (100) found no difference in response when calves were treated with PMSG or FSH. Mickelsen et al. (77) found calves treated with FSH and LH had greater numbers of ovulations and produced more fertile embryos than did calves receiving PMSG alone or in conjunction with LH. Elsdon et al. (37) compared the use of FSH with PMSG in the induction of superovulation in mature cows. They found that ovulation and fertilization were similar for the two products when the FSH was given twice daily over five days.

Some researchers have shown that FSH and LH may be acting synergistically in follicular development. Labsetwar (61) found mice infused with a combination of FSH and LH to have higher ovulation rates than groups receiving either FSH or LH alone. In a study by Laster (63), heifers were continuously infused with FSH-β, purified bovine or ovine FSH. Ovulation rates were similar between the treatment groups.
However, the doses of FSH-p contained considerably less FSH activity. Studies in calves (43, 100) and cows (37) have found a 5:1 ratio of FSH to LH to be satisfactory in the induction of superovulation. Pregnant mare serum gonadotropin has an FSH to LH ratio of 1:1.4 (2) and additional LH activity is not required for superovulation.

Ovulation failures have been a problem with the superovulation of cows (44) and especially calves (43). Many researchers have used additional injections of LH to enhance the ovulation of calves. It is believed calves may be lacking an adequate LH surge to bring about ovulation of the many follicles produced by superovulation treatments. Luteinizing Hormone, HCG and GnRH treatments have been used to mimic or heighten the endogenous LH surge seen prior to ovulation in mature cows. Early work done by Marden (70, 71) in calves showed no benefit in using LH or Human Chorionic Gonadotropin (HCG) to increase the number of ovulations when given 4 to 6 days after the beginning of the superovulation treatment. More recent work (89) has shown LH and HCG injections, 5 days after PMSG, do enhance the superovulation of young calves. Mickelsen et al. (77) increased mean ovulation rates from 2 to 11.3 by the addition of 50 mg of LH given intravenously 72 hours after PMSG. Ovulation rates increased from 2 to 21.9 when the same LH treatment was given 96 hours after the start of FSH injections. They also found that ova recovery and fertilization rates were increased in calves which received the ovulatory dose of LH.

The ovulatory dose of LH seems to be affecting ovulation by bringing about final maturation of the follicle, but also has been shown to have an effect on oocyte maturation. W.D. Foote et al. (45) found
that 20 mg of LH given iv did not increase follicular growth, but did increase the number of oocytes resuming meiosis and reaching metaphase II across all follicle size groups. Bedirian and Baker (12) noted oocyte maturation occurred only when PMSG was followed by 1000 IU of HCG. PMSG or HCG alone did not cause oocytes to mature. The PMSG and HCG group also displayed a higher proportion of oocytes with an expanded cumulus mass. Onuma et al. (87) suggested that an ovulatory dose of LH may be more important when calves are superovulated with FSH because the follicles seemed harder to rupture.

Larger doses of LH have been shown to be more effective in promoting oocyte maturation. Onuma et al. (86) found an increase in the ovulation percentage when the LH dosage was increased from 25 mg to 50-150 mg. Reidel and Rommel (89) found no difference in ovulation rates when the dosage of HCG was increased from 2000 IU to 4000 and 6000 IU. However, the higher doses did increase oocyte maturation and fertilization rates. Their best treatment however, was found to be 1 mg of GnRH given 120 hours following PMSG.

Changing the interval of time from the beginning of the superovulating treatment to the ovulating dose of LH may also be important. Seidel et al. (100) noted a decrease in the ovarian response when LH was given 72 hours rather than 120 hours after PMSG. Lineweaver (66) found no difference in the ovulation rate when LH was given 72, 120 and 168 hours after PMSG. Splitting the LH dose into five equal injections 1 hour apart had no effect on the ovulation rate when compared with a single injection (99).

Regardless of the timing of the ovulating LH injection, ovulation usually begins approximately 24 hours later (65).
In the normal cycling cow, estrus and ovulation are preceded by a period of high progesterone levels in the blood. Prepubertal calves have very low levels of progesterone in the blood prior to stimulation with gonadotropins. Researchers have tried to improve superovulation results by pretreating the calves with progesterone (4,5,14,65,86). The reasoning being that better and more consistent superovulations should result if elevated progesterone levels are mimicked using exogenous sources (i.e., injections, implants or pessaries). Progesterone pretreatments have been beneficial in the superovulation of sheep (59) however has adversely affected the superovulation response of young calves. Researchers have reported no effect (4,5,14) or a negative effect (65,86) on ovulation rates in calves. Progesterone pretreatment also accelerated ovum transport through the oviduct (86). Injections of estradiol at the time of estrus has also been shown to have a negative effect on the ovulation rate of superovulated calves (65,86,89).

Superovulation treatments can significantly increase follicular development. One important question is, where are these follicles coming from? Studies have shown that superovulation treatments stimulate the small tertiary follicles to develop and ovulate (12,111). Testart (111) studied the follicle populations in the superovulated calf and found the 4 to 5 mm follicles to be the critical population. These follicles begin to increase as early as 16 hours after the start of the superovulation treatments at the expense of the 2 to 3 mm follicle population. Although the majority of the follicles in the superovulated animal can be explained by small follicle growth, some can not be accounted for. Some researchers believe that superovulation treatments
may also be causing atretic follicles to be ovulated (38). Tables 1 and 2 illustrate the increases in follicular development which can occur as a result of PMSG and FSH treatments. Note that in most cases the increases in the numbers of large follicles are at the expense of the small follicle population.

Two to four month old calves have considerable follicular development with the ovaries possessing many antral follicles. Attempts at ovulating these follicles with LH or HCG has been ineffective (12,100). For this reason it is believed that both FSH and LH are required for ovulation in the calf (12).

Some researchers (14,86) have found a dose relationship between the amount of FSH or PMSG administered and the numbers of follicles or ovulations produced. In general the greater the quantity of FSH or PMSG given the greater the number of follicles which develop and ovulate. However individual variability makes this relationship imperfect. In the selection of a proper dosage, one is striving for the maximum production of good quality fertilizable oocytes. The dose can be increased until we reach a maximum quantity of follicular development, however this is probably not the point at which the best oocytes are produced. Studies (12,45) show that follicles of the 6 to 10 mm diameter range produce mature oocytes with the majority reaching Metaphase II. The majority of oocytes found in smaller follicles (3 - 5 mm) are immature, while many of the oocytes from larger follicles (< 10 mm) tend to be degenerate. Increasing the dosage of FSH or PMSG also increases follicle size so a dose must be selected in which the maximum number of medium sized follicles are produced.
### TABLE 1

FOLLICULAR RESPONSES TO PMSG AND HCG IN PREPUBERTAL CALVES

*(Bedirian and Baker, 1975)*

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Number of Follicles</th>
<th>3-5mm</th>
<th>6-10mm</th>
<th>10mm</th>
<th>No. CL's</th>
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<tr>
<td>Control</td>
<td>4</td>
<td>45.8</td>
<td>2.8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1000 IU PMSG</td>
<td>4</td>
<td>13.8</td>
<td>10</td>
<td>29.3</td>
<td>0.25</td>
</tr>
<tr>
<td>1000 IU HCG</td>
<td>4</td>
<td>50</td>
<td>2</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>1000 IU PMSG + 1000 IU HCG</td>
<td>4</td>
<td>19.8</td>
<td>28</td>
<td>19</td>
<td>12.5</td>
</tr>
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### TABLE 2

FOLLICULAR RESPONSES TO PMSG, FSH AND LH IN PREPUBERTAL CALVES

*(W. D. Foote et al., 1978)*

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total No. of Follicles</th>
<th>% Small Follicles</th>
<th>% Medium Follicles</th>
<th>% Large Follicles</th>
</tr>
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<tr>
<td>Controls</td>
<td>16</td>
<td>98</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2000 IU PMSG</td>
<td>52</td>
<td>20</td>
<td>66</td>
<td>14</td>
</tr>
<tr>
<td>75 mg FSH</td>
<td>65</td>
<td>49</td>
<td>44</td>
<td>7</td>
</tr>
<tr>
<td>2000 IU PMSG + 20 mg LH</td>
<td>56</td>
<td>29</td>
<td>55</td>
<td>16</td>
</tr>
<tr>
<td>75 mg FSH + 20 mg LH</td>
<td>71</td>
<td>65</td>
<td>27</td>
<td>8</td>
</tr>
</tbody>
</table>
Several studies (54, 57, 70, 99) have been conducted to see if age and/or weight can affect the superovulation response in calves. In general most studies found no effect of age (54, 70, 71) or weight (56) on the numbers of follicles or ovulations produced. Marden (70) found no difference in the superovulatory response of calves ranging from 3 - 20 weeks of age. Seidel et al. (99) showed an age response on follicular development and on the ovulation rate. The calves used were 0, 4 and 8 weeks of age. They found that the 0 and 4 week old calves did not respond as well as the 8 week old calves. They believed the reason for this is a shortage of vesicular follicles at the earlier ages.

Comparisons of different injection regimens are difficult to make and vary with the individual researchers. Ages of the calves and the dosages vary widely. Table 3 is a summary of most of the research work and the responses obtained from the superovulation of prepubertal calves.

The exhibition of estrus in the young calf is similar to that in the postpartum cow because the first ovulation is usually accompanied by a silent estrus. It is believed there is a prerequisite for progesterone before estrus behavior is properly displayed. Morrow (82) found 74% of normal heifers at the time of first ovulation had a silent estrus. Reports (5, 70) in the superovulated calf are similar. Mickelsen et al. (77) reported that only 5 out of 28 superovulated calves showed signs of estrus behavior.

Ovulation rates in superovulated calves are highly variable between individuals given the same treatment. Avery et al. (5) found a range in ovulation rates from 1 to 77, with a mean of 37, in calves receiving
## TABLE 3

**SUMMARY OF THE SUPEROVULATION OF PREPUBERTAL CALVES**

<table>
<thead>
<tr>
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<td>Avery et al. (5)</td>
<td>65-173 Da</td>
<td>43</td>
<td>45 mg FSH 100 mg LH</td>
<td>--</td>
<td>37.8</td>
<td>33 24</td>
</tr>
<tr>
<td>Jainudeen (57)</td>
<td>4-24 wks</td>
<td>13</td>
<td>2000 IU PMS 10 mg NIH*LH</td>
<td>28</td>
<td>16</td>
<td>27-60 6</td>
</tr>
<tr>
<td>Bedirian &amp; Baker (12)</td>
<td>18-21 wks</td>
<td>4</td>
<td>1000 IU PMS 1000 IU LH 1000 IU PMS</td>
<td>66.8</td>
<td>12.5</td>
<td>79</td>
</tr>
<tr>
<td>Black et al. (14)</td>
<td>2-10 wks</td>
<td>32</td>
<td>FSH various sources &amp; HCG</td>
<td>9.8</td>
<td>2.0</td>
<td>35 7</td>
</tr>
<tr>
<td>Fiser et al. (42)</td>
<td>13-15 wks</td>
<td>7</td>
<td>PMS &amp; HCG</td>
<td>54.1</td>
<td>35.9</td>
<td>135 61</td>
</tr>
<tr>
<td>Foote, W. D., et al. (45)</td>
<td>6-9 mo</td>
<td>40</td>
<td>2000 IU PMS 20 mg LH</td>
<td>61</td>
<td></td>
<td></td>
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<tr>
<td>Howe (54)</td>
<td>?</td>
<td>33</td>
<td>PMS and HCG</td>
<td>4.27</td>
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<tr>
<td>Lineweaver (65)</td>
<td>45-90 Da</td>
<td>50</td>
<td>1000-2000 IU PMS 1500-10,000 IU HCG</td>
<td>53</td>
<td>23.5</td>
<td>153 4</td>
</tr>
<tr>
<td>Marden (70)</td>
<td>24-30</td>
<td>17</td>
<td>APH &amp; LH</td>
<td>18.6</td>
<td>15.41</td>
<td>70 4</td>
</tr>
<tr>
<td>Marden (71)</td>
<td>?</td>
<td>20</td>
<td>Ant. Pit Horm.</td>
<td>25.6</td>
<td>1.75</td>
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</tr>
<tr>
<td>Mickelsen et al. (77)</td>
<td>4-7 mo</td>
<td>28</td>
<td>1200 IU PMS or 30 mg FSH with &amp; without 50 mg LH</td>
<td>--</td>
<td>9.3</td>
<td>56 39</td>
</tr>
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TABLE 3 — Continued

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<tr>
<td>Onuma &amp; Foote (84)</td>
<td>17 wks</td>
<td>14</td>
<td>2000 IU PMS</td>
<td>66.5</td>
<td>36.5</td>
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<tr>
<td>Onuma et al. (86)</td>
<td>8-9 wks</td>
<td>29</td>
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<td>53</td>
<td>25</td>
<td>145</td>
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<td></td>
<td>25-150 mg LH or 1500 IU HCG</td>
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<tr>
<td>Onuma et al. (87)</td>
<td>9-11 wks</td>
<td>14</td>
<td>50 mg FSH 10-150 mg LH</td>
<td>30.8</td>
<td>2.6</td>
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<tr>
<td>Reidel &amp; Rommel (89)</td>
<td>6 mo</td>
<td>41</td>
<td>1500 IU PMS HCG or GnRH</td>
<td>--</td>
<td>22.6</td>
<td>--</td>
<td>--</td>
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<tr>
<td>Seidel et al. (100)</td>
<td>2 mo</td>
<td>20</td>
<td>1500 IU PMS 50 or 75 mg LH</td>
<td>44</td>
<td>28</td>
<td>86</td>
<td>38</td>
</tr>
<tr>
<td>Testart (112)</td>
<td>3 mo</td>
<td>7</td>
<td>1800 IU PMS Progesterone Pessary</td>
<td>12.7</td>
<td>14</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
45 mg of FSH and 100 mg of LH. Jainudeen et al. (57) noted similar variability in calves superovulated with PMSG. Most of the early work (14, 20, 54, 71) on the superovulation of calves showed very low numbers of ovulations when compared with the numbers of antral follicles which develop. More recent studies (42, 84, 89) have shown ovulation rates of 70 - 90% when injection schedules are refined and an ovulatory dose of LH is included. Table 3 contains a summary of ovulation results from many studies.

Fertilization rates are usually very low in prepubertal calves when compared with the numbers of follicles which ovulate (44). Table 3 shows the numbers of embryos which were collected in the different studies. Low fertilization rates may have many contributing factors. The first being a general immaturity of the reproductive tract which may be a hostile environment to the developing embryo. Seidel et al. (101) noticed that embryos with four or more blastomeres did not undergo further cleavage when collected and cultured in vitro. They postulated damage may result if embryos are allowed to remain in the tract to the four cell stage. Highly elevated estradiol and progesterone levels in the serum may cause an adverse effect on the oviductal and uterine environments (93, 112). Ovum transport in the oviduct is accelerated in calves administered progesterone with the superovulatory treatment (71, 86). This can place the embryo in a uterus not yet prepared to receive it. The ovaries of the superovulated calf respond by expanding greatly in size, 25 or more times as large as in the unstimulated calf (84). Some researchers (57, 84) believe the immature fimbria cannot adequately cover the ovary and pick up all the oocytes from the
follicles which ovulate. Another problem which may cause low fertilization rates is poor sperm transport to the site of fertilization.

However a study done by Howe and Black (52) showed motile sperm could be found in the oviduct of the calf 13 minutes after insemination in the vagina.

Information on hormone levels in the prepubertal calf are scarce (30,89,93,112). From hormone levels in control groups (10,89) it can be seen that gonadotropins and steroid levels remain relatively constant from birth till about the time of puberty. Progesterone levels may increase gradually throughout this period of life (30). Basal levels of gonadotropins are in the range of 0.5 – 5.0 ng/ml for LH (10,29,58,108, 112) and 1.0 – 30.0 ng/ml for FSH (10,29,112). Basal levels for steroids are about 3 – 20 pg/ml for estradiol (10,98,112) and 0.5 ng/ml for progesterone (98,112).

The patterns of serum gonadotropins in the superovulated calf are similar to those found in the superovulated cow (92,108,112). With the initiation of the superovulation treatment, gonadotropins rise abruptly possibly due to a positive feedback of estrogens from the developing follicles. LH levels peaked at 5 to 7 ng/ml one day after the PMSG injection (108,112). Levels then decline back to basal levels over the next seven to ten days. There was no evidence of an LH surge prior to the time of ovulation. FSH follows primarily the same pattern as LH except, FSH peaks at a higher value of 23 – 40 ng/ml (112). Progesterone levels
in the blood of the superovulated calf follow development of the corpora lutea which form. Peak progesterone levels after ovulation have been found to be positively correlated ($r=0.76, P<0.01$) with the numbers of corpora lutea which develop (108). Treated calves can produce more than 100 grams of luteal tissue with a progesterone concentration similar to that found in the corpus luteum of a mature animal (107). Spilman et al. (107) found that corpus luteum slices from prepubertal calves continue to produce progesterone 20 days past superovulation, while slices from mature cows had stopped producing progesterone between 15 and 20 days after ovulation. In vivo an increase has been seen in the life of the corpora lutea of prepubertal calves (98). The reason for this is unknown but may be due to a lack of luteolytic factor production by the immature uterus or the variation in the ages of the corpora lutea as a result of ovulations over a period of several days.

Progesterone levels remain at basal levels until the time of estrus when they begin to rise. Maximum progesterone levels of 100 ng/ml were reached about six days after the ovulatory LH injection (98,108,112). Levels begin to decrease about 8 to 16 days after ovulation and slowly drop to basal levels by 21 to 30 days after ovulation. Figure 1 shows the variations in progesterone and estradiol levels in the prepubertal calf superovulated with PMSG (98). In the study of Schneider et al. (98) no permanent cyclic activity was produced in the calves and they all returned to their prepubertal condition.

 Estradiol levels in the blood follow follicular development. Estradiol concentrations increase as early as two days after beginning
Progesterone and Estradiol in Blood Plasma of the Calf Following Superovulation.
Schneider et al. (1980)
the superovulation treatment and peak six days after starting the treat-
ment (98). Calves with moderate follicular responses will have peak
estradiol levels of 250 pg/ml but those levels drop precipitously after
the ovulatory LH injection (98). Testart et al. (112) found that
ovulatory estradiol concentrations were highly correlated ($r^2=0.92$;
P<0.01) with the number of ovulations, but were not correlated with the
number of large unruptured follicles. Post-ovulatory estradiol levels
were not correlated with the numbers of ovulations or large unruptured
follicles.

Estradiol and progesterone levels are much higher in the stimulated
prepubertal calf than in the normally cycling adult (95) or in the
superovulated mature cow (51,91,92,94). Estradiol levels may reach
140 pg/ml in the superovulated cow prior to ovulation and progesterone
levels peak at 20 - 50 ng/ml during the mid luteal phase (91,94). Part
of the problem of poor fertility in the superovulated calf may be an
endocrine imbalance affecting the reproductive process.

The Chemical Composition of Follicular Fluid

Follicular fluid is a viscous straw colored fluid which collects
around the oocyte in the developing follicle. It is composed of
exudates of the plasma and products of the follicular tissues. Folli-
cular fluid contains electrolytes, proteins, carbohydrates, mucopoly-
saccharides, vitamins, lipids and hormones. The function of follicular
fluid is unknown but it has been implicated in the control of many
processes at the follicular and cellular levels.
Follicular fluid has a pH of 7.4 which is close to that of blood plasma (76). Levels of most of the ions are also similar to plasma, including Mg, Cl, Ca, Zn, Cu and Phosphate (60). Sodium and potassium have been shown to fluctuate with the stages of the estrous cycle in the cow (33). Scheutz and Anisowicz (97) found that the levels of electrolytes decreased as the size of the follicle increased in the pig.

Follicular fluid also contains many carbohydrates. In the cow 80% of the total carbohydrate content is glucose. Its concentration is about 40mg% (33). Other carbohydrates have been found in the follicular fluid, including fructose, fucose, lactic acid, sialic acid, citric acid (72) and protein bound hexoses (68).

The follicular fluid contains some mucopolysaccharides. Zachariae (118) found that growing follicles with follicular fluid used large quantities of S\(^{35}\) in the formation of follicular fluid. The sulfur taken up by the follicles was used in the making of hyaluronic acid and chondroitin sulphuric acid. These mucopolysaccharides are believed to form a network in the follicular fluid and a coating around the antrum of the follicle (118). The breakdown of this network may be important in the ovulation of the follicle (83).

Follicular fluid also contains many proteins. Most of the proteins found in the fluid are in equilibrium with the blood serum. There is some filtering out of the very large serum proteins such as fibrinogen and the beta lipoproteins. Shalgi et al. (102) found a relationship between the relative concentration of a protein in the follicular fluid and its molecular weight. The larger the protein the more it was excluded from the follicular fluid. Immunoglobulins, even though they
are very large in size were found in very high concentrations and have been implicated in protection of the ova after ovulation (114). Antisperm antibodies present in the follicular fluid may be a reason for reproductive failure in some individuals. Anderson and others (3) found that the mean protein concentration of the follicular fluid was 86.4% of the sera. Specific steroid binding proteins may be important in maintaining very high levels of steroids in the follicular fluid. Cook, Hunter and Kelly (24) found no unusual steroid binding proteins in the follicular fluid of cattle, sheep or swine. Most of the steroids are bound very loosely to albumin much like they are in the blood.

Bovine follicular fluid contains many different enzymes. Many of the enzymes found in the follicular fluid are found in most cells of the body; these include lactate dehydrogenase, aspartate and alanine aminotransferases, alkaline and acid phosphatases (72), plasmin (13), proteinase, hyaluronoglucosidase (72) and acetylcholinesterase (27). Follicular fluid has been shown to contain many hormones including LH, FSH, prolactin, estrogens, androgens and progestagens. McNatty et al. (73,74,75) found that with the exception of prolactin and the androgens, all the rest of the hormones were correlated with plasma levels in humans. They believed the sequence of hormone changes noted in the follicular fluid may be of considerable importance in follicular growth and control.

The major hormone found in the follicular fluid is estrogen. There are theories which try to explain where estrogens are produced in the follicle. In vitro studies (81) using follicular cells support the two
cell theory of estrogen synthesis. It states that the theca produces androgens which diffuse into the follicle where they are aromatized into estrogens by the granulosa cells. The two cell theory is beginning to receive greater support because it does explain the reason for high levels of androgens in the follicular fluid (7). Younglai and Short (117) injected radioactive pregnenolone and androstenedione into the graafian follicle of a mare and followed their movement through the body tissues. They found that most of the radioactivity remained in the follicle. From this they concluded that the majority of the estradiol found in the blood is derived from extrafollicular sources. Studies in swine (40) and sheep (79) examined different parts of the ovarian follicle and the steroids produced by each. It was found the theca is the major source of androstenedione and testosterone whereas the granulosa is the major source of progesterone. Estradiol was not produced by the theca or the granulosa alone (40) but when supplied with the proper intermediates both produced comparable quantities of estradiol (79). Maximum estradiol production required that the theca and granulosa be in close juxtaposition with one another and not simply in coculture. This suggests that a transfer of androgens may be taking place between the theca and granulosa layers of the follicle however this may not be totally essential to estrogen production (40).

Many steroids have been found in very high concentrations in the follicular fluid. Levels in the follicular fluid may be as much as 100 to 100,000 times higher than levels found in the blood stream and 5 to 10 times higher than levels found in the perifollicular capillary network (35). Younglai (110) quantified steroid levels in the follicular
fluid of the mare. He found the following concentrations: progesterone, 35 ng/ml; 17-alpha hydroxyprogesterone, 130 ng/ml; androstenedione, 125 ng/ml; 19 norandrostenedione, 110 ng/ml; epitestosterone, 80 ng/ml estrone, 90 ng/ml; and estradiol, 1510 ng/ml. Short (105) studied concentrations of steroids in a pooled sample of bovine follicular fluid. The results of this study are shown in Table 4. The table shows that progesterone was found to be the major steroid in the follicular fluid however all sized follicles in all stages of development were used. In general estrogens are the major steroids found in the follicular fluid of developing follicles (8). Since most other follicular fluid components are in equilibrium with the blood, the high concentration of estrogen suggest that steroids are selectively held within the follicle.

Bahr (6) took simultaneous measurements of the steroids in the follicular fluid and the ovarian venous blood, in the rabbit. She found the majority of the steroids are secreted directly into the ovarian vein, while only small amounts are secreted directly into the follicular fluid. This suggests the secretion of steroids into the follicle does not contribute significantly to increased ovarian output.

Follicular fluid steroid levels reflect the steriodogenic activity of the follicle (47). Changes in these levels are usually caused by altered function of the theca and granulosa cell layers surrounding the follicle.

Follicular fluid steroid levels change throughout the estrous cycle of an animal and especially about the time of ovulation. Studies in humans (73,74) have found that estrogen levels in small follicles were
<table>
<thead>
<tr>
<th>Steroid</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnenolone</td>
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</tr>
<tr>
<td>Progesterone</td>
<td>233.0</td>
</tr>
<tr>
<td>20 beta-Hydroxypregn-4-en-3-one</td>
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</tr>
<tr>
<td>17 alpha-Hydroxyprogesterone</td>
<td>37.6</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>31.5</td>
</tr>
<tr>
<td>Testosterone</td>
<td>22.1</td>
</tr>
<tr>
<td>Oestrone</td>
<td>5.4</td>
</tr>
<tr>
<td>Oestradiol-17 beta</td>
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</tr>
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Steroids that could not be detected:

<table>
<thead>
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<th>Steroid</th>
<th>Concentration (ng/ml)</th>
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</thead>
<tbody>
<tr>
<td>Cortisol</td>
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</tr>
<tr>
<td>19-Hydroxyandrostenedione</td>
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</tr>
<tr>
<td>19-Norandrostenedione</td>
<td>5.0</td>
</tr>
<tr>
<td>epiTestosterone</td>
<td>5.0</td>
</tr>
<tr>
<td>17 alpha: 20 beta Dihydroxypregn-4-en-3-one</td>
<td>5.0</td>
</tr>
<tr>
<td>6 alpha Hydroxyoestradiol-17 beta</td>
<td>5.0</td>
</tr>
<tr>
<td>Oestradiol-17 alpha</td>
<td>10.0</td>
</tr>
<tr>
<td>17 alpha-Hydroxyprogrenolone</td>
<td>0.5</td>
</tr>
<tr>
<td>Dehydroepiandosterone</td>
<td>0.5</td>
</tr>
</tbody>
</table>

(Short, R. V.; 1962.)
unchanged throughout the menstrual cycle. Levels in large follicles increased in the mid to late follicular phase. Androstenedione decreased in these large follicles probably because of its use as a precursor in estrogen synthesis. Serum steroid levels usually follow steroid levels in the follicular fluid. Brand (15) examined estradiol levels in the follicular fluid of sheep and found levels remained very low throughout the luteal phase of the estrous cycle. However during the follicular phase, levels increased and peaked just prior to ovulation, with a pattern similar to that found in the blood. Eiler and Nalbandov (35) studied concentrations of estrogen, progestagens and androgens in the follicular fluid of swine. The estrogen concentration was found to be 13 ng/ml on Day 8, 180 ng/ml on Day 20 and 72 ng/ml twelve hours prior to ovulation. Progesterone went from 32 ng/ml to 754 ng/ml and 120 ng/ml, while androgens went from 1 ng/ml to 21 ng/ml and 177 ng/ml, on Days 8, 20, and 12 hours before ovulation, respectively.

Follicular levels of steroids change rapidly as ovulation approaches. The follicle suddenly changes from the production of primarily estrogen to a producer of progesterone, under the influence of the ovulatory surge of LH. In rats (47) progesterone levels increased seven fold in the follicular fluid within one hour after an LH injection. Estradiol and androstenedione rose slightly within the first hour and then declined to low levels ten hours after the LH surge. A similar rise in progesterone occurred in cultured large bovine follicles when injected with LH (103). It is believed that LH affects this progesterone rise by elevating cAMP levels which cause an increase in
the activity of 3-beta Hydroxy steroid dehydrogenase which is essential in the conversion of pregnenolone to progesterone (90). England and others (38) have studied the changes which take place in follicular fluid estradiol and progesterone levels prior to ovulation in the ewe. During the luteal phase of the estrous cycle progesterone levels in the follicular fluid are high (47 ng/ml) while estradiol levels were relatively low (3 ng/ml). During the follicular phase prior to the LH surge, progesterone dropped to 7 ng/ml and estradiol rose to 7 ng/ml overall, and higher (26 ng/ml) in large follicles. After the LH surge, progesterone levels rose to 40 ng/ml and estradiol levels dropped to 1 ng/ml. Ireland and Roche (56) found similar patterns in the ovulatory follicle of the cow. On Day 8 of the cycle, concentrations of progesterone, androstenedione, testosterone and estradiol in the follicular fluid averaged 47 ± 5, 2.6 ± 0.6, 2.4 ± 0.5 and 34 ± 8 ng/ml. After a prostaglandin injection, levels of all steroids increased, with estradiol rising to levels 19 fold higher than in follicles on Day 8. Concentrations of steroids remained relatively the same until after the LH surge when progesterone rises and estradiol and the androgens fall rapidly. The final stages of development of the preovulatory follicle are probably determined by the LH patterns and not FSH (9). Episodic LH surges occur after luteal regression and a decrease in blood progesterone levels. LH and FSH cause estradiol levels to rise by stimulating the aromatase enzymes which convert androgens to estrogens. A sustained rise in LH, such as during the LH surge prior to ovulation, inhibits aromatase activity and eventually all steroid secretion in the
follicle (9). The rise in intrafollicular progesterone levels may be important in final oocyte maturation prior to ovulation (96).

The ovaries of animals contain many follicles which vary tremendously in size and may respond differently to gonadotropins. Follicular fluid steroid levels vary widely between follicles of the same size and stage of development. Bartol et al. (14) examined quantities of estradiol produced by the largest and second largest follicles from the bovine ovary. Although there was little difference in size, on the average the largest follicles produced 33% more estradiol than the second largest follicles. In general smaller follicles are less reactive to gonadotropins and respond by producing less estradiol (17,38 103). As the size of the follicle increases so does its steroidogenic activity. Chang et al. (24) analyzed steroids in the follicular fluid of small, medium and large porcine follicles. They found that as size increased so did estrogen, progesterone and testosterone concentrations. England et al. (38) found significant correlations (P 0.01) between progesterone, testosterone and estradiol and the size of the follicle in the ewe, these values were +.72, −.70 and +.68, respectively. Ireland and Roche (56) found similar correlations in bovine follicular fluid (progesterone, +.55; testosterone, −.63; estradiol, +.69). Developing follicles display these correlations, very small follicles do not (38).

It has been noted that there are generally two populations of developing follicles present on the ovaries (17). The first being a population of relatively small inactive follicles with moderate levels of progesterone and relatively low levels of estradiol. These follicles will not respond to LH treatments and have decreased aromatase activity. As the
follicle begins to grow beyond a certain diameter, there is an increased response of the follicle to gonadotropin stimulation and estradiol increases rapidly at the expense of androgens.

The increase in estrogens in developing follicles, is intimately related to normal follicular development and function (80). In addition to the two populations of follicles mentioned above, another should be added. This group is the atretic follicles in which estrogen production is limited. Atretic follicles show similar steroid profiles in the follicular fluid to those found in small follicles. Atretic follicles generally have reduced steroidogenic function especially in the production of estrogens and have reduced aromatase activity (17). Androgens have been associated in follicular atresia in that they occur in greater quantities in atretic follicles and when added to cultured follicles do increase the incidence of atresia (18). Other researchers (17, 119) believe increased androgen levels may not cause atresia but are simply the result of decreased utilization as precursors in estrogen synthesis. Researchers (56) have used the criteria that if estradiol levels in the follicular fluid are lower than the sum of progesterone and testosterone levels the follicle is considered atretic. Moore (80) has also related follicle atresia with a dissociation of the granulosa layer from the follicular wall. The truth is that little is known about the reasons why some follicles continue to develop and ovulate while others regress. Recent findings (56) in the bovine indicate that the largest follicle present on the ovary up to Day 17 of the cycle is not the follicle that ovulates. That follicle regresses while another follicle rapidly takes its place and ovulates.
Follicular fluid steroid levels do show changes when ovarian function is altered.\(^{(115)}\). Friedrich et al.\(^{(46)}\) found cystic follicles in humans had lower levels of progesterone than normal graafian follicles. McNatty and Baird\(^{(68)}\) found that cystic follicles had higher amounts of androgens than estrogens in the follicular fluid. In normal follicles the ratio was reversed. Short\(^{(104)}\) found that concentrations of estrogen were lower in bovine cyst fluid. He also found cyst fluid had higher levels of androstenedione and progesterone than follicular fluid from normal cows.

Follicular fluid is believed to be involved in the control of maturation, ovulation and luteinization of ovarian follicles. Hunter, Cook and Baker\(^{(55)}\) found a dissociation of the response to injected gonadotropins between the follicle and the oocyte in swine. They proposed rising intrafollicular estrogen levels in the oocyte's response to gonadotropins. Darga and Riechert\(^{(26)}\) found that follicular fluid caused inhibition of radioactive FSH binding to bovine granulosa cells. Trafriri and Channing\(^{(22,34,113)}\) found that follicular fluid, when added to oocyte cultures inhibits spontaneous maturation past the dictyate stage of first meiosis. Stone et al.\(^{(110)}\) tried to isolate this factor from porcine follicular fluid and found it to be protein in nature. Ferrando et al.\(^{(41)}\) found injections of bovine follicular fluid reduced ovulation rates in rats. Miller et al.\(^{(78)}\) found that im injections of follicular fluid were effective in inhibiting ovulation in the bovine, when injected after PG administration. They believed follicular fluid was delaying follicular development and not luteolysis. Heat treated follicular fluid had the same effect. Ledwitz and Rigby
(64) found that follicular fluid stopped spontaneous luteinization which normally occurs in \textit{in vitro} cultures of granulosa cells. It acts to inhibit maturation of LH receptors on the granulosa cells and LH stimulation of cAMP. Channing (22,34) found follicular fluid added to anterior pituitary cell cultures inhibited basal and LH-RH stimulated FSH release. She concluded follicular fluid is a good source of ovarian inhibin. Although follicular fluid constituents change and have been implicated in many reproductive functions its physiological functions are still unknown.

Follicular fluid also has effects on other tissues of the body. Eiler et al. (36) found that bovine follicular fluid increased contractility of rat uteri and alveoli of the bovine mammary gland. Breur and Wells (15) found that follicular fluid also sped up capacitation of spermatozoa \textit{in vitro}.

**Radioimmunoassay**

The radioimmunoassay is a very common and accurate method of measuring small amounts of hormone in body fluids. The technique involves creating a competitive binding situation in which radioactive and non-radioactive hormone compete for antibody sites. With the separation of bound from unbound hormone, we can measure the amount of radioactivity bound to the antibody. By including a number of standard tubes, with known amounts of hormone in them, we can develop a curve of standard concentrations versus radioactivity and interpolate the concentrations in the unknowns from the curve using the radioactivity
measurements obtained from the unknowns (1). Sensitivity of the radioimmunoassay varies with the technician, laboratory, technique, hormones, antibody and isotope used. Steroid sensitivity levels generally fall in the range of 20-50 pg/ml, however, concentration of the samples prior to the assay enable the researcher to quantify levels much lower. The radioimmunoassay always has some variability, even under ideal conditions.
The Superovulation Procedure

Thirty-four dairy and beef heifer calves of various breeds were used in this study. Calves were obtained from The Ohio State University, Waterman Dairy Complex, The Animal Science Beef Barns and the State of Ohio Institutional Herd in Tiffin, Ohio. The calves ranged in age from one to six months old and 130-315 lbs. in weight at the time of surgery. All the calves were in good condition and were fed a ration consisting of good quality alfalfa hay and a concentrate mix, consisting largely of corn and soybean meal. Three of the calves were not yet weaned at the time of the study. The surgeries were conducted during the months of July and August 1981.

Superovulation was accomplished using injections of FSH-p (potency: 49.6 mg per 10 ml., Burns Biotech). This preparation is a semi-purified FSH extract obtained from swine pituitaries, and contains substantial LH activity. Each of the calves was allotted to one of three treatment groups: 1) 30 mg, 2) 19 mg or 3) 16 mg of FSH-p over several injections. The individual injection schedules are shown in Table 5. Twenty four hours prior to the expected time of surgery, each calf received a single intravenous injection of 2500 IU of HGC (lypo-Med.) to mimic the ovulatory surge of LH.
### TABLE 5

**INJECTION SCHEDULES**

<table>
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<tr>
<th>Treatment</th>
<th>Day</th>
<th>30 mg</th>
<th>19 mg</th>
<th>16 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, AM</td>
<td>5 mg FSH</td>
<td>1 mg FSH</td>
<td>1 mg FSH</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>5 mg FSH</td>
<td>2 mg FSH</td>
<td>2 mg FSH</td>
<td></td>
</tr>
<tr>
<td>2, AM</td>
<td>4 mg FSH</td>
<td>3 mg FSH</td>
<td>3 mg FSH</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>4 mg FSH</td>
<td>4 mg FSH</td>
<td>4 mg FSH</td>
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</tr>
<tr>
<td>3, AM</td>
<td>3 mg FSH</td>
<td>4 mg FSH</td>
<td>3 mg FSH</td>
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<tr>
<td>PM</td>
<td>3 mg FSH</td>
<td>3 mg FSH</td>
<td>2 mg FSH</td>
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<tr>
<td>4, AM</td>
<td>2 mg FSH</td>
<td>2 mg FSH</td>
<td>1 mg FSH</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>2 mg FSH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5, AM</td>
<td>1 mg FSH</td>
<td>2500 IU HCG</td>
<td>2500 IU HCG</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>1 mg FSH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6, AM</td>
<td>Surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Prior to surgery, feed was withheld for twelve hours to decrease rumen size.

Each calf was weighed on the day of surgery and then anesthetized using Rompun (.065 mg/lb., Cutter-Lab) and Ketamine (2 mg/lb.; Vetalar). Additional Ketamine was administered if the surgery required more time than usual. A line block was also given along the incision site using approximately 30 cc of a 2% Lidocaine solution.

A vertical incision 5-6" long was made in the left flank perpendicular to the backbone, approximately 2" cranial to the thurl. The ovaries were located and retracted to the incision site where ovarian size and follicular response could be visually assessed. Individual follicles were counted and their diameters measured. A 20 gauge hypodermic needle (1½ or 3" long) was introduced into the follicle near its base and the follicular contents were removed from all follicles greater than or equal to 1 mm in diameter. Follicular fluid samples were collected separately from small follicles (<5mm) and large follicles (>5 mm). One half ml was collected from both large and small follicles if possible. Any additional follicular fluid was collected and searched for oocytes to be used in an oocyte maturation and in vitro fertilization study (25).

Blood samples were collected from each calf by puncture of the jugular vein. Samples were allowed to clot and then centrifuged at 2500 RPM (1240g) for 20 minutes. Serum samples were collected prior to the first FSH injection, during the middle of the FSH treatments, at the time of surgery (just prior to the expected time of ovulation) and 2, 4,
6, and 21 days post surgery. Samples were stored frozen at -20 C until steroid analysis could be done.

Radioimmunoassay

The radioimmunoassay was performed in the steroid laboratory of the Department of Obstetrics and Gynecology, The Ohio State University (88). Samples were thawed at room temperature and mixed thoroughly prior to extraction. A single serum sample of 2 ml was dispensed for progesterone and estradiol analysis. Follicular fluid samples of 10 or 100 ul were used depending on the size of the sample. The aliquot was placed into a test tube for extraction. The levels of follicular fluid were brought up to 0.5 ml with isotonic saline for more efficient extraction. Five ml of fresh ether was added to each tube; it was then mixed for 10 seconds and allowed to settle out. The tubes were then placed in a dry ice and ethanol bath to freeze the aqueous layer. The ether was decanted off into another tube. Follicular fluid samples were extracted a second time with an additional 5 ml of ether. Four samples out of each fifty contained radioactive steroid in addition to the serum and were used to calculate recovery rates for the extraction. The samples were allowed to dry over night.

Antibodies were supplied by the Department of Obstetrics and Gynecology. The progesterone antibody was synthesized in a rabbit using bovine serum albumin (BSA) bound to progesterone at the 11-alpha position, as the antigen. The cross reactivity of the antibody was found to be 6.5% with 17-alpha progesterone and 0.01% with 20-alpha Hydroxy-4-pregn-3-one and 3-beta Hydroxy-5-pregnen-20-one. Cross reactivity was
undetectable with other steroids. The antibody for 17-beta Estradiol was synthesized using a BSA-6-carboxymethyl oxime of estradiol. Cross reactivity of the estradiol antibody was less than 0.01% with estrone and estriol (88).

Samples were resuspended in 0.1% Knox Gelatin Phosphate Buffered Saline (KG-PBS) and allowed to sit overnight. Serum samples were resuspended in 2.0 ml of 0.1% KG-PBS, follicular fluid samples in 0.5 ml.

Aliquots of different doses were pulled from each sample and placed into assay tubes. The levels of each tube were brought up to 0.5 ml to maintain equal concentrations throughout the assay. Different dose levels were used to test parallelism of the standard curve to the dose response. Duplicate standard tubes, total count and nonspecific binding tubes were added to each assay. Four "Zero" dose tubes were added to the assay, two placed with the standard curve, one before the unknowns and one at the end of the assay. This is used to measure differences in technique throughout a single assay.

Labeled antigen was added to all tubes at a rate of 0.1 ml per tube and mixed. Antibody was then added to all except the total count and non-specific binding tubes, at a rate of 0.1 ml/tube. All tubes were incubated overnight at 4 C.

The next day, 0.1 ml of 0.5% of KG-PBS was added to each tube, to stabilize the charcoal pellet. Charcoal suspension (1%) was added to all except the total count tube, at a rate of 0.5 ml/tube. Tubes were mixed and incubated at 2 C for 20 minutes, to absorb all the unbound steroid. Tubes were then centrifuged for 15 minutes at 1000g. The tubes were removed sequentially and the supernatant decanted from the
charcoal pellet into scintillation vials. Counting cocktail was added to each vial, the vials were then mixed and placed into the counter. The vials were equilibrated for at least an hour before counting for 2 minutes.

Dose levels, sample size and counts per minute, for each tube were entered into the computer. The program used corrected the CPMs for chemical quench and then finds the best fit standard curve. From this standard curve, the unknown concentrations are interpolated using the corrected CPM figures for the unknowns.

Ten samples were added to three estradiol assays to determine the interassay variation.

Statistical Analysis

Serum progesterone and estradiol levels were analyzed using a mixed model least squares analysis designed by W. R. Harvey of The Ohio State University. In the analysis, sums of squares were partitioned out for treatment effects, day of collection and any interactions between treatment and day of collection.

The General Linear Models Procedure (proc GLM) of the Statistical Analysis System (SAS) (48) was used to test the significance of treatments effects and age and weight regressions on each of the dependent variables (responses, follicular fluid levels, and serum levels) in the study. A step-down procedure was used to determine the best fit model (including treatment, age, weight and all possible interactions) for each of the independent variables. Independent variables were eliminated from the model when the probability of significant differences
was greater than 0.15. Partial correlation coefficients were computed for each dependent variable with all other dependent variables after the variation due to the model was removed.

Simple correlations were calculated between all the variables studied. Students t-test was used to test the differences between group means.
RESULTS

Effect of Exogenous Gonadotropins on Ovarian Size and Follicular Response

Table 6 is a summary of the ovarian sizes and responses found in this study as reported by Longo (66,67). Ovarian size was computed by multiplying the length of the ovary by the width as measured at the time of surgery. Average ovarian size was 6.66 ± 1.07 cm² and the average number of follicles per calf was found to be 36.1 ± 6.0. Treatments had a significant effect (P<0.05) on both ovary size and the numbers of follicles produced. The smaller doses of FSH over a shorter period of time (16 and 19 mg treatments) produced smaller ovaries than did the 30 mg treatment given over 5 days. Numbers of follicles showed a dose relationship in which the 19 mg group produced significantly (P<0.05) more follicles than did the 16 mg group. A positive correlation (r=.579; P<0.001) was found between the number of follicles produced and the ovarian size.

Effect of Exogenous Gonadotropines on Follicle Sizes

Table 7 is a summary of the follicle size data from this study as reported by Longo (66,67). The average follicle size for the entire study was found to be 5.61 mm in diameter and the percentage of small follicles (<5 mm in Dia.) was 58%. Treatments had a significant effect.
TABLE 6

OVARIAN AND FOLLICULAR RESPONSE TO FSH TREATMENTS

<table>
<thead>
<tr>
<th>Treatment mg of FSH</th>
<th>n</th>
<th>Ovarian Size</th>
<th>Total # of Follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>9</td>
<td>11.54 ± 2.32a</td>
<td>29.9 ± 6.6</td>
</tr>
<tr>
<td>19</td>
<td>17</td>
<td>5.53 ± 1.25</td>
<td>44.9 ± 9.8a</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>3.46 ± 1.25</td>
<td>17.2 ± 6.9</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>6.66 ± 1.07</td>
<td>36.1 ± 6.0</td>
</tr>
</tbody>
</table>

Mean ± SEM.

*a p 0.05.

Longo, K. L.; (61,62)

---

TABLE 7

EFFECT OF FSH TREATMENT ON AVERAGE FOLLICLE SIZE AND PERCENT SMALL FOLLICLES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Follicle Size</th>
<th>% Small Follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mg</td>
<td>7.69 ± 0.60</td>
<td>36 ± 10</td>
</tr>
<tr>
<td>19 mg</td>
<td>4.25 ± 0.44a</td>
<td>72 ± 7a</td>
</tr>
<tr>
<td>16 mg</td>
<td>6.48 ± 1.17</td>
<td>48 ± 14</td>
</tr>
<tr>
<td>Total</td>
<td>5.61 ± 0.44</td>
<td>58 ± 5.9</td>
</tr>
</tbody>
</table>

Mean ± SEM.

*a p 0.05.

Longo, K. L.; (61,62).
(P<0.05) on average follicle size, with the 19 mg treatment group producing the largest numbers of follicles but, of a much smaller size (4.25 mm). The 19 mg treatment group also had a significantly (P<0.05) greater percentage of small sized follicles. As would be expected, there was a negative correlation (r=.925; P<0.0001) between average follicle size and the percent small follicles.

Age and Weight Effects on the Follicular Response to Gonadotropins

No age or weight effects were found on the numbers of follicles produced, percentage of small follicles, average follicle size or relative ovarian size (P>0.05).

Serum Estradiol Concentrations

Estradiol levels found in this study were highly which made any patterns or relationships involving estradiol difficult to ascertain. The overall mean concentration of estradiol in the blood serum was 117.8 ± 4.83 pg/ml with a range in concentrations from 3 pg/ml to 1625 pg/ml. Estradiol levels averaged 81.4 ± 11.4 pg/ml on Day 1 prior to the start of the superovulation treatments. No age or weight effects were found in Day 1 estradiol concentrations. Fluctuations in overall mean estradiol levels throughout the superovulation are shown in Figure 2. After the FSH injections were started, estradiol rose to a maximum of 177 ± 27.5 pg/ml on the day of surgery. Levels declined after surgery to 118 ± 32 pg/ml on Day 6 post-surgery and to 40 ± 6.7 pg/ml on Day 21 post-surgery.
Figure 2

Serum Estradiol and Progesterone Concentrations Averaged Over All Treatments

SO — Superovulation.
Appendix Table 10 shows the analysis of variance table for the serum estradiol concentrations. It shows treatments had no effect on serum estradiol levels. However, there was a significant (P<0.05) Day effect which was quartic in nature. These equations account for most of the variation seen in serum estradiol concentrations illustrated in Figure 2.

No age or weight effects were found overall, however there was an effect of age on estradiol levels in samples collected during the middle of the superovulation treatment (P<0.005).

There was no significant (P>0.05) relationships between serum estradiol levels and ovarian size or the numbers of follicles which were produced. There was a relationship (r=.38; P<0.05) between estradiol on Day 1 prior to the FSH treatments and the average follicle size.

**Serum Progesterone Concentrations**

The overall mean progesterone concentration in the serum was found to be 1.57 ± 0.36 ng/ml with a range in values from a low of 0.021 ng/ml to a high of 50.606 ng/ml. Figure 2 shows the fluctuations which were found in mean progesterone values throughout the study. The average concentration on Day 1 prior to the start of the superovulation treatments was 0.170 ± 0.032 ng/ml. The Day 1 concentrations were significantly related to the age of the animal. In general an increase of one day of age caused an increase in progesterone in the serum on Day 1 of 0.0023 ng/ml. Mid-superovulation levels averaged 0.148 ± 0.018 ng/ml. Levels began to rise after the middle of the superovulation to a high on
Day 6 post-surgery of 5.267 ± 1.908 ng/ml. Levels returned to near basal levels by Day 21 post-surgery.

Appendix Table 11 is the analysis of variance table for serum progesterone concentrations found in this study. It illustrates a significant treatment effect (P<0.001) on progesterone in the blood serum. Figure 3 shows the patterns of progesterone levels for each treatment group. It can be seen that the longer treatment (30 mg) caused greater levels of progesterone in the post-surgery period. Appendix Table 11 also indicates a Day effect which is cubic in nature.

Serum progesterone concentrations also showed a significant (P<0.0001) treatment-by-day interaction which can be illustrated in Figure 3. A treatment-by-day interaction means the different treatments affected serum progesterone levels differently on the different days of the study. For example in Figure 3 it can be seen that progesterone concentrations are increased in the 30 mg group on Day 6 post-surgery. However, this increase is much less between the treatment groups on the day of surgery.

**Follicular Fluid Estradiol Concentrations**

The overall estradiol concentration in the follicular fluid was found to be 366.7 ng/ml. The levels of estradiol in the follicular fluid were highly variable. See Raw Data, Table 14. Concentrations in large follicles ranged from 12.7 to 1825.7 ng/ml and in small follicles from 6.7 to 946.6 ng/ml in large follicles. These levels are 1000 to 100,000 times higher than levels found in the blood serum. Table 8
**Figure 3**

Effect of FSH Treatments on Serum Progesterone Concentrations

SO — Superovulation.
shows the mean levels of estradiol in the follicular fluid of both large and small follicles for each of the three treatment groups. Treatments had an effect on follicular fluid estradiol (P<0.01).

Appendix Tables 12 and 13 are the analysis of variance tables for estradiol in the follicular fluid of both large and small follicles, respectively. They both show that treatment groups had significant effects (P<0.01) on estradiol concentrations in the follicular fluid. In small follicles, the 19 mg treatment group had the highest concentrations of estradiol (479.9 ± 73.0 ng/ml) and it was different from both the 16 and the 30 mg treatment groups. In large follicles, the highest concentrations were found in the 16 mg group (730.2 ± 555.2 ng/ml) and the 30 mg group had the lowest concentrations (216.8 ± 106.5 ng/ml). The 30 mg group was significantly different (P<0.01) from both the 16 and 19 mg groups.

Age effects were found to be significant in affecting the estradiol concentrations of both large and small follicles. Estradiol concentrations in small follicles increased 8.3 ng/ml per day of age (P<0.05). Concentrations in large follicles increased 22.7 ng/ml per day of age.

A weight effect was found in estradiol concentrations of large follicles. As the weight of the animal increased, estradiol decreases in large follicles at a rate of 9.3 ng/ml per pound (P<0.001). No weight effects were found in small follicles.

As the average follicle size increased estradiol concentrations in small follicles decreased (r=-0.458; P 0.05). A negative correlation (r=-0.604; P 0.05) between estradiol in the serum and estradiol levels in large follicles was observed on the day of surgery.
### TABLE 8
FOLLICULAR FLUID ESTRADIOL CONCENTRATIONS

<table>
<thead>
<tr>
<th>Treatment mg of FSH</th>
<th>Small Follicles ng/ml</th>
<th>Large Follicles ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>162.0 ± 73.8</td>
</tr>
<tr>
<td>19</td>
<td>11</td>
<td>479.9 ± 73.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>140.3 ± 119.2</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>317.0 ± 58.3</td>
</tr>
</tbody>
</table>

Mean ± SEM.

<sup>a</sup> <i>p</i> 0.01.

### TABLE 9
FOLLICULAR FLUID PROGESTERONE LEVELS

<table>
<thead>
<tr>
<th>Treatment mg of FSH</th>
<th>Small Follicles ng/ml</th>
<th>Large Follicles ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>24.54 ± 0.75&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>19</td>
<td>11</td>
<td>16.88 ± 1.25</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>15.65 ± 3.36</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>*19.10 ± 1.18</td>
</tr>
</tbody>
</table>

*<i>k</i> = SEM.

<sup>a</sup> <i>p</i> 0.01.

<sup>a</sup> <i>p</i> 0.05.
Follicular Fluid Progesterone Concentrations

The average progesterone concentration in the follicular fluid was found to be 21.28 ng/ml. Progesterone levels showed less variation than estradiol and ranged from 6.29 to 26.61 ng/ml in small follicles and from 15.39 to 28.00 ng/ml in the follicular fluid of large follicles. Table 9 shows the average progesterone concentrations in the follicular fluid for each treatment group. Progesterone in both large and small follicles was dose related with the 30 mg treatment having the largest concentrations. There was a significant treatment effect (P<0.05) in which the longer treatment regimen (30 mg) produced significantly greater progesterone concentrations.

Small follicles averaged 19.10 ± 1.18 ng/ml which was different (P<0.001) from concentrations in large follicles which averaged 23.46 ± 0.71 ng/ml.

No age or weight effects were found in progesterone concentrations in large or small follicles.

Correlations of .62, .43, .46, .46 and .52 (P<0.05) were found between progesterone concentrations in small follicles and serum progesterone levels during mid superovulation, on the day of surgery, and on Days 2, 4, and 6 post-surgery, respectively. No relationships were found between progesterone concentrations in blood serum and follicular fluid from large follicles.

Correlations were also found between progesterone in small follicles and the average follicle size (r=+.547; P<0.01) and the percent small follicles (r=+.461; P<0.05).
DISCUSSION

Superovulation of the prepubertal calf offers many possibilities by enabling the animal breeder to increase the number of offspring which can be obtained from an outstanding female in a lifetime. This study is an attempt to quantify the concentrations of estradiol 17-beta and progesterone in the blood serum and follicular fluid of the superovulated calf. By evaluating the steroid concentrations in the calf one can gain a better understanding of follicular development and the endocrine environment created in the superovulated animal.

Follicular Response

It has been known for many years that exogenous FSH injections can increase follicular development and cause cattle, both mature and immature to superovulate (20). When results of this study are compared with the amount of follicular development in the normal prepubertal calf (39) it becomes evident that ovarian activity was increased. Ovarian size and the numbers of follicles produced were greater than values found in the nonsuperovulated calf. The doses of FSH given in this study were lower than levels given in most other studies (5,45,77,87). As a result the average number of follicles per calf (36.1) was considerably lower than numbers reported by W. D. Foote et al. (45). However there was considerable variability with a range in follicle
numbers from 3 to 148 for a single calf. Average follicle size was also lower than values reported in other studies (12,45) probably due to the lower levels of FSH used in this study. Seidel et al. (99) reported the age of the calf had a significant effect on the ovulation rate. However this study showed no age or weight effects on the numbers of follicles produced, follicle size, ovarian size and the percentage of small follicles.

Serum Estradiol and Progesterone Concentrations

There has been considerable research reported on steroid concentrations in superovulated mature cows (51,91,92,94). However information on steroid concentrations in the superovulated prepubertal calf is scarce (93,98,112). The largest study reported (112) represents data accumulated on only seven calves. The data summarized here, on 34 calves, is the largest study examining serum steroids in the superovulated calf.

Serum estradiol concentrations in the superovulated calf closely follow follicular development (91). Estradiol concentrations in the serum were highly variable as represented by very high standard deviations, approximately equal to the means on the given days of collection (Figure 2). This makes the fluctuations and levels difficult to compare within this study and with other studies. Reports by Testart (112) and Schneider et al. (98) indicated estradiol levels rise between the PMSG injection and the ovulatory dose of LH. After the ovulating LH injection estradiol levels decline abruptly then slowly return to basal levels over a period of five to seven days. The same general estradiol
patterns were noted in this study however the absolute concentrations were in disagreement with other studies (93,98,112). Levels of estradiol found prior to superovulation and after surgery were two to four fold higher than concentrations found by Testart (112) and Schneider et al. (98). Treatments were not found to have an effect on serum estradiol concentrations in this study, as has been found in studies using mature cows (51).

Peak levels of estradiol on the day of surgery were similar to levels reported in the prepubertal calf (98,112). However, the concentrations found are much higher than levels reported in the superovulated cow. There are many explanations for the higher levels including a dilution theory proposed by Testart (112). If estradiol production in the superovulated cow and calf are similar, the calf being a much smaller animal will have greater concentrations of estradiol in its body fluids. Larger calves would also have lower concentrations of estradiol in the serum than smaller calves. However, no weight effect was found in serum estradiol levels of animals given the same treatment.

Serum progesterone levels follow corpora lutea development in the superovulated prepubertal calf. Serum progesterone concentrations were less variable than estradiol. Progesterone levels reported by Testart (112) and Schneider et al. (98) remain low until the ovulatory LH injection and then they increase to a maximum 6 to 8 days after ovulation. Basal levels are reached 20-30 days after ovulation. Similar patterns have been noted in mature cattle which have been superovulated (51,92,94). As indicated in Figure 2 the pattern of serum progesterone found
in this study was similar. However, a slight decrease was noted during the middle of the superovulation treatment, this is possibly due to a negative feedback of estradiol from the developing follicles on progesterone production by ovarian follicles.

Serum levels of estradiol and progesterone in individual calves showed similar fluctuations to the means shown in Figure 2. Absolute concentrations varied greatly between individuals. However the fluctuations within individual calves remained relative throughout the study, as shown by high correlations between the hormones on different days of collection.

In the study by Schneider et al. (98), plasma progesterone concentrations on Day 6 post-ovulation ranged from 75-140 ng/ml. Serum progesterone levels in this study were significantly lower in the postsurgical period, especially in the 3½ day treatment regimes (16 and 19 mg). Mean progesterone concentrations were 0.869 ± 0.240 and 0.950 ± 0.673 ng/ml on the sixth day post-surgery for the 16 and 19 mg treatment groups, respectively. The low levels suggest poor corpus luteum development.

There are many possible reasons for the poor corpus luteum development. One reason may be the smaller doses of FSH given in the shorter treatment regimens. Follicular fluid concentrations indicate significantly higher levels of estradiol and lower levels of progesterone in the follicles of the calves from the 16 and 19 mg treatment groups. The follicles from these groups may be slightly immature and upon rupturing do not respond to form active corpora lutea. Another reason is probably due to the surgery. The calves were under additional
stress after surgery which may have inhibited corpus luteum development. In addition the follicles were not allowed to ovulate normally but were ruptured with a needle and the contents were aspirated. Destroying many of the small follicles on the ovary could have interfered with normal corpus luteum development. Edwards (33) and McNatty (72) have implicated follicular fluid as an important component in luteinization of the follicle and the formation of the corpus luteum. Channing (22,34) has shown that removal of the follicular fluid from a follicle causes spontaneous luteinization of the granulosa cells. This has led to the belief of the presence of an inhibitor of granulosa cell luteinization in porcine follicular fluid (22). Premature removal of the follicular fluid in this study along with the injection of HCG did cause luteinization of the follicles, as indicated by the rise in progesterone levels in the serum after surgery. However corpus luteum development was incomplete suggesting that the presence of follicular fluid is necessary for the proper initiation of luteinization. Hansel et al. (50) has reported estrogen injections in cattle to be luteolytic in the post-estrus period. Serum estradiol concentrations found in this study did not show the abrupt drop reported by Saumande (94), Testart (112) and Schneider et al. (98), but remained elevated in the post-estrus period. These higher concentrations found after ovulation may have had a luteolytic effect or negative feedback on corpus luteum development and caused the lower progesterone concentrations found Day 2–6 post-surgery.
Follicular Fluid Estradiol and Progesterone Concentrations

In recent years there has been increased interest in the steroid concentrations of follicular fluid and their relationships to reproductive processes in the female. Staligrammer (109) and Ireland and Roche (56) have documented changes in steroids in bovine follicular fluid as ovulation approaches. The changes which take place are similar to changes in the blood about the time of ovulation (23,95). Results in this study show progesterone concentrations in the blood serum on the day of surgery were only correlated ($r=+.43; P<.05$) with progesterone in the follicular fluid from small follicles. However serum estradiol concentrations were negatively correlated ($r=-.604; P<0.05$) with estradiol in the follicular fluid of large follicles after treatment effects were removed, indicating there may be a loss of estradiol from large follicles as ovulation approaches and the follicles shift toward the production of progesterone. Estradiol and progesterone concentrations in the follicular fluid are representative of the steriodogenic activity of the follicles from which they are taken.

Estradiol is the major steroid present in bovine follicular fluid throughout much of the life of the follicle (80). However progesterone may be found in largest quantities in the preovulatory follicle (109) and atretic (80) or cystic follicles (104). Follicular fluid estradiol concentrations in this study were found to be highly variable (see Raw Data, Appendix Table 14). Estradiol concentrations increase with follicle size, probably due to smaller follicles being less responsive to gonadotropin stimulation and have less steriodogenic activity (38,103).
Consistent increases in follicular fluid estradiol are intimately associated with normal follicular development (80). This study is in agreement with the findings of Chang et al. (21) and England et al. (38) that estradiol and progesterone concentrations are lower in small follicles ($E = 316.9 \pm 58.3 \text{ ng/ml}; P = 19.10 \pm 1.18 \text{ ng/ml}$) than large follicles ($E = 416.7 \pm 95.8 \text{ ng/ml}, P < 0.1; \text{Prog} = 23.46 \pm 0.71 \text{ ng/ml}, P < 0.001$).

Dufour et al. (31) showed that synthesis of steroids by follicles can influence the growth of neighboring follicles in sheep. It was found that ovaries containing a functional corpus luteum had more follicles than ovaries containing a dominant follicle. This suggests that there is a local inhibition of secondary follicular development by the large follicle population. Correlations found in this study also implicates a local inhibitory effect of large follicles on steroidogenesis in small follicles. Correlations were found between progesterone in small follicles and the percent small follicles ($r = +.461; P < 0.05$). As the percent large follicles increases (% small follicles decreases) progesterone in small follicles increases and estradiol decreases. Large follicles on the ovary may be inhibiting the small follicles by decreasing estrogen synthesis.

Treatment effects have been shown to affect the endocrine environment of the superovulated mature cow (51). In this study treatments had a significant effect on progesterone in the blood serum of immature heifers. See Figure 3. The 30 mg treatment produced higher levels of progesterone in the post-surgery period. Treatments also had an effect on estradiol and progesterone concentrations in the follicular fluid of both large and small follicles. The 30 mg treatment again was different
from the shorter treatment groups. The 30 mg treatments produced follicles with greater concentrations of progesterone and lower concentrations of estradiol in the follicular fluid of small follicles. During the surgery there were indications that the follicles produced by the 30 mg group were abnormal, and possibly atretic. The follicular fluid from these animals looked cloudy and bloody and the samples contained more cellular debris than samples from other groups. The oocytes collected were used in the in vitro maturation and fertilization portion of this study (Dahlhausen et al., 25). Many of the oocytes from the 30 mg group showed degenerate cumulus masses. Superovulation treatments have been shown to have a great effect on the numbers of follicles which are produced (12,45), the ovulation and fertilization rates (43,65) and the numbers of oocytes which mature (12,45). Results given here show treatments also have an effect on the follicular fluid concentrations of estradiol and progesterone. This indicates many of the differences seen in the response to different superovulation treatments may be caused by alterations in the local hormonal environment within the follicle. It also suggests that superovulation treatments giving the best results may be those which provide the best environment for follicles to develop. Selection of a treatment in the future may be in terms of those treatments which produce the proper changes in follicular fluid steroid concentrations.
SUMMARY AND CONCLUSIONS

The immature bovine is a potential source of embryos. If techniques can be developed for the collection of these embryos, the young calf can be used to decrease the generation interval while increasing the number of offspring a female can produce throughout a lifetime. This would enable the animal industry to practice more intensive selection and make faster genetic progress provided the trait can be measured early in life. There are many problems with the present techniques involved in the superovulation of immature females. Some of these problems are related to the immaturity of the animals while other problems stem from our lack of knowledge of the reproductive events that take place from birth to maturity.

There has been recent interest in the concentrations of steroids in the follicular fluid. Research has shown that there is a precise sequence of endocrine changes which occur within individual developing follicles. Little is known about what makes certain follicles ovulate while others do not, but presumably it has to do with differences in the local hormonal environment the follicles are exposed to. Follicular growth is controlled from both inside and outside the follicle. The follicular fluid is an important part of this internal follicular control.
This study is an attempt to quantitate the concentrations of estradiol and progesterone in the follicular fluid of the superovulated pre-pubertal calf. Little is known about the local endocrine environment of the calf. This study is a starting point from which to build with additional research in an attempt to elucidate the factors which control the development of individual follicles.

One of the most important findings of this study is that treatment regimes had an effect on serum and follicular fluid concentrations of estradiol and progesterone. It is known that different treatments can affect follicle numbers, ovulation rates, oocyte maturation and fertilization. This suggests these treatment differences may be the result of altered steroid production by ovarian follicles. The superovulation treatments giving the optimum results are probably those which produce the best endocrine environment for follicular development and should be studied more closely.

Age and weights of the calves were found to have significant effects on the follicular fluid steroid levels. This suggests that there are differences in follicles, which vary with the age of the animal, in their ability to respond to superovulation treatments and produce steroids. Treatments may have to be tailored to the age of the animal used.

Follicular fluid progesterone levels were found to be closely related to progesterone levels in the serum prior to ovulation. However estradiol concentrations in the follicular fluid were negatively related to estradiol levels in the serum. This indicates serum levels do not
always reflect follicular fluid steroid concentrations even though the follicle is the major source of steroids in the body prior to ovulation.

Small follicles were found to have lower levels of estradiol and progesterone concentrations than did large follicles. This indicates larger follicles have greater steriodogenic activity and responsiveness to exogenous gonadotropins.

Several relationships were found between the follicular fluid steroid concentrations and the superovulation response. Percent small follicles was positively correlated with estradiol and negatively correlated with progesterone concentrations in small follicles. This is evidence to support a local effect of large follicle's ability to inhibit estradiol production in smaller follicles.

The potential use of prepubertal calves as a source of offspring is unlimited, however there are still many problems to be solved before it will become practical. This study is an examination of the follicular development in the superovulated prepubertal calf from an endocrinological viewpoint, to aid in the determination of the factors which govern follicular development. These data strongly support the theory that peripheral blood concentrations of hormones do not necessarily reflect effective local levels in many internal structures such as the follicle.
APPENDIX A

ANALYSES OF VARIANCE
TABLE 10
ANALYSIS OF VARIANCE
Serum Estradiol Concentrations

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<th>Source</th>
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<th>F</th>
<th>Prob.</th>
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TABLE 11
ANALYSIS OF VARIANCE
Serum Progesterone Concentrations

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TABLE 12
ANALYSIS OF VARIANCE FOR ESTRADIOL IN THE FOLLICULAR FLUID OF LARGE FOLLICLES
### TABLE 13

**ANALYSIS OF VARIANCE FOR ESTRADIOL IN THE FOLLICULAR FLUID OF SMALL FOLLICLES**

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### TABLE 14

**FOLLICULAR FLUID CONCENTRATIONS**

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