Comparison of corpus luteum function, uterine environment and embryo quality in heifers and second/third parity lactating cows

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Abstract

The aim of this study was to compare embryo quality, uterine environment and corpus luteum function in heifers and second/third parity cows. After superovulation and embryo recovery at day seven, 78 embryos (*n* = 17 from cows and *n* = 61 from heifers) were scored morphologically for quality and developmental stage. Significant differences were observed in fertilization rate (p =0,01) and transferability (p < 0,01) between heifers and cows.

Blood was collected on 11 different days in the cycle for progesterone (P_4) analysis from every heifer (*n* = 6) and cow (*n* = 7) that was involved in this experiment. Day 0 is the start of the Ovsynch protocol. The plasma P_4 levels on days 13, 15, 17, 19, 26, 28 and 30/31 were significantly higher (p < 0,05) in heifers than in cows. There was a tendency for this difference on day 11 (0,05 < p <0,10). There were no significant differences in plasma P_4 on day 9, day 21 and day 24.

1. General Introduction

Dairy cow fertility has been declining for the last decades (Lucy, 2001). Genetic selection for high milk yields as well as nutritional stressors have resulted in much lower pregnancy rates of about 30% in second and third parity lactating cows. Yet as milk production increased, pregnancy rates steadily declined.

Many factors which can contribute to the poor fertility rates in dairy cows have been acknowledged, such as poor oviductal and uterine environments, compromised health and immune system, altered follicular dynamics and oocyte quality, reduced corpus luteum function and increased embryo mortality (McNeill *et al.*, 2006). However, the pregnancy rate of heifers (60%) has not shown a dramatic decrease due to intense genetic selection (Pursley *et al.*, 1997), compared to second and third parity dairy cows.

Since different reproductive technologies have been used in this study, the female reproductive hormonal cycle will shortly be addressed for better insight to understand superovulation and Ovsynch, a protocol designed to synchronize ovulation in dairy herds.

1.1 Estrous Cycle

The female reproductive hormonal cycle will shortly be addressed for better understanding of superovulation and Ovsynch, a protocol designed to synchronize ovulation in dairy herds. Both reproductive technologies were used during this study

The estrous cycle provides females with repeated opportunities to copulate and become pregnant. Copulation and sexual receptivity are the primary behavioural events during estrous. The estrous cycle is divided into two phases, the follicular phase and the luteal phase. The follicular phase is relatively short, about 20% of the estrous cycle, the other 80% is called the luteal phase (see figure 1). The follicular phase is dominated by high estradiol (E_2) levels, which is produced mainly by preovulatory follicles, and low progesterone (P_4) levels, due to the regression of the corpus luteum (CL). During the luteal phase P_4 is the dominant hormone, which is produced by the luteal cells of the CL. P_4 suppresses production of luteinization hormone (LH) and follicle stimulating hormone (FSH) (see figure 1). This is done directly by negative feedback on the pituitary gland and indirectly by suppressing production of gonadotrophin releasing hormone (GnRH) in the hypothalamus.

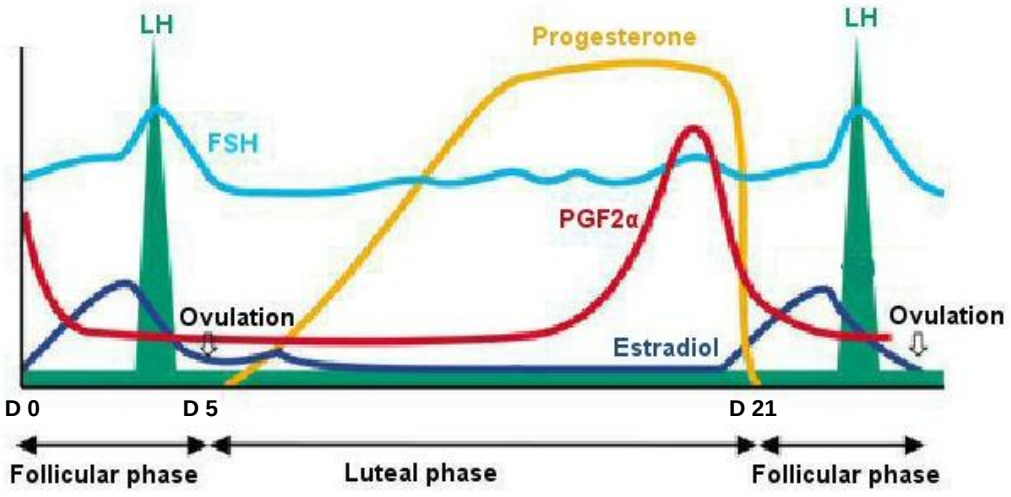


Figure 1: Changes in relative hormone levels during normal estrous cycle in cows/cattle (modified from Intervet)

1.1.1 Follicular phase

The follicular phase starts after luteolysis of the CL due to the decrease of P_4. There is no negative feedback on the hypothalamus by P_4 due to the luteolysis. Therefore the GnRH pulse frequency starts to increase in the hypothalamus. As a result an increase in FSH and LH production by the anterior pituitary will occur. Follicles are recruited from the gonadotrophin sensitive pool in the ovaries and start to grow as a result of the increasing levels of FSH. The process of follicular growth and degeneration occurs continuously throughout the entire estrous cycle, so called follicular waves. Each follicular wave consists of three stages of development: recruitment, selection and dominance (see figure 2). The first stage (recruitment) is characterized by the development of small follicles which start to grow and produce small increasing amounts of estradiol (E_2), which have a positive feedback on the hypothalamic surge center and produce small amounts of inhibin, which begin to inhibit the FSH release by the anterior pituitary. During the process from recruitment to dominance the selected dominant follicle produces increasing amounts of E_2 and inhibin. Inhibin has a direct negative feedback on the FSH secretion in the pituitary gland.

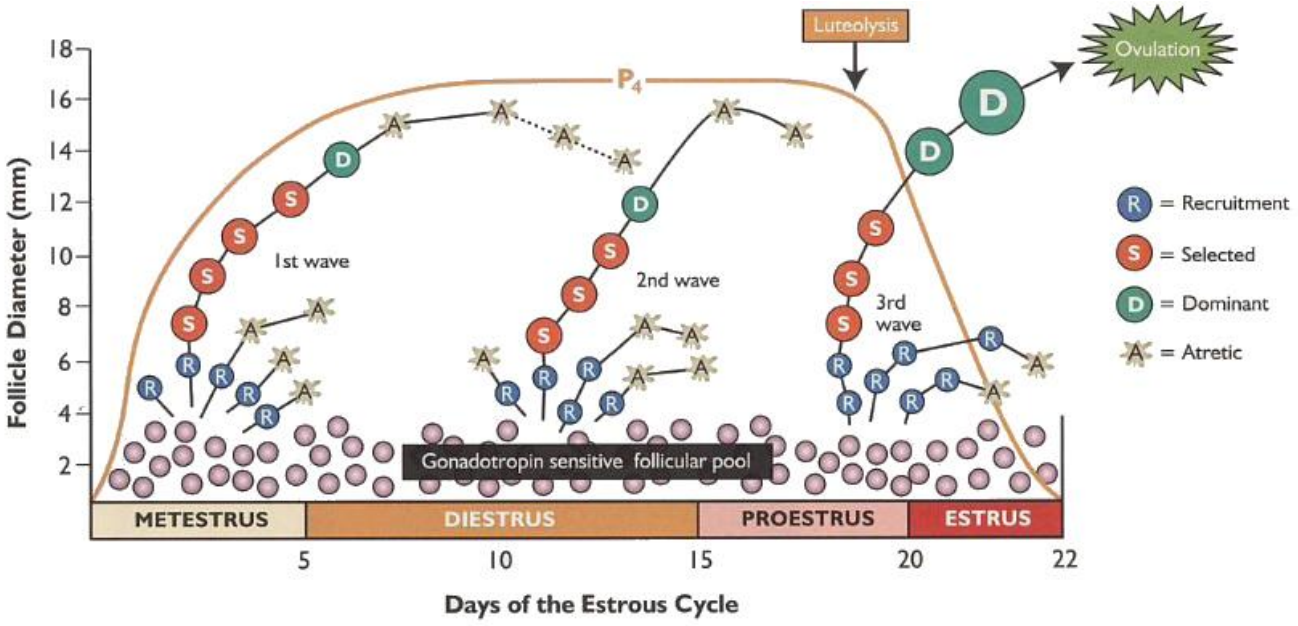


Figure 2: Follicular waves during cycle (Senger, 2003)

The third follicular wave results in a dominant follicle which will ovulate. The first two waves occur during P_4 elevation, the third follicular wave will occur after luteolysis (see figure 2).

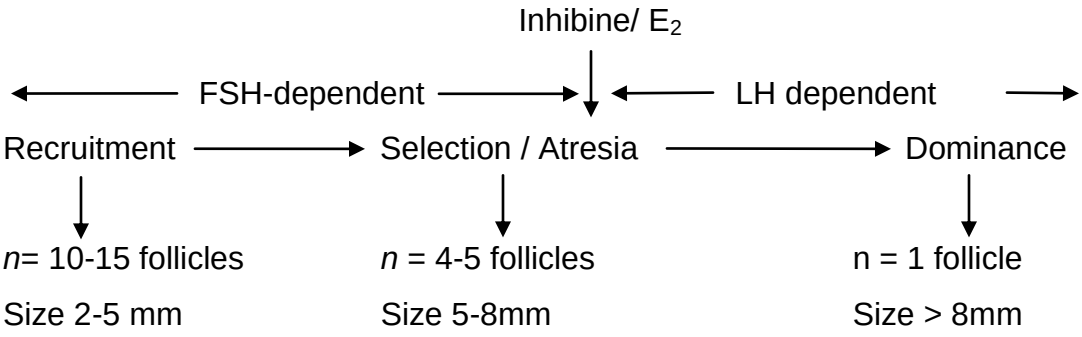


Figure 3: Follicles are recruited, selected and either become atretic or become dominant

The suppression of FSH concentrations in the blood by inhibin results in a reduced blood supply to the subordinate follicles, which causes atresia. The recruitment of other follicles stops. In the next stage (selection) some small follicles (<8mm) undergo atresia, some large potentially ovulatory follicles become selected. The final stage of dominance is characterized by a further decrease in FSH and an increase in LH and inhibin. Some selected follicles undergo atresia, except one which becomes dominant and continues to grow (see figure 3). Estradiol produced by the dominant follicle is approaching the threshold and the dominant follicle reaches its maximum size. Estradiol initiates a preovulatory LH surge after reaching the threshold. The granulosal cells acquire LH receptors which are essential for the follicle to ovulate. This LH surge will stimulate ovulation, induces resumption of oocyte maturation and initiates luteinization of the granulosal and thecal cells of the follicle which includes the switch from E_2 to P_4 production. Follicles generally ovulate after they reach a maximum diameter of 13-20 mm at an interval of 24-48 h after an LH surge. (Wathes *et al.* 2003)

1.1.2 Luteal phase

The luteal phase starts immediately after ovulation of the dominant follicle. The result of the ovulation is the corpus hemorrhagicum (CH), which can be observed 1 to 3 days after ovulation. The thecal interna and granulosal cells from the CH transform to a CL under influence of LH. This process is called luteinization. At day 3 to 5 the CL increases in size until mid-luteal phase and produces an increasing amount of P_4. During the mid-luteal phase the CL is fully functional and produces the maximum amount of P_4. The primary target organs are the uterus, the hypothalamus and the mammary gland. Progesterone prepares the endometrium for implantation of the embryo by increasing activity of secretory glands in the endometrium. Progesterone also inhibits the uterine motility by reducing the contractions of the myometrium. Progesterone has a negative feedback on the secretion of GnRH by the hypothalamus, which will decrease FSH and LH secretion. When conception is lacking the luteal phase ends with the degeneration of the CL under the influence of $PGF_{2\alpha}$ produced by the endometrial cells of the uterine endometrium. This process is called luteolysis. Luteolysis is controlled by oxytocin from the CL and $PGF_{2\alpha}$ from the endometrium. Luteal oxytocin triggers the $PGF_{2\alpha}$ synthesis in endometrium cells. When sufficient numbers of oxytocin receptors are recruited during the late luteal phase, pulsatile release of $PGF_{2\alpha}$ occurs, which will induce luteolysis.

1.1.3 Pregnancy

When conception occurs, the female enters a period of anestrous. Between 3-4 days after ovulation the fertilized oocyte, now called embryo, enters the uterus. The trophoblastic cells of the embryo produce interferons (IFN-τ). Interferons inhibit the production of oxytocin receptors by endometrial cells, so there is no stimulation of $PGF_{2\alpha}$ synthesis, which will provide the signal for prevention of luteolysis. This process is called maternal recognition and is essential for maintaining pregnancy, which prevents luteolysis.

1.2 Ovsynch: a timed insemination protocol

The Ovsynch protocol is designed to synchronize ovulation in dairy herds and to reduce the variability in time of ovulation. Cows are first injected intramuscularly (i.m.) with GnRH on day 0 of the experiment. The first injection is designed to manipulate ovarian follicular development by ovulating and/or luteinizing the existing dominant follicle and initiating the growth of a new cohort of follicles so that a new dominant follicle emerges by day 7, or extend the lifespan of the existing CL in late-luteal phase cows, so that the CL will still be responsive to prostaglandin injection 7 days later. The second injection (i.m.) of $PGF_{2\alpha}$ is given 7 days after the GnRH injection which induces luteolysis. The third injection, 48 hours after the injection of $PGF_{2\alpha}$, contains GnRH to induce ovulation by initiating the pre-ovulatory LH surge. Timed insemination of cows is executed 16 hours after the second GnRH injection (see figure 4). This protocol of timed artificial insemination (TAI) makes it possible to breed heifers and cows without the need of heat detection and could therefore be a useful management tool.

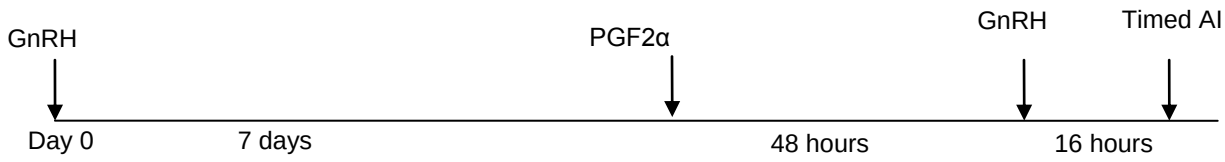


Figure 4: Schematic presentation of the OvSynch protocol

1.3 Superovulation (SO)

A method to generate more ovulations hence more released oocytes after ovulation is called superovulation (SO). This technique was used to increase the number of embryos. Superovulation is induced by administering exogenous doses of hormones during mid-luteal-phase that have FSH activity/activity similar to FSH to overcome the natural mechanism that would normally allow one follicle to become dominant and ovulate. The treatment is used in embryo transfer technology (ET). There is a wide variety in the effect on ovulation numbers, ranging from 0 to more than 50 ovulations per cow (Seidel *et al.*, 1989). In cattle SO treatment can be done between day 9 and 14 (mid-luteal phase) after estrous, when P_4 concentrations are dropping naturally. The

injected FSH is still effective and will cause the follicles to keep growing. Because of the natural variation in estrous cycle, it is difficult to optimize the timing of FSH injections. Eight FSH injections are given every 12 hour for 4 days. FSH is injected both before and after the $PGF_{2\alpha}$ injection to foster follicular growth at the right time (see figure 5).

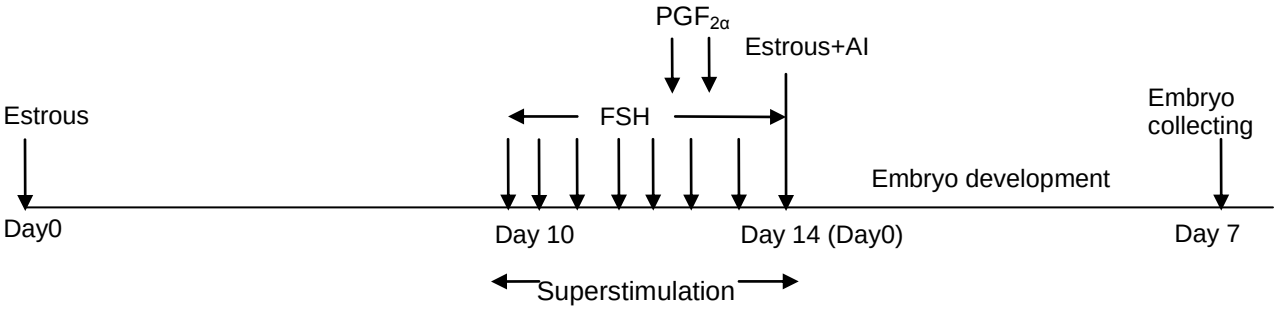


Figure 5: Standard superovulation protocol

1.4 Embryo recovery

The ovulated oocytes become an embryo after fertilization. After 5 to 6 days post-estrous the fertilized oocyte is called a morula. After 7 to 8 days embryo is called blastocyst, it contains a fluid-filled cavity and about 80 cells. The embryo leaves the oviduct and enters the uterus horn on day 4 to 5 after estrous. From this time on the recovery of embryos becomes feasible by flushing the uterus. After hatching of the embryo, 8-9 days after estrous, elongation of the trophoblast starts to occur. The embryo becomes more difficult to recognize. From this moment the embryo grows more rapid and fills the entire uterus by day 20.

1.5 Endometrium genes

The endometrium plays a central role in early embryo-maternal communication and pregnancy (Bauersachs *et al.*, 2006). One of the primary causes of embryonic mortality in cattle is failure of the conceptus to develop an adequate trophectoderm and produce a significant amount of interferon-tau (IFNτ). Recent studies indicate a postovulatory rise in P_4 promotes blastocyst growth and development. Different genes are involved in the regulation of IFNτ and the fine-tuning of the endometrium. The functions of these factors are briefly explained in the next paragraphs.

1.5.1 Cytokines

Leukemia Inhibitory factor (LIF) is one of the most important cytokines in the reproductive tract. The implantation of a blastocyst cannot be successful without expression of LIF in the uterus (Kimber, 2005).

1.5.2. Growth factors

Insulin increases the ovarian response to gonadotrophins and has a positive effect on recruitment of small follicles and better follicle growth. IGF-1 is necessary for full functional development of a follicle (Gong *et al.*, 2001; Thatcher *et al.*, 2006). Besides it IGF-1 stimulates proliferation of endometrial cells and IGF-2 is implicated in early embryonic and placental development. Blastocyst development can be stimulated by injections of exogenous growth hormone (GH), which increases IGF (Satterfield *et al*., 2008).

Epidermal Growth Factor (EGF) plays an important role in embryo development and implantation. The endometrial mitotic activity is affected by the mediation of EGF binding with the endometrial EGF receptors (Wynn *et al.*, 1989)

Fibroblast Growth Factor (FGF) is present in uterine lumen during early pregnancy. FGF is a key regulator in the development of trophectoderm and increases IFNτ in trophectoderm, which suggests that this factor plays an important role in maintaining pregnancy (Michael *et al.*, 2006). Interferon-τ interacts with the endometrial epithelium to decrease the pulsatile release of $PGF_{2\alpha}$. IFNτ also controls the expression of different uterine-derived factors that prepare the uterus for placental attachment and early conceptus development (Ealy *et al.,* 2009). FGF is expressed by the luminal and glandular epithelium throughout the estrous cycle and early pregnancy. In rodents and primates P_4 on itself increases endometrial FGF-2 mRNA (Michael *et al*., 2006). Inhibition of P_4 decreased the FGF synthesis in the endometrium (Satterfield *et al.* 2008). Therefore, P_4 may regulate functions of endometrial epithelia and trophectoderm differentiation and growth.

1.6 Experimental objectives

In lactating cows, embryonic loss is considered to be a major factor in delaying the calving length. The economic loss is valued at $4.70 per cow for each day beyond 12 months of delayed calving. This amounts to losses of $25 million per year in Alberta and British Columbia (Rajamahendran, 2007).

The main problem in modern dairy cow fertility is a delay in the onset of postpartum estrous and ovulation in second and third parity cows (Leroy *et al.*, 2005).

The production of a viable embryo requires ovulation of a good quality/competent oocyte, adequate P_4 levels and an adequate uterine environment. Reduced fertility rates in lactating dairy cows have been shown to be associated with low or abnormal levels of P_4 after breeding, and/or with suboptimal uterine environments (Diskin *et al.*, 2006; Hommeida *et al.*, 2004).

Another factor which suppresses the fertility rate is the fact that high yielding dairy cows experience a major adaptation in their energy balance postpartum to sustain the level of milk production. Nutritional requirements shift at parturition as milk production increases. This shift results in a negative energy balance (NEBAL) for the cow. NEBAL during the first 3-4 weeks postpartum is negatively correlated with the onset of ovulation. With loss of Body Condition Score (BCS), the reduction in conception rate increases (Butler, 2000). During estrous cycles in the first 2 to 3 weeks postpartum P_4 increases; however the rate of P_4 levels is moderated by NEBAL in the early postpartum period (Butler, 2000). The metabolic change has a negative effect on the growing and maturing follicle hence the quality of the oocyte.

It appears that early embryonic loss following insemination is higher (up to 20%) in high-producing dairy cows and that a much higher proportion of embryos die before day 7 following insemination in comparison to heifers and low yielding cows (Diskin *et al.*, 2006; Sartori *et al.*,2002).

1.6.1 Object of the study

There are several objectives of this study. First, comparing progesterone synthesis in corpora lutea by measuring the P_4 concentrations in bloodplasma from heifers and

lactating cows. Secondly, testing oocyte / embryo quality of lactating cows by determining the pregnancy rate following embryo recovery from superovulated lactating cows and heifers. Finally, we attempted to compare the uterine environment of heifers and lactating cows by determining the pregnancy rate, following transfer of embryos obtained from heifers and cows.

Heifers are not in production yet. So they are not influenced by the negative energy balance. Because of the absence of these factors they form an ideal control group.

The results of this study will determine whether the embryo quality, uterine environment, and / or progesterone production ability of corpora lutea are compromised in 2^{nd} and 3^{rd} parity lactating dairy cows. Based on the findings, appropriate reproductive / management technologies may be adopted to overcome the problem of reduced pregnancy rate in lactating dairy cows.

In this experiment three objectives were defined to compare heifers and lactating dairy cows:

1. Compare P_4 concentration (corpus luteum function) by measuring the P_4 concentrations in plasma between heifers and 2^{nd} and 3^{rd} parity cows during the superovulation treatment until the day of collection of embryos.
2. Test embryo quality collected from heifers and 2^{nd} and 3^{rd} parity cows.
3. Compare uterine environment of heifers and 2^{nd} and 3^{rd} parity cows by comparing pregnancy data following the transfer of embryos collected from heifers to recipient heifers and 2^{nd} and 3^{rd} parity cows.

2. Materials and methods

This experiment was carried out at the University of British Colombia (UBC) Dairy Education & Research Centre at Agassiz, British Columbia in Canada. The facilities contain 300 free-stalls, a 24-cow milking parlor, limited dry lab and public reception areas. It helps to meet the Faculty's expanding programs in large animal nutrition, reproduction, behavior and welfare.

2.1. Experimental design and animals

This experiment was carried out in 2 runs due to logistic reasons. The first run (batch 1) was performed between 13th of August 2007 and 17th of September 2007, using 11 animals. The second run (batch 2) was performed between 16th of October 2007 and 16th of November 2007, using 35 animals (see table 1 and 2). The animals used for flushing were also used for CL function. The results of the CL function are based on the second batch. The results of the embryo quality are based on both batches.

Table 1: Numbers of animals used for embryo flushing

		Heifers (*n*)	**Cows (*n*)**	**Total**
Batch 1	**Used**	6	5	11
	Not used	0	0	0
Batch 2	**Used**	6	7	13
	Not used	10	12	22
Total		22	24	46

Table 2: Numbers of animals used for CL function

		Heifers (*n*)	**Cows (*n*)**	**Total**
Batch 1	**Used**	0	0	0
	Not used	0	0	0
Batch 2	**Used**	6	7	13
	Not used	10	12	22
Total		16	19	35

Thirty-five Holstein Friesian dairy cows (*n*=19) and heifers (*n*=16) involved in the second batch of the experiment. The lactating dairy cows were cycling roughly 50 to 60 days post-partum checked using an ultrasound scanner (Aloka SSO-500, Aloka Co) with a 7.5 MHz transrectal transducer. The heifers were cycling 14 to 15 months of age. All animals were treated with Ovsynch protocol (see figure 4, paragraph 1.2), using GnRH (100µg i.m.; Fertiline®, Vetoquinol Canada Inc.), followed by $PGF_{2\alpha}$ (25µg i.m.; Estrumate®, Bayer, Shawnee Mission, KS, USA) 7 days later only to synchronize the animals, so no data will be used in this experiment. The group received a second GnRH injection 48 hours after the $PGF_{2\alpha}$ injection to synchronize/induce the ovulation (figure 6). Not all animals (*n*=22) were used for the experiment. The animals which were cycling normally were selected for the experiment. Those animals with gynaecological deviations, like a deviated size uterus and/ or the presence of cystic or inactive ovaries, were excluded for this experiment.

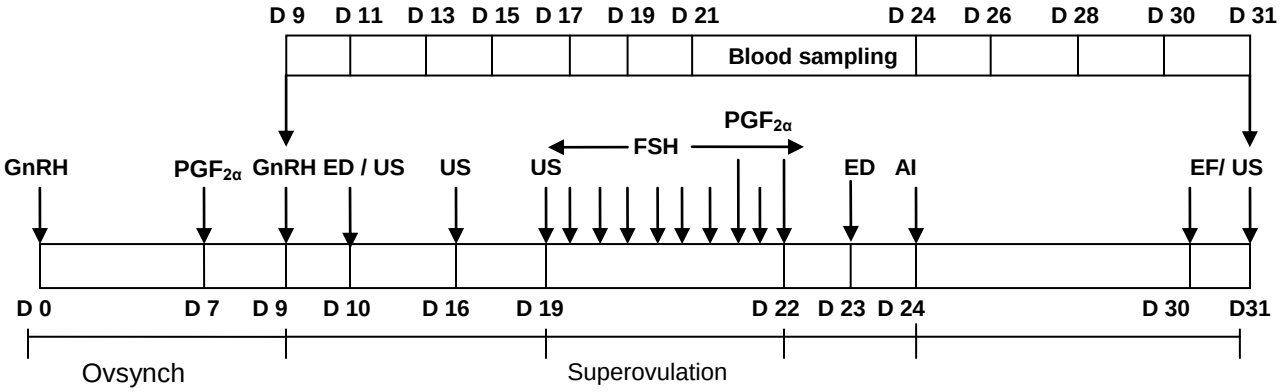


Figure 6: Time schedule of the experiment (batch 2) (**ED** =Estrous Detection; **US**=ultrasound; **AI**=Artificial insemination am/pm; **EF**=embryo flushing; **D**= day)

2.1.1 Blood sampling and progesterone analysis (Corpus Luteum function)

Blood samples were collected from the coccygeal vein during the procedure on 12 different days in the cycle for subsequent progesterone analysis from every heifer (*n*=6) and cow (*n*=7) that was involved in the experiment. Blood sampling was done every other day starting at day 9 till day 21 of the experiment. And from day 24 (AI) till day 30/31(flushing) of the experiment (figure 6) . The animals (*n* =13) from batch 2 were

divided in 2 groups for flushing due logistic reasons: 7 animals on day 30 (day 6 after AI) and 6 animals on day 31 (day 7 after AI).

Blood samples were centrifuged (2000x g, 10 min) and the plasma was stored at -20°C pending hormonal determination in order to determine the possible differences between the progesterone plasma concentration between cows and heifers.

Concentrations of P_4 (ng/mL) in plasma were determined using a commercially available solid-phase radioimmunoassay (Coat-A-Count Progesterone, Diagnostic Products, Los Angeles, CA). This kit was previously validated at the laboratory of the University of British Columbia for the measurement of P_4 in milk and blood (Rajamahendran *et al.*, 1993). Briefly, sample or reference standard (0.1mL) was added to tubes coated with a specific P_4 antibody. Reference standards contained between 1ng/ml and 40ng/ml P_4. Buffered I-125-labelled P_4 (1.0ml) was added to all tubes, which were shaken on a vortex and left to incubate for three hours. Tubes were decanted after the incubation period. The tubes were counted for radioactivity on a gamma counter for one minute. Coefficient of variation within (intra) and between (inter) assays were 7% and 9%, respectively. The sensitivity of the assay was 0.03ng/ml.

2.1.2 Embryo recovery and evaluation of embryo quality

Standard SO and non-surgical embryo recovery procedure was used to obtain embryos from 24 animals (12 heifers and 12 2^{nd} and 3^{rd} parity cows from batch 1 and batch 2) . The same procedures during the experiment for batch 1 and batch 2 were used, so the data could be used for analysis. Embryos will be graded and afterwards used in other lab-experiments, none were used for transfers.

On day 19 of the experiment all animals were rectally palpated on the day of expected estrous for the presence of an ovulatory follicle and 7 days later for the presence of a CL. All animals were normally cycling and gynaecologically normal as judged by rectal exploration before starting the SO treatment. The SO treatment was started 10 days after second GnRH injection (Ovsynch). Superovulation involved 8 intramuscular injections of FSH (Folltropin-V®, Bioniche, Ontario, Canada) administrated over a 12-h interval in decreasing doses. Two Prostaglandin analogues (Estrumate®, Bayer, Shawnee Mission, KS, USA) are injected intramuscular on day 22 along with 2 FSH

injections (see table 3). Forty-eight hours after the second $PGF_{2\alpha}$ injection the animals were inseminated twice within a 12-h interval.

Due logistic reasons the uteri were non-surgically flushed on day 6 (*n*=12) or day 7 (*n*=12) after insemination by non-surgical procedure to collect embryos. Immediately before the flushing, blood was collected from the coccygeal vein. Embryo collection can be performed more accurately and with less strain if the donor cow's rectal squeeze is inhibited. This is accomplished by epidural anesthesia using 6 ml of lidocaine injected in the intervertebral space between the vertebrae of the tail-head region. The vulva and rectum were thoroughly washed before flushing. The ovaries were scanned on days 10, 16, 19, 30 and 31 by an operator using an ultrasound scanner (Aloka SSO-500, Aloka Co) with a 7.5 MHz transrectal transducer to count the number of CL. The results on day 10, 16 and 19 were not recorded. Embryos were transcervically collected from each uterine horn, involves a catheter fixed within a small balloon in one of the uterine horns, which is then inflated so that it forms a snug seal against the uterine horn wall. Using about 500 ml of phosphate buffered saline (PBS) solution, each horn is repeatedly filled and emptied. Following flushing, the recovered lavage was filtered through an embryo filter (Miniflush Embryo Recovery System, mesh 44µm, Minitub, Germany) and the fluid was examined for oocytes / embryos under a stereomicroscope. The embryos were graded for quality according to the criteria of the International Embryo Transfer Society (IETS,1998). From each cow and heifer used for flushing, the following data were recorded: number of CL, recovery rate (RR), total embryos and oocytes per flush, the transferability and the developmental stage of the embryo. The transferability is determined by observing the inner cell mass (shape of cells), the shape and thickness of the zona pellucida and the colour of the embryo. Embryos not selected were excluded from further experiments.

Table 3: Schedule of superovulation in this experiment

	6 am	**6 pm**
Day 19	4 ml FSH*	4ml FSH
Day 20	3 ml FSH	3 ml FSH
Day 21	2 ml FSH	2 ml FSH
Day 22	1 ml FSH	1 ml FSH
Day 22	2 ml $PGF_{2\alpha}$**	2 ml $PGF_{2\alpha}$

* FSH= Folltropin-V®, Bioniche, Ontario, Canada

** $PGF_{2\alpha}$ = Estrumate®, Bayer, Shawnee Mission, KS, USA

2.2 Endometrium environment

In the mid-luteal phase of the estrous cycle, endometrium biopsies were taken by a veterinarian. The uterine samples were transcervically collected from each uterine horn. The vulva and its surroundings were cleaned with water and iodine solution. The biopsy instrument was covered with a plastic sheath. For some heifers a dilator was used because the cervix was too narrow. The endometrial tissue (100mg) was collected about 7cm beyond the uterine bifurcation. All these handlings were performed under rectal exploration. The collected tissue was preserved in an alcohol solution. In the lab, the samples were stored at -80°c for RNA isolation for analysis of growth factors (EGF, IGF and FGF) steroid hormones and cytokines (TNFα and LIF) using a semi-quantitive Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) procedure using specific primers. The procedure was used to measure the amount of mRNA present. The total RNA was isolated from the endometrial samples using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturing instructions. After the reverse transcription of total RNA using Cells-to-cDNA II kit (Applied Biosystems, Canada), the samples are ready for the RT-PCR. The RT-PCR was performed using JumpStart RED Taq Ready Mix PCR (Sigma-Aldrich) reaction mix and gene-specific primers. PCR products were analysed by gel electrophoresis using ethidium bromide (0.2lg/ml) stained 2% agarose gel and photographed under ultraviolet illumination. The relative optical density of each gene was normalized to housekeeping gene (Glyceraldehyde-3-phosphate dehydrogenase: G3PDH) and was compared between cows and heifers.

Unfortunately I did not get to work on the results of this part of my project, due the time constraints.

2.4 Statistical procedure

For statistic analysis, the data were analysed using the computer software package SPSS 16.0.

The rates of embryo recovery with recovery rate, fertilization rate and transferability were compared with a χ^2 test and T-test. Progesterone concentrations as a measure for CL function were analyzed by a T-test. Level of significance was defined at $p < 0.05$.

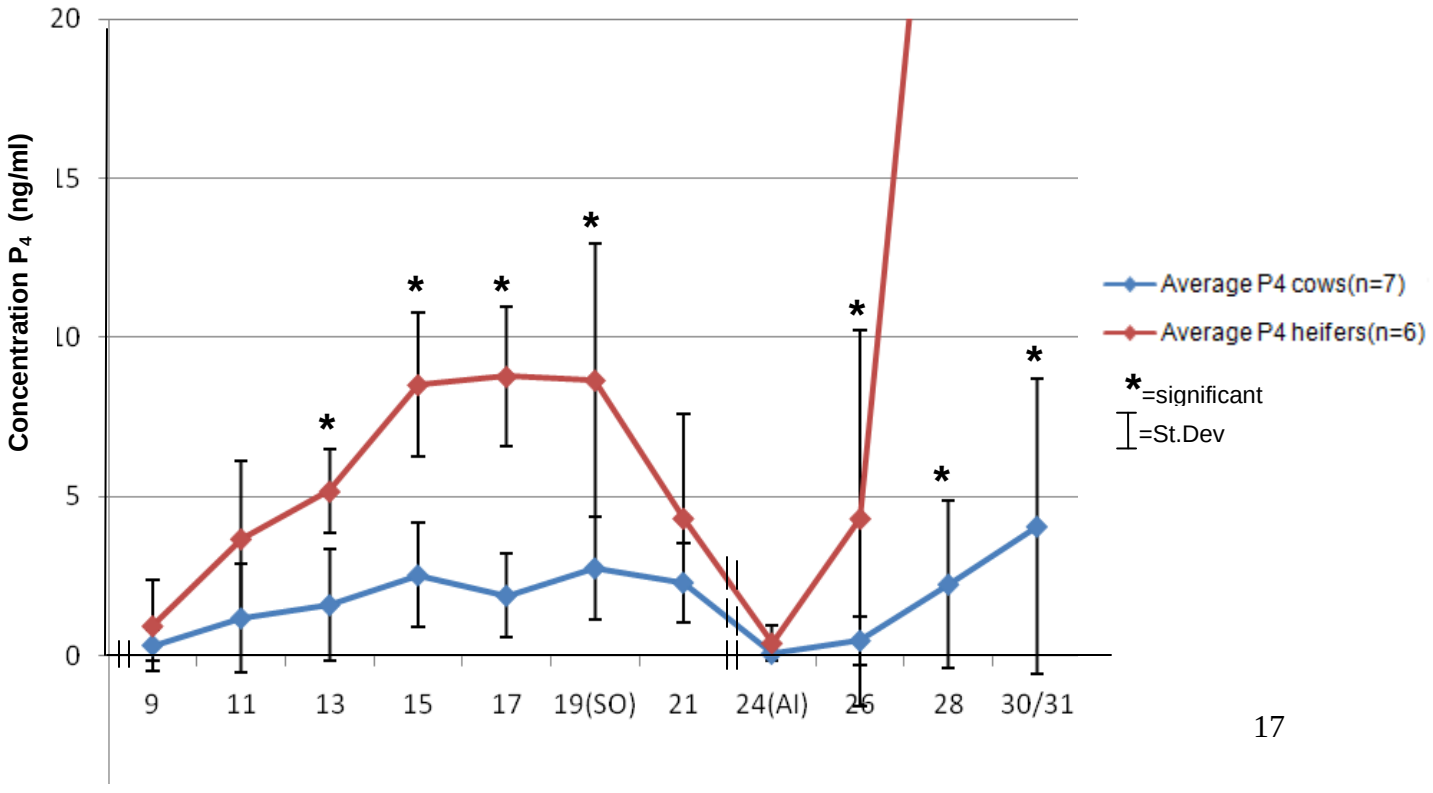
3. Results

3.1 Corpus Luteum function

The results presented are based on the animals (*n*=13) from the second batch. Heifers produced significantly more P_4 during the experiment than cows. Days 13, 15, 17, 19, 26, 28 and 30/31 were significantly higher ($p < 0,05$) in heifers than in cows. There was a tendency for this difference on day 11 ($0,05 < p < 0,10$) (see table 4). There were no significant differences in plasma P_4 on day 9, day 21 and day 24.

Table 4: Average progesterone levels (ng/ml) in cows and heifers on 11 collection days.

	D9	D11	D13	D15	D17	D19(SO)	D21	D24 (AI)	D26	D28	D30/31
Cow (n=7)	***0.318***	***1.173***	***1.589***	***2.529***	***1.884***	***2.747***	***2.295***	***0.056***	***0.466***	***2.238***	***4.060***
St. Dev. (±)	*0.497*	*1.709*	*1.764*	*1.636*	*1.324*	*1.610*	*1.255*	*0.051*	*0.772*	*2.617*	*4.618*
Heifer (n=6)	***0.943***	***3.677***	***5.170***	***8.521***	***8.775***	***8.663***	***4.323***	***0.404***	***4.317***	***32.704***	***62.013***
St. Dev. (±)	*1.428*	*2.455*	*1.319*	*2.247*	*2.202*	*4.294*	*3.277*	*0.552*	*5.907*	*25.912*	*57.601*
P-value	*0.346*	*0.066*	*<0.001*	*<0.001*	*<0.001*	*0.018*	*0.201*	*0.184*	*0.014*	*<0.001*	*<0.001*



Days of experiment

Figure 7: P_4 concentrations (ng/ml) in cows (blue line) and heifers (red line) on collection days (figure 6).

3.2 Flushing results and embryo quality

The results of the embryo quality are based on the data of first (*n*=11) and second (*n*=13) batch. The total number of CL were significantly (p<0,01) higher in heifers compared to the cows. The embryo quality is expressed by 3 variables: the ratio of fertilized embryos, the number of transferable embryos and the number of morula/blastula. The proportion of transferable embryos and the proportion of blastocysts recovered were higher in heifers compared to the cows. Heifers produced significantly (p=0,01) more fertilized embryos compared to cows. Moreover, recovery ratio (RR) did not show a significant (p=0,64) difference between heifers and cows. The transferability was also significant (p=<0,01) comparing heifers to cows (see table 5).

Table 5: Outcome of the flushing results[1] on day30/31 of the experiment

ID	CL#	Tot. O/E	RR (%)	Fertilized	UFO	Degen.	Transfer.	Embryo stages	
								Morula	Blastula
Cow (n=12)	**52**	**22**	**42.3**	**17**	**5**	**9**	**8**	**8**	**0**
St. Dev. (±)	4.274	7.132		2.021	0.51	1.215	0.985	0.674	
Heifer (n=12)	**145**	**83**	**57.2**	**61**	**22**	**28**	**33**	**21**	**12**
St. Dev. (±)	3.288	4.295		2.275	2.368	2.188	1.215	1.193	1.595
P-value	<0.01	0.01	0.64	0.01	<0.01	0.03	<0.01	0.269	

[1] CL# = Number of Corpora Lutea
Tot. 0/E = Total of recovered ova-embryos
RR (%) = Recovery Rate of ova-embryos (number of structures recovered/number CL)
Fertilized = Number of fertilized ova
UFO = Number of unfertilized ova
Degen. = Number of degenerated embryos
Transfer. = Number of transferable embryos
Morula = Number of morula
Blastula = Number of Blastula

4. Discussion

The aim of the experiment was to study differences between heifers and second/third parity cows for corpus luteum function and the quality of embryos.

4.1 Corpus Luteum function and Superovulation

Before the SO treatment started, heifers produced since day 13 significant more P_4 than 2^{nd} and 3^{rd} parity cows till the end of the experiment, except day 21 and day 24. Different studies observed a difference in P_4 values between cows and heifers during first days of pregnancy (Wolfenson *et al.*, 2003; Chagas e Silva *et al.*, 2002ab).

During early lactation, negative energy balance (NEBAL) is metabolically similar to undernutrition and seems to cause a disturbance in pulsatile secretion of LH. The LH pulse rate and P_4 concentrations are also reduced in higher yielding cows. As a result of this disturbance, ovarian inactivity may occur in cattle (Kafi *et al.* 1997; Wathes *et al.*2003). The mean plasma concentration of P_4 from day 4 to day 6 after ovulation increased significantly with increasing diameter of the ovulatory follicle and decreased with increasing time of luteolysis. Dairy cows that ovulate small oocytes developed smaller CL and secreted less P_4 (Perry *et al.*, 2005)

Mann *et al.* (1999) demonstrated a highly significant 10,3% improvement in pregnancy rates when P_4 was administered before day 6 compared with only 1,4% increase when P_4 was administered after day 6.

Desaulniers *et al.* (1995) found that during the luteal phase the synchronized estrous cycle in cows showed a lower LH pulse amplitude, and lower plasma E_2 and FSH concentrations than heifers. The timing of the increase in P_4 in the early luteal phase (days 4-5) appears to be a key determinant of fertility, because it alters the secretory activity of the reproductive tract, thus influencing the embryonic growth and IFN-τ production (Wathes *et al.* 2003).

Different studies found a significant relationship between the concentration of milk progesterone on days 4-6 after ovulation and the probability of embryo survival (McNeill *et al.*, 2006; Stronge *et al.*, 2005). This suggests that besides blood samples also milk samples can be used for CL function screening.

Second/third parity cows responded poorer to SO than heifers in this experiment. Variability in SO response in cattle remains a problem (Desaulniers *et al.*, 1995; Lopes da Costa *et al.*, 2001). Lower SO response, fertilization rate and viable embryo yield of 2^{nd} and 3^{rd} parity cows was associated to significantly lower P_4 concentrations either before or after the SO treatment (Chagas e Silva *et al.* 2002). In Holstein cows the maximal response to SO occurs at 5,6 years of age. Increasing the doses of FSH in older cows may support to increase the numbers of oocytes and embryos in an unpredictable and variable outcome (Desaulniers *et al.*, 1995)

Abnormal P_4 profiles are negatively related to the SO response, embryo yield and quality (Chagas e Silva *et al.*, 2002b). Plasma P_4 concentrations increased during the SO treatment and the mean P_4 concentration was higher in heifers than in low response cows (Desaulniers *et al.*, 1995). This could explain the difference in SO response between cows and heifers.

The low recovery rate can possibly be explained by overrating the number of corpora lutea while scanning the ovaries. For the exact number of CL, the animals must be euthanized to get the ovaries.

4.2 Embryo recovery and quality

The cows did produce fewer embryos than the heifers. This was associated with the low response to SO. A low response means less ovulations, which results in less CL and less P_4 and less embryos. Starbuck *et al.* (2001) showed that cows with adequate milk P_4 (> 3ng/ml) had pregnancy rates of approximately 50-55%, whereas cows with concentrations of < 1 ng/ml had pregnancy rates <10%, this could explain the differences in embryo recovery between cows and heifers. The increase in P_4 on day 4 and day 5 is critically important for embryo development (Wathes *et al.* 2003)

Flushing procedures have been done on day 6 and day 7. The differences in developmental stage could influence the flushing results.

The cows and heifers were inseminated with sperm of different bulls. In the last 2 batches, the quality of the used semen was better than sperm used in the first batch. Chenoweth (2007) and Samardzija *et al.* (2006) indicated that bulls used for AI differ in embryo mortality rates. Greater losses occurred with low fertility bulls as compared to high fertility bulls.

For future studies it is essential to increase the number of animals in the experiment to get a more reliable result. In this experiment the sample size after selection proved to be too small.

5. Conclusion

The study confirmed that heifers produced since day 13 significantly more P_4 than 2nd and 3rd parity cows till the end of the experiment. This association was not evident on day 9, 11, 21 and 24. High milk production appears to exert a negative effect on embryo production and CL function.

For future studies it is essential to increase the number of animals in the experiment to get a more reliable result. In this experiment the sample size after selection proved to be too small.

Many studies have shown that ovarian cyclicity in postpartum dairy cows is often irregular. The increase in milk yield has placed cows under increasing metabolic demands. This manifests as inhibition of pulsatile LH secretion, reduced follicular and luteal growth, which results in a lower oocyte quality and a poor environment in the reproductive tract. Abnormal P_4 profiles are negatively related to the SO response, embryo yield and quality. This problem is not seen in heifers, because they are not lactating.

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Appendix

Cow (n=7)	D9	D11	D13	D15	D17	$D19_{SO}$	d21	$D24_{AI}$	D26	D28	D30/31
3051	1,059	4,338	3,545	5,754	4,001	5,804	4,014	0,021	0,037	4,221	6,112
3056	0,041	0,324	0,35	0,976	0,483	1,898	1,559	0,088	0,775	5,22	8,236
4028	0	0,046	0,767	3,388	0,916	3,13	2,868	0,058	0,002	0,228	0,673
4078	0,025	0,702	2,006	2,608	1,298	3,513	3,058	0	0,023	0,032	0
4079	0,032	0,020	0,027	1,595	1,687	2,247	0,903	0,132	0,305	0,331	0,906
4087	1,03	2,779	4,363	2,026	3,456	0,855	0,676	0,091	2,098	5,56	11,67
9037	0,038	0,000	0,067	1,354	1,348	1,785	2,986	0	0,022	0,076	0,825
Average	0,318	1,173	1,589	2,529	1,884	2,747	2,295	0,056	0,466	2,238	4,060
Heifer (n=6)	**D9**	**D11**	**D13**	**D15**	**D17**	$D19_{SO}$	**D21**	$D24_{AI}$	**D26**	**D28**	**D30/31**
6081	0,027	3,762	4,736	8	7,679	8,405	0	0,74	3,492	77,604	163,922
6082	0,016	2,058	4,451	11,91	9,152	13,372	7,588	0	1,198	10,23	25,8
6083	0,085	2,351	4,259	6,517	11,519	10,025	6,119	0	3,794	40,205	91,956
6084	3,126	6,727	7,236	9,361	10,15	0,604	0,45	1,381	16,035	38,958	54,85
6088	0,008	0,716	3,967	5,759	5,114	9,226	6,669	0,141	0,98	21,173	21,702
6092	2,398	6,450	6,373	9,575	9,036	10,347	5,112	0,16	0,402	8,051	13,849
Average	0,943	3,677	5,170	8,521	8,775	8,663	4,323	0,404	4,317	32,704	62,013

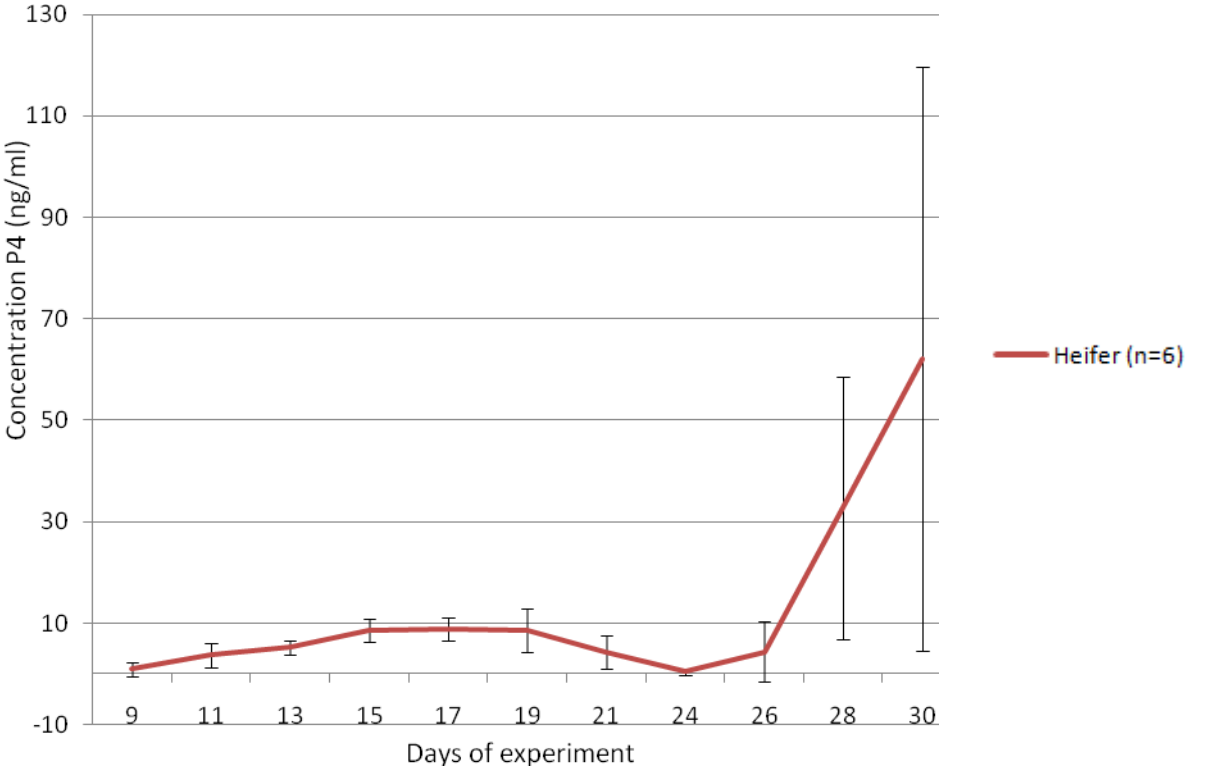
Appendix 1: Progesterone concentrations (ng/ml) in cows and heifers on 11 collection days (batch 2)

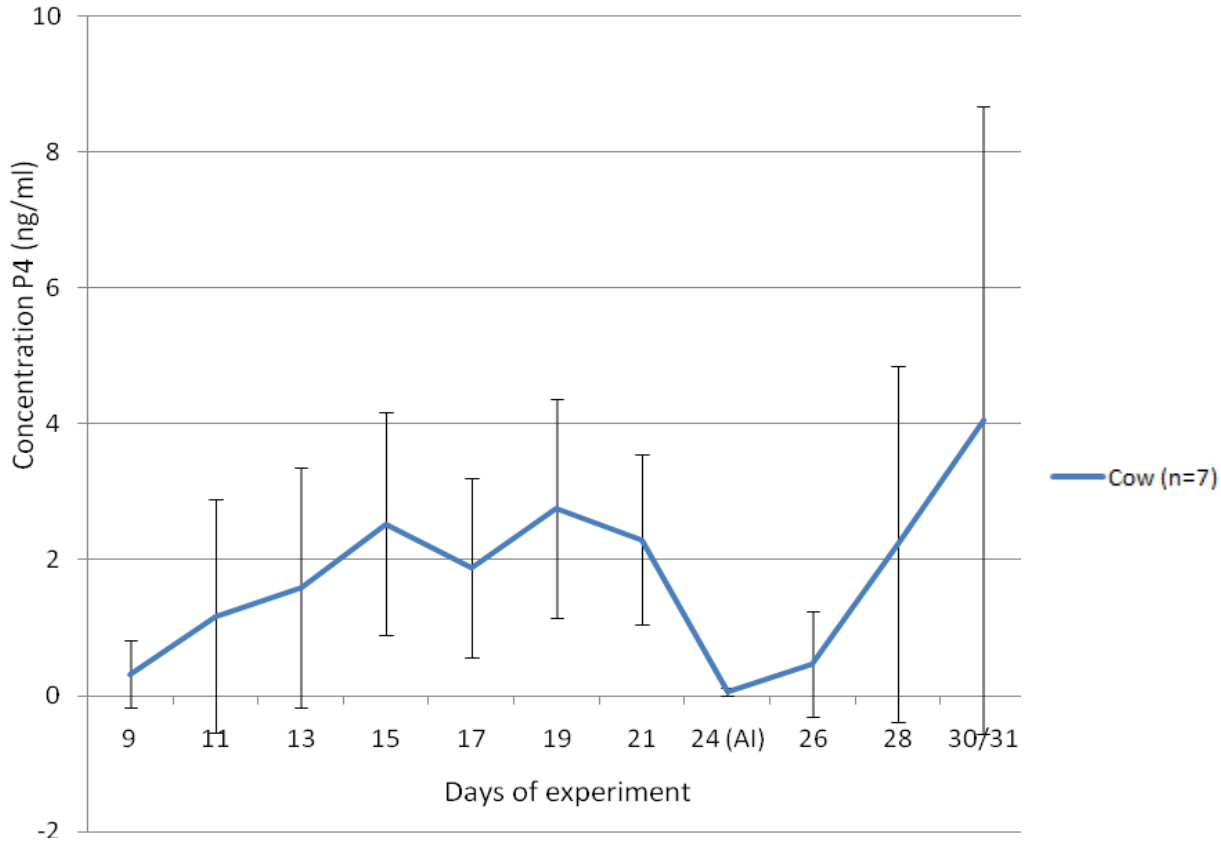
ID	Parity	CL#	Tot. O/E	RR (%)	Fertilized	UFO	Degen.	Transfer.	Morula	Q.	Blastula	Q.
Cows (n=12)												
3036[1]	3	1	1	100	0	1	0	0	0		0	
3051[2]	3	13	7	53,8	6	1	4	2	2	c	0	
3055[1]	3	3	3	100	2	1	1	1	1		0	
3056[2]	3	7	5	71,4	5	0	2	3	1	a	0	
									2	c		
3126[1]	3	5	2	40	1	1	0	1	1	a	0	
3133[1]	3	1	0	0	0	0	0	0	0		0	
4028[2]	2	5	1	20	1	0	1	0	0		0	
4055[1]	2	1	0	0	0	0	0	0	0		0	
4078[2]	3	3	0	0	0	0	0	0	0		0	
4079[2]	2	1	0	0	0	0	0	0	0		0	
4087[2]	2	12	2	16,7	1	1	1	0	0		0	
9037[2]	7	1	1	100	1	0	0	1	1	b	0	
Total		52	22	42,3	17	5	9	8	8		0	
Heifers (n=12)												
6059[1]	0	12	10	83,3	7	3	4	3	0		3	
6062[1]	0	10	10	100	6	4	4	4	4		0	
6064[1]	0	5	2	40	2	0	0	2	1		1	
6065[1]	0	16	15	93,8	9	6	5	3	3		0	
6070[1]	0	11	7	63,6	7	0	2	5	0		5	
6071[1]	0	12	12	100	7	5	5	1	1		0	
6081[2]	0	15	2	13,3	2	0	0	2	1	a	1	a
6082[2]	0	9	3	33,3	3	0	0	3	3	b	0	
6083[2]	0	17	6	35,3	6	0	4	2	2	b	0	
6084[2]	0	14	9	64,3	5	4	4	1	1	b	0	
6088[2]	0	11	3	27,3	3	0	0	3	1	a	2	a
6092[2]	0	13	4	30,8	4	0	0	4	2	a	0	
									2	b		
Total		145	83	57,2	61	22	28	33	21		12	

Appendix 2: Analysis of the flushing[*] (1 = batch 1 and 2 = batch 2)

[*] CL# = Number of Corpora Lutea
Tot. 0/E = Total of recovered ova-embryos
RR (%) = Recovery Rate of ova-embryos (number of structures recovered/number CL)
Fertilized = Number of fertilized ova
UFO = Number of unfertilized ova
Degen. = Number of degenerated embryos
Transfer. = Number of transferable embryos

Morula = Number of morula
Blastula = Number of Blastula





Appendix 3: P_4 concentrations (ng/ml) in cows (blue line) and heifers (red line) on collection days with standard deviations.